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Trans-sialidase from *Trypanosoma cruzi* catalyzes sialoside hydrolysis with retention of configuration

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The *trans*-sialidase from *Trypanosoma cruzi* is a member of the sialidase superfamily that functions as a sialidase in the absence of a carbohydrate acceptor. We have used ^{1}H nuclear magnetic resonance (NMR) spectroscopy to investigate the stereospecificity of the hydrolysis of two substrates, namely, 4-methyl-umbelliferyl-*N*-acetylneuraminic acid and α(2–3)-sialyllactose, catalyzed by a recombinant *T.cruzi trans*-sialidase. We demonstrate that, in aqueous solution, the thermodynamically less stable α-form of *N*-acetylneuraminic acid is the initial product of the hydrolysis; subsequent mutarotation leads eventually to an equilibrium mixture of the α and β forms, in molar ratio 8:92. In a mixed water/methanol solution, the hydrolysis reaction produces also the α-methyl sialoside but not its β-methyl counterpart. We also show that 4-methyl-umbelliferyl-*N*-acetylneuraminic acid is a significantly better substrate for the sialidase than α(2–3)-sialyllactose. Prolonged incubation of α(2–3)-sialyllactose with an excess of *trans*-sialidase produced a trace of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid, as identified by NMR spectroscopy and by gas liquid chromatography/mass spectrometry. In conclusion, this study shows that the stereoselectivity of the sialidase activity of *T.cruzi trans*-sialidase is identical to that of bacterial, viral, and mammalian sialidases, suggesting a similar active-site architecture.

Key words: enzyme mechanism/NMR spectroscopy/sialidase/*trans*-sialidase/*Trypanosoma cruzi*

Introduction

The protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, is unable to synthesize sialic acid, instead scavenging it from exogenous glycoconjugates and transferring it to mucin-like acceptor molecules on the parasite surface by means of a *trans*-sialidase (TS) (Previato *et al.*, 1985). The *T.cruzi* TS differs from mammalian sialyltransferases that use exclusively CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) as the sialic acid (Sia) donor. *T.cruzi* TS-mediated surface sialylation is thought to be necessary for trypomastigote forms of the parasite to attach to and enter host cells (Burleigh and Andrews, 1995).

In vitro studies have shown that *T.cruzi* TS preferentially catalyzes the transfer of sialic acid residues from Siaα2–3Galβ1-*x* containing donors and attaches them in α2–3 linkage to terminal β-galactopyranosyl (β-Gal*p*) containing acceptors (Vandekerckhove *et al.*, 1992). Terminal α-Gal, Galβ1–4(Fucα1–3)GlcNAc and Galβ1–3(Fucα1–4)GlcNAc are not acceptors (Scudder *et al.*, 1993). Incorporation of one *N*-acetylneuraminic acid (Neu5Ac) residue onto an acceptor appears to hinder entry of a second residue when two potential acceptor sites are present on the same oligosaccharide (Previato *et al.*, 1995). In the absence of a suitable carbohydrate acceptor, *T.cruzi* TS irreversibly transfers sialic acid to a water molecule, thus functioning as a sialidase similar to viral, mammalian and bacterial sialidases (Scudder *et al.*, 1993). Sequencing of *T.cruzi* TS genes shows that, although the enzyme differs from those sialidases in acceptor specificity, it is a member of the sialidase superfamily (Roggentin *et al.*, 1993).

Members of the sialidase family display only a moderate degree of sequence homology. Firstly, the sequence motif S-X-D-X-G-X-T-W (the so called Asp-box) is found repeated three to five times in the sequences of bacterial, trypanosomatid, and mammalian sialidases, though it is barely recognizable in most viral sialidases (Roggentin *et al.*, 1989, 1993). N-terminally from the Asp-box one finds the X-R-X-P (or FRIP) region, which includes one arginine residue out of the three arginines that are known to bind the carboxylate group of sialic acid (Garskell *et al.*, 1995). Crystallographic studies of bacterial and viral sialidases have shown that the overall fold of these molecules and the spatial arrangement of key amino acids at the active sites are similar (Crennell *et al.*, 1993). Furthermore, ^{1}H nuclear magnetic resonance (NMR) spectroscopy has demonstrated that members of the sialidase family hydrolyze the sialyl glycosidic bond with retention of configuration at the anomeric center of sialic acid (Friebolin *et al.*, 1981a,b; Chong *et al.*, 1992; Wilson *et al.*, 1995, 1996; Kao *et al.*, 1997).

Inhibition by the transition-state analog 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) appears to be a characteristic feature of most members of the sialidase family (Schauer and Kamerling, 1997). Furthermore, it has been shown by gas liquid chromatography/mass spectrometry (GC/

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MS) (Burmeister *et al.*, 1993) and by ^{1}H NMR spectroscopy (Janakiraman *et al.*, 1994) that small amounts of Neu5Ac2en are formed during prolonged incubation of Neu5Ac or α(2–3)-sialyllactose (SL3) with influenza-B virus sialidase, but Neu5Ac2en has not been detected in reactions catalyzed by bacterial sialidases (Schauer and Kamerling, 1997).

The catalytic site region in the N-terminal domain of *T.cruzi* TS contains all amino acid residues that are known to be involved in sialic acid binding for other sialidases studied (Pereira *et al.*, 1991; Roggentin *et al.*, 1993; Crennell *et al.*, 1993, 1994; Garskell *et al.*, 1995). The *T.cruzi* TS N-terminal domain also contains the FRIP motif and five conserved Asp box sequences (Campetella *et al.*, 1994; Schenkman *et al.*, 1994). The C-terminal domain consists of a variable number of 12-amino-acid repeats (Cazzulo and Frasch, 1992). It is not required for TS activity; rather, it enables oligomerization and binding of the enzyme to the parasite surface (Schenkman *et al.*, 1994).

In this study we use NMR spectroscopy to investigate the mechanism of the sialidase activity of a recombinant *T.cruzi trans*-sialidase (*T.cruzi* rTS). We show that the initial product of hydrolysis of a sialyl donor catalyzed by rTS is the α-anomer of sialic acid, and that the reaction can be partially inhibited by Neu5Ac2en; we also investigate the effect of a nucleophile other than water on the hydrolysis reaction. On the basis of our results we suggest a mechanism for the sialidase activity of *T.cruzi* TS.

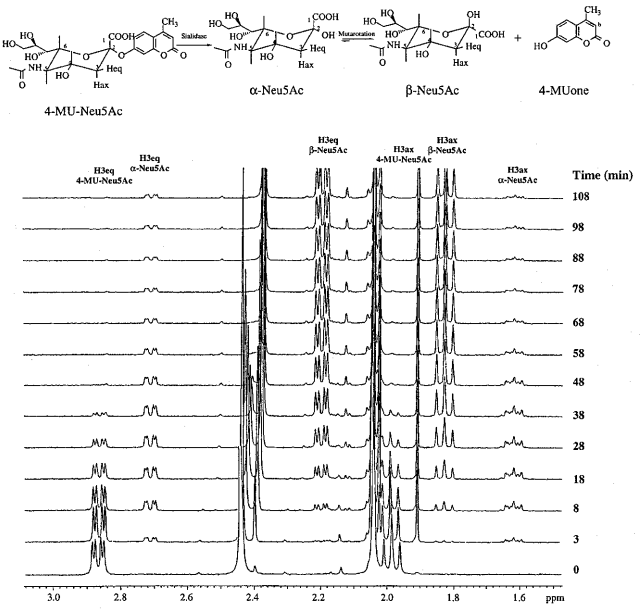


Fig. 1. Reaction scheme and time course of hydrolysis of 4-MU-Neu5Ac (10 mM) by *T.cruzi* rTS (1 U) in deuterated PBS, pH 5.8 at 37ºC, monitored by 500 MHz ^{1}H NMR spectroscopy. The times at which spectral data were acquired refer to the addition of *T.cruzi* rTS as t = 0.

Results

Purification of T.cruzi *rTS*

A recombinant analog of *T.cruzi* TS that has a six-tandem histidine N-terminal tail was purified from *E.coli* lysates using iminodiacetic metal affinity chromatography as described by Buschiazzo *et al.* (1996), followed by sequential ion exchange chromatography on Mono Q and Mono S. The final yield of recombinant enzyme was 1 mg of protein per liter of bacterial culture. The purified *T.cruzi* rTS migrated on a 10% polyacrylamide denaturing gel as a diffuse band of 90 kDa, exhibited kinetic properties similar to those of the native enzyme (Scudder *et al.*, 1993; Ribeirao *et al.*, 1997) (results not shown) and was stable for extended periods of time at 4°C without loss of activity.

*Hydrolysis of 4-methyl-umbelliferyl-*N*-acetylneuraminic acid by* T.cruzi *rTS*

Figure 1 shows the progress of the hydrolysis of 4-methyl-umbelliferyl-*N*-acetylneuraminic acid (4-MU-Neu5Ac) catalyzed by *T.cruzi* rTS as monitored by ^{1}H NMR spectroscopy. The time course of the reaction was monitored by observing the emergence of the H3 resonance signals of free sialic acid. The "time-zero" spectrum shows the spectral region from 1.5 to 3.1 p.p.m. of 4-MU-Neu5Ac (10 mM) at 37°C in deuterated phosphate-buffered saline (PBS), pH 5.8, before addition of enzyme. The H3eq and H3ax signals of 4-MU-Neu5Ac are observed at 2.87 and 1.98 p.p.m., respectively, not obscured by other resonances. The remaining spectra show the course of the hydrolysis reaction after addition of 1 unit (U) *T.cruzi* rTS. At t = 3 min, in the first spectrum recorded after the start of the incubation, the H3eq and H3ax signals corresponding to the α-anomer of sialic acid are observed at 2.72 and 1.62 p.p.m., respectively. These signals increase in intensity as the reaction proceeds, with a concomitant decrease in the intensity of the H3 signals of the substrate. The H3 signals of the Neu5Ac α-anomer continually increase for about 28 min, after which they progressively decrease (Figure 1) as mutarotation takes place. The mutarotation starts immediately after liberation of α-Neu5Ac and at t = 3 min the H3eq and H3ax signals of β-Neu5Ac are visible, albeit barely, at 2.21 and 1.84 p.p.m., respectively (Figure 1). Interestingly, the H3ax signal of α-Neu5Ac appeared as a multiplet rather than a well-defined triplet (Figure 1). This phenomenon is due to the chemical shifts of H4 and H5 being about 3.80 and 3.82 p.p.m., respectively, with $J_{4,5}$ being 10.1 Hz (Rensch *et al.*, 1983). With H4 and H5 nearly coinciding, Δδ between H4 and H5 is of the same magnitude as $J_{4,5}$, causing H3ax to experience a virtual coupling to H5. That virtual coupling, in concert with the scalar couplings to H3eq and H4, leads to the multiplet pattern of the H3ax resonance.

The hydrolysis of the substrate was found to be complete after 58 min. As shown in Figure 2, mutarotation of α-Neu5Ac continues for an additional 40 min until final equilibrium values of 92% β and 8% α sialic acid are reached, as determined by integration of the H3 signals. The rate constants for the Neu5Ac mutarotation were calculated. Under the conditions employed (pH 5.8 and 37°C) the k_f (α→β) and k_b (β→α) were found to be 6.27×10^{-4} s^{-1} and 6.43×10^{-5} s^{-1}, respectively.

Hydrolysis of α(2–3)-sialyllactose by T.cruzi *rTS*

The time course of the rTS-catalyzed hydrolysis of SL3 was monitored by observing the emergence of the resonance signal (at 4.45 p.p.m.) of the Gal H1 proton of the released lactose and

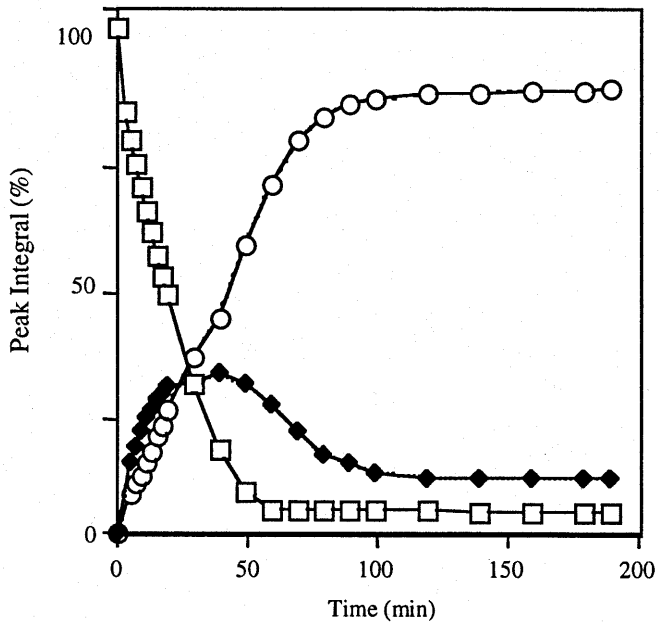


Fig. 2. Kinetics of the hydrolysis of 4-MU-Neu5Ac (10 mM) by *T.cruzi* rTS (1 U) in deuterated PBS, pH 5.8 at 37ºC. The reaction was monitored by 500 MHz ^{1}H NMR spectroscopy. Shown are the normalized peak area integration values for H3eq of 4-MU-Neu5Ac (open squares), α-Neu5Ac (solid diamonds), and β-Neu5Ac (open circles).

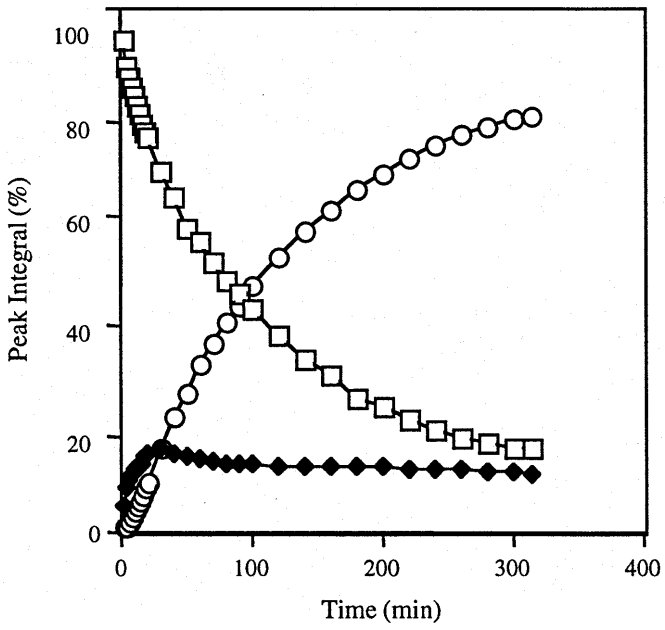


Fig. 3. Kinetics of the hydrolysis of SL3 (10 mM) by *T.cruzi* rTS (1 U) in deuterated PBS, pH 5.8 at 37°C, monitored by 500 MHz ^{1}H NMR spectroscopy. Shown are the normalized peak area integration values for the signals arising from Gal H1 in SL3 (squares); H3eq of β-Neu5Ac (diamonds) and H3ax of α-Neu5Ac (circles).

the concomitant decrease of the substrate Gal H1 resonance (at 4.53 p.p.m.). Hydrolysis of SL3 by *T.cruzi* rTS followed a course similar to that observed for 4-MU-Neu5Ac except that the reaction was significantly slower. Figure 3 shows the progress of the hydrolysis of SL3 (10 mM) in the presence of *T.cruzi* rTS (1 U) at 37°C in deuterated PBS, pH 5.8. The signals for H3eq and H3ax of α-Neu5Ac were visible after the first 3 min of the reaction, at 2.72 and 1.62 p.p.m., respectively. The signal for H3eq of the β-anomer was first detected after about 10 min of incubation. The product signals increase in intensity as the reaction proceeds, with a concomitant decrease in the signal intensity of protons from the substrate. Since SL3 acts as a donor in the TS reaction, some of the released lactose is resialylated in a *trans*-sialidase catalyzed side reaction. An equilibrium between acceptor and donors was established after about 5 h of incubation.

Competition of 4-MU-Neu5Ac and α(2–3)-sialyllactose hydrolysis by T.cruzi *rTS*

4-MU-Neu5Ac (5 mM) and SL3 (5 mM) together were incubated with 1.2 U *T.cruzi* rTS in deuterated PBS, pH 7.6 at 37°C and ^{1}H NMR spectra were recorded every 2 min. Monitoring the 4-methyl-umbelliferone (4-MUone) Hb signal (for definition, see inset in Figure 1) at 6.09 p.p.m. revealed that the enzymatic hydrolysis of 4-MU-Neu5Ac goes to completion in about 60 min. In contrast, the doublet corresponding to Gal H1 of SL3 at 4.53 p.p.m. only disappears after 180 min. Figure 4 illustrates that in a competition reaction 4-MU-Neu5Ac is a better substrate for the *T.cruzi* rTS-catalyzed hydrolysis reaction than SL3. The rate of hydrolysis of 4-MU-Neu5Ac was found to be 90 nmol·min^{-1} while the rate of hydrolysis of SL3 was 25 nmol·min^{-1}.

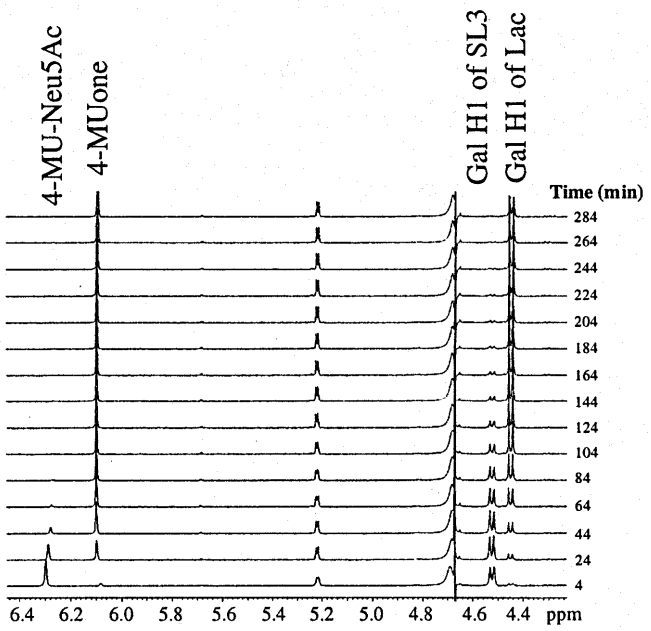


Fig. 4. Competition between the hydrolysis of SL3 (5 mM) and 4-MU-Neu5Ac (5 mM) by *T.cruzi* rTS (1.2 U) in deuterated PBS, pH 7.6 at 37°C, monitored by 500 MHz ^{1}H NMR spectroscopy. Spectral data were acquired at the times indicated on the spectra.

Effect of Neu5Ac2en on the sialidase activity of T.cruzi *rTS*

The hydrolytic activity of *T.cruzi* rTS toward 4-MU-Neu5Ac (1 mM) was determined in the absence and the presence of

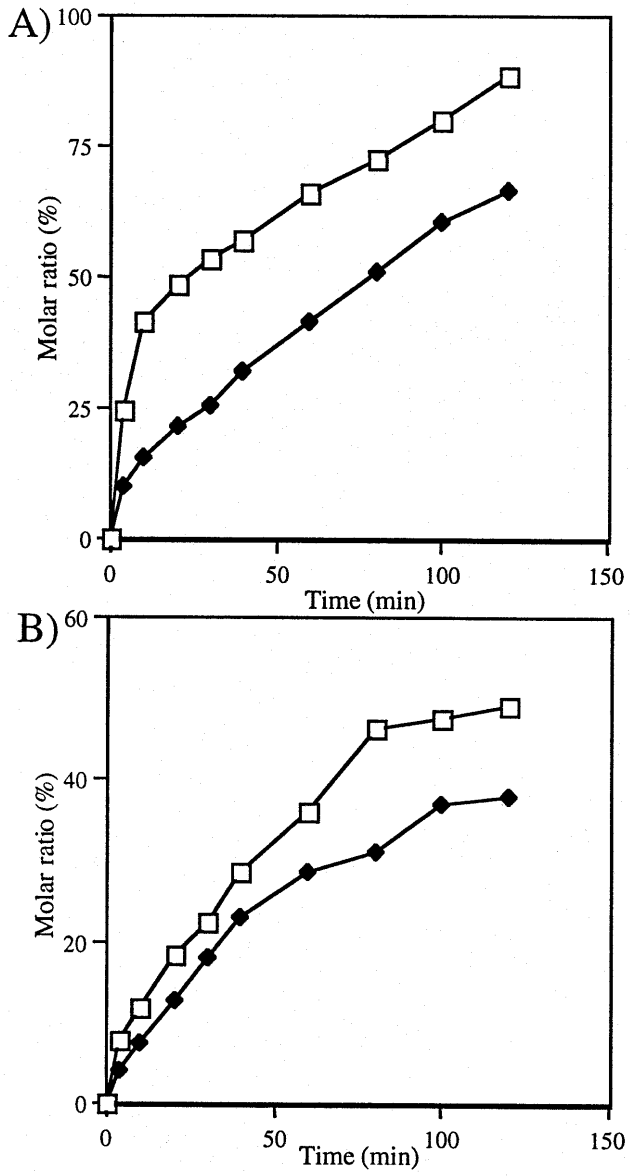


Fig. 5. Effect of Neu5Ac2en on the kinetics of the hydrolysis of (**A**) 4-MU-Neu5Ac (1 mM) and (**B**) SL3 (1 mM), by *T.cruzi* rTS (0.2 U) in deuterated PBS, pH 7.6 at 37°C. The reactions were monitored by 500 MHz ^{1}H NMR spectroscopy in the absence (squares) and presence (diamonds) of Neu5Ac2en (10 mM). Shown are the normalized peak area integration values for Gal H1 of SL3 and Hb of 4-MUone (see inset in **Figure 1**).

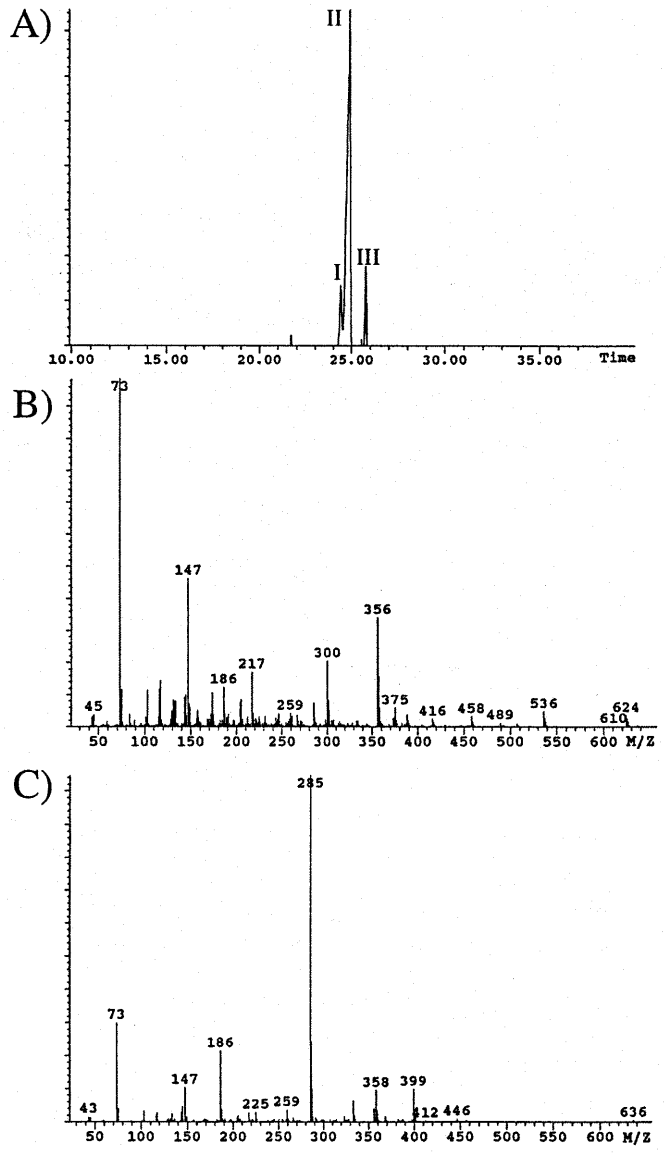


Fig. 6. (**A**) Reconstructed *m/z* 285 fragment ion gas chromatogram of the per-*O*-trimethylsilyl derivatives of the products of incubation of SL3 (1 mM) with *T.cruzi* rTS (10 U), pH 5.8 at 37°C for 48 h. The peaks are attributed to α-Neu5Ac (I), β-Neu5Ac (II), and Neu5Ac2en (III). (**B**) EI-mass spectrum of per-*O*-TMS I and II; (**C**) EI-mass spectrum of per-*O*-TMS III.

Neu5Ac2en (10 mM). The same experiment was carried out with SL3 (1 mM) as substrate. As shown in Figure 5, Neu5Ac2en partially inhibited the sialidase activity of rTS under the conditions described. The hydrolysis reactions of 4-MU-Neu5Ac and SL3 by rTS were followed for 120 min (Figure 5A,B). The relative intensities of the 4-MUone Hb signal at 6.09 p.p.m. and the SL3 Gal H1 signal at 4.53 p.p.m. each were compared in the spectra recorded in the absence and in the presence of Neu5Ac2en. It was found that Neu5Ac2en reduced the rTS sialidase activity by 25% and 23%, respectively (Figure 5A,B).

Production of Neu5Ac2en during T.cruzi *rTS-catalyzed hydrolysis of α(2–3)-sialyllactose*

We investigated the possibility of production of Neu5Ac2en during *T.cruzi* rTS-catalyzed hydrolysis of SL3 first by NMR spectroscopy. SL3 (10 mM) was incubated with *T.cruzi* rTS (1 U) in deuterated PBS, pH 5.8 at 37°C. After 48 h, a trace of Neu5Ac2en was detected, as indicated by the appearance of the characteristic H3 signal at 5.805 p.p.m. and by the presence of the signal at 2.063 p.p.m. attributed to the 5Ac protons of Neu5Ac2en (result not shown) (Vliegenthart *et al.*, 1982; Reuter and Schauer, 1994).

In an independent experiment the production of Neu5Ac2en during hydrolysis of SL3 by rTS was verified by GC/MS. The EI-mass spectrum of the per-*O*-trimethylsilylated (per-*O*-TMS) derivative of Neu5Ac2en is known to be characterized by a prominent fragment at *m/z* 285 (Burmeister *et al.*, 1993; Reuter and Schauer, 1994; Schauer and Kamerling, 1997). Upon electron impact, per-*O*-TMS-Neu5Ac also produces a fragment ion at *m/z* 285, however less abundant than that of per-*O*-TMS-Neu5Ac2en. Moreover, the literature suggests that per-*O*-TMS-Neu5Ac has a shorter retention time on GC than per-*O*-TMS-Neu5Ac2en. Figure 6 shows the reconstructed ion chromatogram (i.e., the abundance of fragment *m/z* 285 as a function of time) of the TMS-derivatized mixture resulting from the incubation of SL3 (1 mM) with *T.cruzi* rTS (10 U) for 48 h at 37°C, pH 5.8. Three peaks were observed. The first two, at 24.52 and 24.69 min, correspond to the α and β Neu5Ac anomers, respectively. The third peak, appearing at 25.75 min, corresponds to Neu5Ac2en. These assignments were verified by measuring the GC retention times of the per-*O*-TMS-derivatives of authentic Neu5Ac and Neu5Ac2en standards in separate experiments. In a nonenzymatic control experiment, no GC peaks producing a fragment ion at *m/z* 285 were observed.

Effect of methanol on the hydrolysis of 4-MU-Neu5Ac by T.cruzi *rTS*

In order to study the *T.cruzi* rTS-catalyzed hydrolysis in the presence of a nucleophile other than water, the effect of addition of methanol to an aqueous solution of 4-MU-Neu5Ac on its enzymatic hydrolysis was examined by ^{1}H NMR spectroscopy. In the presence of methanol, the products of 4-MU-Neu5Ac hydrolysis were identified as 4-MUone, α-Neu5Ac, and β-Neu5Ac, and α-methyl sialoside (α-Neu5Ac2Me). An increase in methanol concentration (from 0 to 20%) did not affect the relative rate of release of 4-MUone but increased the formation of α-Neu5Ac2Me with concomitant decrease of the amount of free β-Neu5Ac produced. Figure 7 shows the ^{1}H NMR spectra of the products of hydrolysis of 4-MU-Neu5Ac in the absence and in the presence of methanol (20%, v/v). The H3ax signal of α-Neu5Ac2Me was observed at 1.62 p.p.m. as a well-defined triplet, and the H3eq signal of the glycoside was detected at 2.73 p.p.m., in accordance with Vliegenthart *et al.* (1982). No β-Neu5Ac2Me was produced, by virtue of the absence of H3ax and H3eq resonances at 1.65 and 2.34 p.p.m., respectively (Vliegenthart *et al.*, 1982).

Discussion

NMR spectroscopy is a powerful tool for studying the kinetics of enzymatic reactions, because levels of both substrates and products can be simultaneously measured without perturbing the system. Moreover, the stereoselectivity of the action of sialidases can be monitored by ^{1}H NMR spectroscopy, as the technique is eminently suited for determination of the anomeric configuration of the released sialic acid (Friebolin *et al.*, 1981a), provided that the H3ax and H3eq resonances characteristic of the Neu5Ac α and β anomers are resolved from substrate resonances. Those conditions were satisfied with 4-MU-Neu5Ac and SL3 as substrates. However, at equilibrium

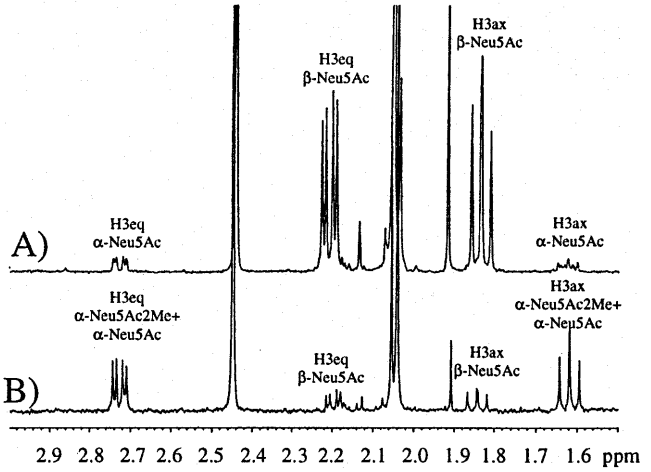


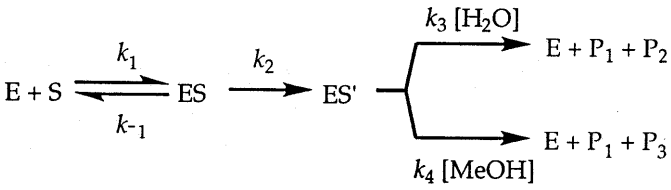
Fig. 7. (**A**) 500 MHz ^{1}H NMR spectrum of the products of hydrolysis of 4-MU-Neu5Ac (1 mM) by *T.cruzi* rTS (0.2 U) in 10 mM deuterated PBS, pH 7.6 at 37°C in aqueous (D_2O) solution; (**B**) as in (**A**), but in a D_2O:CD_3OD solution (4:1, v/v). Please note that the H3ax and H3eq signals of α-Neu5Ac virtually coincide with their counterparts of α-Neu5Ac2Me.

in aqueous solution the β-anomer of free sialic acid is known to be the predominant isomer. Thus, α→β mutarotation could hamper observation of hydrolytic release of α-Neu5Ac as initial product from the substrate. However, the rate of α→β mutarotation is known to be pH-dependent; it was established to be at a minimum around pH 5.4 (Friebolin *et al.*, 1981b). Therefore, we chose a pH value of 5.8 for most of the rTS-catalyzed hydrolysis experiments in this study, a value close to the pH that gives the best opportunity to observe initial release of the α-anomer of Neu5Ac. Furthermore, the amount of enzyme added was chosen such that substrate hydrolysis was complete before equilibrium between the anomers was established. Under these conditions we have shown that the α-anomer of sialic acid is released from 4-MU-Neu5Ac and SL3 by *T.cruzi* rTS. Thus the stereoselectivity of the sialidase activity of *T.cruzi* TS is identical to that of bacterial, viral, and mammalian sialidases (Friebolin *et al.*, 1981a; Kao *et al.*, 1997).

The hydrolysis of SL3 by *T.cruzi* rTS was investigated by observing the resonance signal arising from the Gal H1 proton of the liberated lactose at 4.45 p.p.m., which is distinct from the Gal H1 signal of the substrate at 4.53 p.p.m. Since the aromatic proton signals of 4-MU-Neu5Ac are resolved from those of the product 4-MUone and are found in a region of the spectrum that is free of glycosyl proton signals, we were able to investigate the competition for rTS-catalyzed hydrolysis between 4-MU-Neu5Ac and SL3. Our experiment showed that 4-MU-Neu5Ac is a better substrate for *T.cruzi* rTS than SL3, a result in agreement with that reported by Ribeirao *et al.* (1997). Similar substrate specificities have been reported for *T.rangeli* sialidase (Reuter *et al.*, 1987), *Arthrobacter sialophilus* sialidase (Kessler *et al.*, 1982) and for a KDN sialidase from *Sphingobacterium multivorum* (Terada *et al.*, 1997). These results suggest that the cleavage rate is influenced by the nonsialic acid part of the molecules. The observed preferential cleavage of 4-MU-Neu5Ac over SL3 may be attributed to the fact that the 4-methyl-umbelliferyl aglycone is a better leaving group than the carbohydrates of natural substrates. Furthermore, the electronic characteristics of the 4-methyl-umbelliferyl

moiety could permit a better stabilization of the positive charge possibly formed in the transition state (Tiralongo *et al.*, 1995; Terada *et al.*, 1997).

The hydrolysis of 4-MU-Neu5Ac in the presence of methanol has been documented for other sialidases (Kessler *et al.*, 1982; Bouwstra *et al.*, 1987). Our data indicate that the amount of free Neu5Ac produced decreases with increasing methanol concentration, due to the simultaneous formation of α-Neu5Ac2Me. This means that nucleophiles other than water can attack the enzyme-substrate complex, which leads us to propose the following kinetic scheme for this reaction:



where E is the free enzyme, S is the substrate (4-MU-Neu5Ac), ES is the enzyme-substrate complex, ES' is the transition state, P_1 is 4-MUone, P_2 is Neu5Ac, and P_3 is Neu5Ac2Me. Since methanol has no effect on the release of 4-MUone, k_2 must be smaller than k_3 and k_4. Consequently, $K_{cat,P1} = k_2$, $K_s = K_{m\,(app)}$ [where K_{cat} is the catalytic rate constant, K_m the Michaelis constant, and K_s the dissociation constant of the ES complex] and the release of 4-MUone is the rate-determining step in the solvolysis of 4-MU-Neu5Ac. These results suggest a reaction that involves an oxocarbonium ion as key intermediate formed in a slow step followed by a fast attack by the nucleophile (Carey and Sundberg, 1990).

X-Ray crystallographic and mechanistic studies of influenza virus sialidase suggest a reaction mechanism involving a sialosyl cation transition-state complex (Figure 8) (Chong *et al.*, 1992; Burmeister *et al.*, 1993; Janakiraman *et al.*, 1994). During this reaction, planarization of the sialic acid around the ring oxygen, C1, C2, and C3, in the active center of the sialidase, induces the release of the ketoside partner of Neu5Ac along with the glycosidic oxygen at C2. This release generates a sialosyl cation intermediate, which is rapidly attacked by nucleophiles such as water or methanol. When the sialosyl cation is attacked from beneath the plane formed, the product is the α-anomer, which is consistent with our observations that free α-Neu5Ac is initially formed in the presence of water as the only acceptor, and α-Neu5Ac2Me is formed in the presence of methanol.

While the exact catalytic mechanism of the sialidase activity of *T.cruzi trans*-sialidase cannot be deduced from the data obtained in this study, our results with respect to the role of Neu5Ac2en may provide additional insight in the matter. Similar to influenza virus sialidase, but different from bacterial sialidases, *T.cruzi trans*-sialidase catalyzes hydrolysis of SL3 with the production of both α-Neu5Ac and a tiny amount of Neu5Ac2en. The finding that Neu5Ac2en is a by-product of the sialidase activity of *T.cruzi* recombinant *trans*-sialidase is remarkable. The latter compound is a transition-state analog of the oxocarbonium ion intermediate proposed to be formed during sialoside hydrolysis by viral sialidases. It has been speculated (Burmeister *et al.*, 1993) that Neu5Ac2en is produced by proton elimination at C3 of the oxocarbonium ion intermediate.

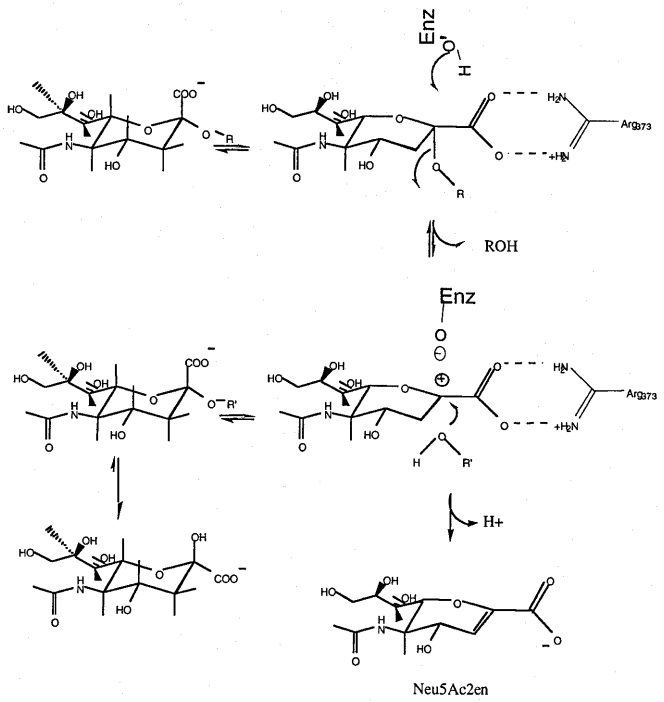


Fig. 8. Proposed reaction mechanism for the sialidase activity of *T.cruzi* rTS.

Neu5Ac2en was found to be only a weak inhibitor for the sialoside hydrolysis reactions catalyzed by rTS. Its inhibiting activity was in the range of 10^{-2} M; this value differs from the inhibiting effect of Neu5Ac2en on bacterial sialidases by several orders of magnitude. As proposed in Figure 8, catalytic hydrolysis by *T.cruzi* rTS potentially leads to the formation of an oxocarbonium ion intermediate positively charged at C2. This implies that the Neu5Ac C2 geometry becomes planar during the course of the reaction. Therefore, in the putative transition state during sialoside hydrolysis catalyzed by *T.cruzi* rTS, the ketoside structure becomes similar to that of Neu5Ac2en. Furthermore, gene sequencing analysis has shown that *T.cruzi* TS and *Salmonella typhimurium* sialidase share the same conserved amino acid residues at the catalytic site that are implicated in the ring distortion of Neu5Ac from $^2C_5$ to the boat conformation of the substrate during the sialidase catalyzed reaction (Cremona *et al.*, 1995, Schauer and Kamerling, 1997).

The apparent conservation of the sialidase mechanism in viral, bacterial, trypanosomal, and mammalian enzymes is consistent with a common origin and supports the hypothesis that the occurrence of related enzymes in phylogenetically distant organisms is the consequence of extensive horizontal gene transfer (Roggentin *et al.*, 1993; Schauer *et al.*, 1995)

Our studies suggest that transition-state analogs may provide a strategy for the rational design of *trans*-sialidase inhibitors which could be highly effective agents for the chemotherapy of acute phase *T.cruzi* infections. Studies are currently in progress to further understand the catalytic mechanism of *T.cruzi trans*-sialidase.

Materials and methods

Materials

SL3 was kindly supplied by Dr. G.Strecker, Laboratoire de Chimie Biologique, Université des Sciences et Technologies de Lille, France. 4-MU-Neu5Ac was purchased from Sigma (St. Louis, MO), Neu5Ac was from Kantoishi Pharmaceuticals (Tokyo, Japan), and Neu5Ac2en was from Calbiochem (La Jolla, CA). D_2O (99.996% D) and CD_3OD (99.9% D) were purchased from Cambridge Isotope Laboratories (Andover, MA). Chromatographic media (Mono S, Mono Q and a HiTrap chelating column) were from Pharmacia Biotech (Uppsala, Sweden). All other reagents were of the highest available quality.

Expression and purification of T.cruzi *rTS*

T.cruzi rTS was obtained from *E.coli* MC 1061 electrotransformed with a plasmid containing TS inserts (TSREP.C in pTrcHisA) (Buschiazzo *et al.*, 1996). The *T.cruzi trans*-sialidase plasmid was a gift from Dr. A.C.C. Frasch, Instituto de Investigaciones Biotecnologicas, UNSAM, San Martin, Provincia de Buenos Aires, Argentina. Bacteria were grown in a medium (TB) (Tartof and Hobbs, 1987), containing (per l) 12 g bacto-tryptone, 24 g bacto-yeast extract, and 4 ml glycerol supplemented with 100 μg/ml ampicillin at 28°C. When the culture reached an optical density of 1.5 at 600 nm, 30 mg/l of isopropyl-β-D-thiogalactoside (IPTG) was added and incubation continued at 28°C with shaking (110 r.p.m.) overnight. Bacteria were lysed at 4°C in 20 mM Tris–HCl containing 2 mg/ml lysozyme, 2% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 0.1 μM iodoacetamide. Deoxyribonuclease I (0.1 mg/ml) was added in order to reduce the viscosity. The suspension was centrifuged at 21,000 × *g* for 30 min and the pellet was discarded. The *T.cruzi* rTS containing a six-tandem histidine tag was purified using Ni^{2+} chelating chromatography on a HiTrap column equilibrated with Tris–HCl 20 mM (pH 7.2) and NaCl 0.5 M, and eluted with an imidazol gradient (0–1 M) (Buschiazzo *et al.*, 1996). Subsequently, the eluate was dialyzed against Tris–HCl 20 mM and applied to Mono Q (HR 16/10) and Mono S (HR 5/5) columns, respectively. The enzyme was eluted with a linear gradient of NaCl (0–1 M). The homogeneity of the enzyme was evaluated by 10% SDS–polyacrylamide gel electrophoresis. The *T.cruzi* rTS was stored in 20 mM Tris–HCl buffer, pH 7.4, at 4°C, until use.

T.cruzi *rTS activity measurements*

The *T.cruzi* rTS *trans*-sialidase activity was assayed by incubating the purified enzyme in 200 μl of cacodylate buffer (5 mM, pH 7.0) in the presence of 0.25 μmol SL3 and [D-glucose-1–^{14}C]-lactose 0.25 μmol (40,000 c.p.m.) as described previously (Scudder *et al.*, 1993). After incubation at 37°C for 30 min, the reaction mixture was diluted with 1 ml of water and applied to a column containing 1 ml of Dowex 2X8 (acetate form) equilibrated with water. The [D-glucose-1–^{14}C]-lactose was eluted by washing the column with 3 ml of distilled water. The sialylated [D-glucose-1–^{14}C]-lactose was eluted with 9 ml of 0.8 M ammonium acetate, and the radioactivity was determined by liquid scintillation counting on a Beckman LS 6500 instrument.

The *T.cruzi* rTS sialidase activity was determined by measuring the fluorescence of 4-MUone released by the hydrolysis of 0.1 mM 4-MU-Neu5Ac in a 200 μl volume containing 5 mM cacodylate buffer, pH 7.0 at 37°C for 30 min (Potier *et al.*, 1979). The reaction was stopped with 1.8 ml of distilled water and the fluorescence of 4-MUone was measured in a Cyto Fluor II instrument.

Preparation of samples for NMR spectroscopy

Saccharides (4-MU-Neu5Ac, SL3, and Neu5Ac2en) were lyophilized from D_2O (99.996% D) three times and dissolved in deuterated PBS, pH 5.8 or 7.6 (these values are not corrected for isotope effects). A *T.cruzi* rTS solution in 20 mM Tris–HCl was exchanged several times with deuterated PBS in a concentrator with a cut-off range of 30 kDa. The enzyme concentration was adjusted based on the sialidase activity of *T.cruzi* rTS (1 U is defined as the amount of enzyme required to catalyze the hydrolysis of substrate at 1 μmol/min at 37°C).

NMR spectroscopy

500 MHz ^{1}H NMR spectroscopy was conducted on a Varian Unity Inova 500 spectrometer, at 37°C in sample volumes of 0.6 or 0.7 ml in 5 mm tubes (Wilmad 528-PP or better). A Varian 5-mm PFG-ID probe was used for all experiments; data acquisition was controlled by a SUN Sparc5 computer running Varian's VNMR software (version 5.3B). Acetate was used as internal standard for chemical shift calibration; its chemical shift was set to 1.908 p.p.m. Prior to an enzyme-catalyzed reaction, a spectrum of the substrate was acquired as a "zero time" spectrum at 37°C in a total sample volume of 0.6 ml. Next, the sample was removed from the probe and the reaction was initiated by mixing 0.1 ml of *T.cruzi* rTS solution with the substrate at 37°C, and then the tube was swiftly placed back into the magnet. Hydrolysis reactions were monitored as a function of time. The first spectrum was taken within 2–3 min after the reaction had begun, and from then on spectra were acquired every 1–2 min over 3–5 h. The HDO signal was suppressed with a low-power transmitter pulse of 1.0 s during the relaxation delay.

The hydrolysis reactions of 4-MU-Neu5Ac (10 mM) and SL3 (10 mM) were performed in 10 mM deuterated PBS, pH 5.8, in the presence of *T.cruzi* rTS (1.0–1.2 U). The competition hydrolysis experiment between 4-MU-Neu5Ac (5 mM) and SL3 (5 mM) was performed at pH 7.6. Hydrolysis reactions in the presence of methanol (CD_3OD) and Neu5Ac2en (10 mM) were carried out with 4-MU-Neu5Ac (1 mM) and 0.2 U of *T.cruzi* rTS.

For each spectrum, 16 transients were acquired in 16K data points. No line broadening and no zero filling were applied before Fourier transformation. Proton peaks were integrated relative to the amount of free acetate in the sample and the values were plotted as a function of time of reaction using Cricket Graph (version 1.53) software on a Macintosh 5400/120 computer.

Mutarotation rate constants were determined using the peak integrals of the H3 signals of the α and β anomers of Neu5Ac after 60 min when the 4-MU-Neu5Ac signals had dropped below the detection limit. The points were fitted for a two-state kinetic-model (Noggle, 1988): $A = A_0 - \{(k_f \times A_0 - k_b \times B_0)/(k_f + k_b)\} \times \{1 - \exp[-(k_f + k_b)t]\}$ and $B = B_0 + \{(k_f \times A_0 - k_b \times B_0)/(k_f + k_b)\} \times \{1 - \exp[-(k_f + k_b)t]\}$, where A_0 and B_0 represent the

initial H3 peak integrals of α and β anomers at t = 60 min (the time at which substrate disappears, i.e., time-zero for the study of the mutarotation process), and k_f (α→β) and k_b (β→α) are the mutarotation rate constants (*f*, forward; *b*, back). The fitting was done using Jandel's Sigma Plot Scientific Graph System software (version 4.17) on a Macintosh 5400/120 computer, with k_f and k_b as adjustable parameters.

GC/MS analysis of the reaction product of incubation of α(2–3)-sialyllactose with T.cruzi *rTS*

SL3 (1 mM) was incubated with *T.cruzi* rTS (10 U) in PBS at pH 5.8 in a total volume of 0.5 ml for 48 h at 37°C. After incubation, the reaction mixture was dried under a stream of nitrogen gas and the resulting products were derivatized with 100 µl of Tri-Sil (Pierce, Rockford, IL) for 1 h at room temperature. The precipitate was removed by centrifugation, and 2 µl of the supernatant was immediately subjected to GC/MS analysis. GC of the TMS-derivatives was carried out with a Hewlett-Packard 5890 gas chromatograph, using a DB-5 fused silica column (30 m × 2.25 mm internal diameter) with helium as carrier gas. The column temperature was programmed from 50–200°C at 20°/min and 200–300°C at 2°/min. Electron impact (EI) mass spectra were recorded with a Hewlett-Packard 591 mass selective detector at an ionization energy of 70 eV. In order to optimize detection of Neu5Ac2en, the fragment ion at *m/z* 285 was selectively monitored throughout the GC elution.

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Abbreviations

ax, axial; D, deuterium; EI, electron impact; eq, equatorial; Fuc, fucose; Gal, galactose; Gal*p*, galactopyranose; GC, gas liquid chromatography; GlcNAc, *N*-acetylglucosamine; IPTG, isopropyl-β-D-thiogalactoside; J, scalar coupling constant; Lac, lactose; Me, methyl; MS, mass spectrometry; 4-MU-Neu5Ac, 4-methyl-umbelliferyl-*N*-acetylneuraminic acid; 4-MUone, 4-methyl-umbelliferone; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; Neu5Ac2Me, *N*-acetylneuraminic acid methylglycoside; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; rTS, recombinant *trans*-sialidase; SDS, sodium dodecyl sulfate; Sia, sialic acid; SL3, α(2–3)-sialyllactose; TB, terrific broth; TMS, trimethylsilyl; TS, *trans*-sialidase; UCSD, University of California San Diego; UFRJ, Universidade Federal do Rio de Janeiro.

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