Glycobiology vol. 20 no. 8 pp. 982–990, 2010
doi:10.1093/glycob/cwq056
Advance Access publication on April 7, 2010

Continuous nonradioactive method for screening trypanosomal *trans*-sialidase activity and its inhibitors

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Received on December 7, 2009; revised on March 31, 2010; accepted on April 1, 2010

***Trypanosoma cruzi*, the agent of American trypanosomiasis is unable to synthesize sialic acid (SA). Instead of using the corresponding nucleotide sugar as donor of the monosaccharide, the transfer occurs from α-2,3-linked SA in the host sialoglycoconjugates to terminal β-galactopyranosyl units of the parasite mucins. For that purpose, *T. cruzi* expresses a glycosylphosphatidylinositol-anchored *trans*-sialidase (TcTS) that is shed into the milieu, being detected in the blood during the acute phase of the infection. The essential role of TcTS in infection and the absence of a similar activity in mammals make this enzyme an attractive target for the development of alternative chemotherapies. However, there is no effective inhibitor toward this enzyme. In vitro, 3′-sialyllactose (SL) as donor and radioactive lactose as acceptor substrate are widely used to measure TcTS activity. The radioactive sialylated product is then isolated by anion exchange chromatography and measured. Here we describe a new nonradioactive assay using SL or fetuin as donor and benzyl β-D-Fuc-(1→6)-α-D-GlcNAc (1) as acceptor. Disaccharide 1 was easily synthesized by regioselective glycosylation of benzyl α-D-GlcNAc with tetra-*O*-benzoyl-D-fucose followed by debenzoylation. Compound 1 lacks the hydroxyl group at C-6 of the acceptor galactose and therefore is not a substrate for galactose oxidase. Our method relies on the specific quantification of terminal galactose produced by *trans*-sialylation from the donor to the 6-deoxy-galactose (D-Fuc) unit of 1 by a spectrophotometric galactose oxidase assay. This method may also discriminate sialidase and *trans*-sialylation activities by running the assay in the absence of acceptor 1.**

Keywords: galactose oxidase/inhibitors/*trans*-sialidase/*Trypanosoma cruzi*

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Introduction

Trypanosomatids are flagellate protozoa, some of them being causative agents of diseases such as *Trypanosoma cruzi* and *Trypanosoma brucei* producing the American (Chagas disease) and African (sleeping sickness and nagana) trypanosomiasis, respectively. Although different in many aspects at the biological and cellular levels, they are unable to synthesize sialic acids (SAs) even when this sugar is highly required to complete their life cycles. Parasites solve this gap by expressing enzymes known as *trans*-sialidases (TS) that are able to transfer sialyl residues among glycoconjugates. In the case of *T. brucei*, the SA incorporation through the TS into the glycosylphosphatidylinositol anchor of the procyclin surface protein cover is essential for parasite survival in the tsetse fly (Nagamune et al. 2004). *T. cruzi trans*-sialidase (TcTS) transfers α(2,3)-linked sialyl residues present in host sialoglycoconjugates to terminal β-galactopyranosyl units of mucins (Schenkman et al. 1991; Ferrero-Garcia et al. 1993; Frasch 2000; de Lederkremer and Agusti 2009) that are widely distributed on the parasite surface (Tomlinson et al. 1994; Pereira-Chioccola et al. 2000). Sialylated mucins are in turn involved in the invasion of mammalian host cells and in the protection against lysis by serum factors (Tomlinson et al. 1994; Pereira-Chioccola et al. 2000). The TcTS is anchored to the membrane by glycosylphosphatidylinositol (Agusti et al. 1997, 1998) and is shed to the medium, then it can be detected in the blood during the acute phase of the infection (Leguizamón, Campetella, González Cappa et al. 1994; Buscaglia et al. 1999; Alvarez et al. 2004) and may produce several abnormalities in the immune system (Leguizamón et al. 1999; Mucci et al. 2002, 2005, 2006; Risso et al. 2007). Animals that survive infection and chronic Chagas disease patients both elicit TcTS-neutralizing antibodies that inhibit the enzymatic activity and have been used on the detection of *T. cruzi* infections (Leguizamón, Campetella, González Cappa et al. 1994, Leguizamón, Campetella, Russomando et al. 1994; Blejer et al. 2008). The essential role of TcTS in the infection and Chagas disease pathogenesis together with the absence of a similar activity in mammals makes this enzyme an attractive target for the development of new chemotherapies.

A high throughput-screening test is required to search for these putative inhibitors. A radioactive method has been frequently used to assay TS activity and to detect TcTS-neutralizing antibodies (Schenkman et al. 1991; Leguizamón, Campetella, González Cappa et al. 1994; Leguizamón, Campetella, Russomando et al. 1994; Blejer et al. 2008). In this method, α-(2,3)sialyllactose (SL) is used as SA donor and radioactive lactose as acceptor. After SA transference, the sialylated radioactive product is captured with anionic resins

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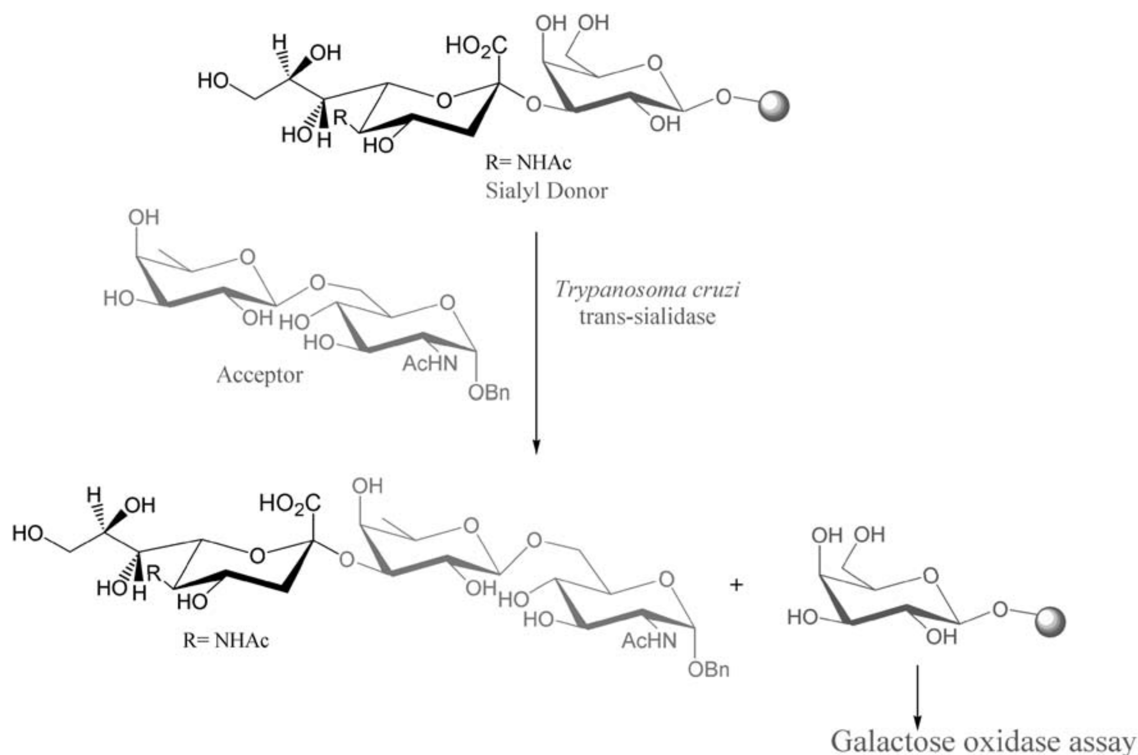

Scheme 1. Transfer of SA by TcTS from a sialyl donor to a D-Fuc*p* unit in the acceptor substrate. Quantification of the terminal galactose residues generated in the donor after the removal of the SA was performed by a coupled colorimetric/fluorimetric galactose oxidase test.

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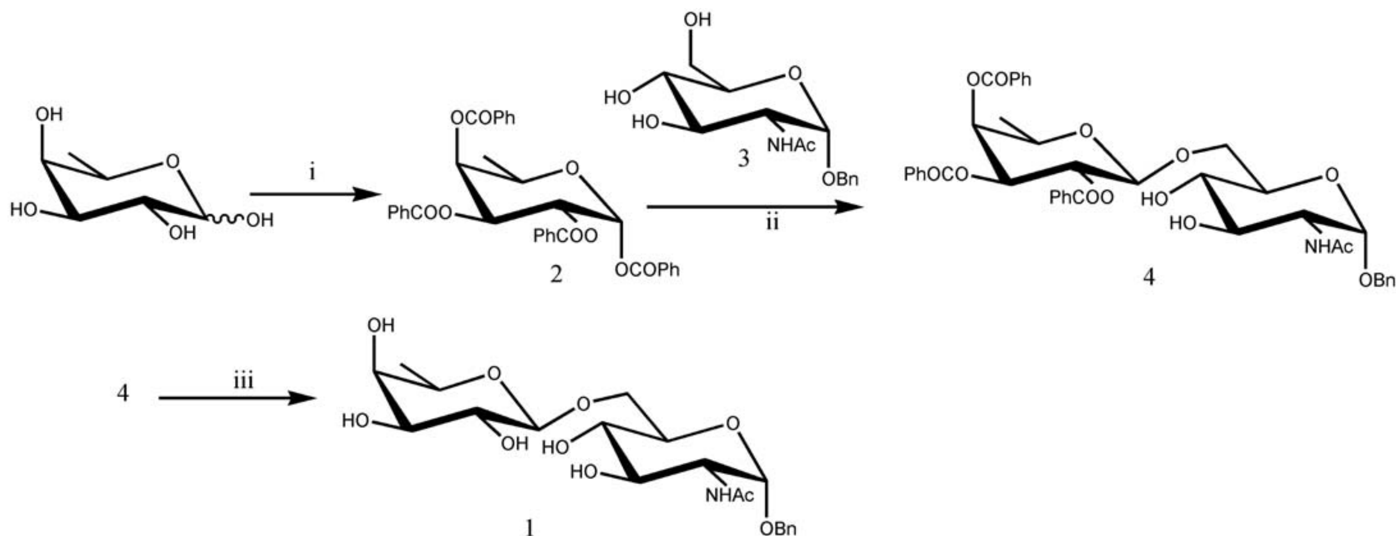
and quantified. Although very sensitive, this method generates radioactive waste, demands hands-on time and is difficult to escalate. On the other hand, several nonradioactive methods have been proposed for TS activity determination (Lee and Kim 2000; Agusti et al. 2004; Neres et al. 2006; Schrader and Schauer 2007). The available nonradioactive methods have different limitations. They continue being hands-on demanding techniques, and the continuous monitoring of the *trans*-sialylated product is not yet possible given that most of them require a stopping step. This, together with the fact that most of the products are also substrates of the enzyme and allow the reversible reaction, the accurate determination of the enzymatic activity and some kinetic properties is hampered. A major drawback is that the available nonradioactive methods are limited in terms of sensitivity. Therefore, the development of sensitive one-step and easy to escalate assays for monitoring TS activity is required to perform the screening of TS inhibitors and also for diagnostic purpose. Here we describe a new nonradioactive assay for TS activity that uses a SA donor, such as fetuin or SL, and an acceptor resistant to galactose oxidase activity such as a D-fucosylated sugar. Then, the *trans*-sialylation reaction is determined by a coupled colorimetric/fluorimetric galactose oxidase test, which allows the continuous quantification of the terminal galactosyl residues generated after the removal of the SA. The reaction used is shown in Scheme 1. Because the exposed galactose is oxidized by the galactose oxidase, the TS reaction proceeds only in one direction. The galactose oxidase assay is currently proposed as a tool for detection of galactose and galactosamine residues on biological samples (Kinoshita et al. 2000) and as a test for determination of neuraminidase from influenza virus and bacteria (Nayak and Reich 2004; Williamson et al. 2008). The method is based on the oxidation of the C-6 of galactose that generates H_2O_2. The amount of H_2O_2 is then measured by a highly sensitive method involving the use of horseradish peroxidase (HRP) and Amplex Red Reagent (10-acetyl-3,7-dihydroxyphenoxazine), rendering resorufin that is in turn quantified by absorbance or fluorescence emission determination (Mohanty et al. 1997).

Results

*Synthesis of benzyl β-D-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-α-D-glucopyranoside (**1**, Scheme 2)*

In order to avoid interference from the acceptor substrate for the galactose oxidase reaction, a 6-deoxy-galactose (D-Fuc) analog of the common disaccharides (lactose or *N*-acetyllactosamine) used for the TS reaction was necessary. Benzyl β-D-Fuc-(1→6)-α-D-GlcNAc (**1**) was chosen because the synthesis was easier than that for the lactosamine analog, and it was previously shown that β-Gal*p*-(1→6)GlcNAc, in particular the benzyl glycoside, was a good acceptor in the TcTS reaction (Agusti et al. 2007).

The starting synthons, tetra-*O*-benzoyl-α-D-fucopyranose (**2**) and benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**3**), were easily prepared from the free commercial sugars. Glycosylation of **3** with a limited amount of the fucose derivative **2**, promoted by tin(IV) chloride, afforded, selectively, the β-(1→6) linked disaccharide (Scheme 2). Analysis of the ^{1}H and ^{13}C NMR spectra confirmed the structure. As expected,



Scheme 2. Synthesis of benzyl β-D-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-α-D-glucopyranoside (**1**). Reagents and yield: (i) benzoyl chloride, pyridine, yield 75%; (ii) $SnCl_4$, CH_2Cl_2, yield 42%; (iii) NaOMe, MeOH, yield 94%.

the primary OH-6 of **3** was regioselectively glycosylated as shown by the downfield shift (6 ppm) of the C-6 resonance with respect to the same signal in benzyl α-D-GlcNAc. The signal at δ 102.1 ppm in the anomeric region of the ^{13}C NMR and at δ 4.85 ppm with J 7.8 Hz in the ^{1}H NMR spectrum indicated the β-configuration for the new glycosidic linkage.

Although the yield was only moderate (42%), the selective formation of **4** by glycosidation of the nonprotected benzyl glycoside **3**, followed by the quantitative deprotection reaction of the galactose unit are points in favor for this approach for the preparation of **1**.

Testing of the synthetic disaccharide as SA acceptor in the TS reaction

By HPAEC-PAD Analysis. Evaluation of disaccharide **1** as acceptor in the TS reaction was performed using SL as donor and recombinant TcTS (Buschiazzo et al. 1996). The reaction was followed by high pH anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) (Figure 1). Transfer of SA was fast and reached equilibrium in about 15 min. Sialylation was very effective, with 57% transfer of SA from SL to **1** (Figure 1B). Since lactose generated from sialyllactose is also present, this value indicates that both acceptors display similar efficiency. A K_M value of 0.13 mM was obtained for **1**. The minor peak at 8.0 min corresponds to SA and is already present in the sample of SL (Figure 1A). The activity as neuraminidase of the TcTS is low in the presence of an appropriate acceptor (Agusti et al. 2004). Transfer of SA to compound **1** was almost completely inhibited (Figure 1C) by previous incubation of the enzyme with a neutralizing antibody (Risso et al. 2007).

TS Activity Detection by a Colorimetric/Fluorometric Assay. Kinoshita et al. (2000) have introduced a test for the determination of galactose/*N*-acetylgalactosamine in biological samples. They employed a galactose oxidase method in combination with the detection of H_2O_2 by a highly sensitive fluorometric/colorimetric assay (Mohanty et al. 1997). The same principle was used in our work to detect the galactose residues exposed by the TS catalytic activity. In order to adapt this method for TS detection, we used fetuin or SL as SA donor and the novel disaccharide **1** as the acceptor considering that this disaccharide cannot be oxidized by galactose oxidase.

The optimal conditions for detection of TcTS activity were empirically settled (data not shown). The sensitivity of the assay was determined by measuring either the optical density or fluorescence emission (Figure 2A, B). The activity of less than 10 ng of TcTS could be determined. Taking into account the optimal temperatures for the enzymes involved, the colorimetric reaction (Figure 2A) was performed incubating first, TcTS with SL and **1** at room temperature (Riberao et al. 1997) and increasing the temperature to 37°C after the addition of galactose oxidase and HRP. The fluorometric method, more sensitive, allowed the determination in one step at room temperature (Figure 2B). On the other hand, the optical density remained constant when the amount of TcTS was higher than 0.3 μg. As expected, the radioactive method allowed the detection of even smaller amounts of TcTS than the novel colorimetric assay now described (Figure 2C). Nevertheless, the small amount of enzyme required together with the possibility to easily and fast assay multiple inhibitors in a multiwell array render this new method of high interest.

Time course of TS reaction and sialidase activity

The TS colorimetric reaction was allowed to proceed by adding all components, from time zero incubating the mixture at 37°C and evaluating the optical densities at different time points. The sialidase activity was also evaluated by performing the assay without any acceptor. As expected, the color generated by the sialidase activity was significantly lower than that measured for the transferase activity (Figure 3A). The kinetic curve for both activities showed good linearity along the time points tested (r=0.99 in both cases). A control assay by the radioactive method showed no inhibition of TS activity in the presence of Amplex Red Reagent, resorufin, H_2O_2 and

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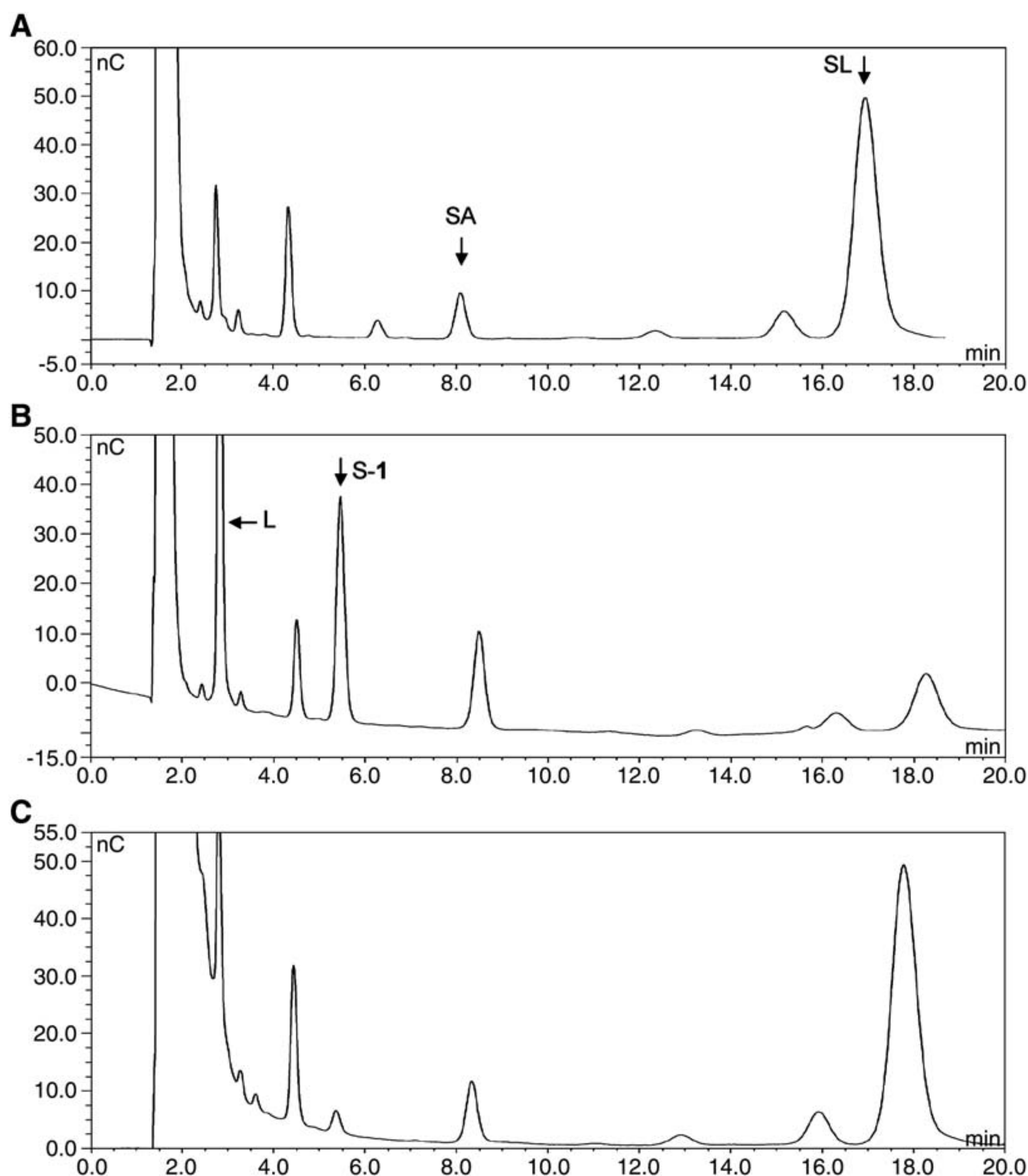

Fig. 1. Analysis of benzyl β-D-Fuc-(1→6)-α-D-GlcNAc (**1**) as acceptor substrate in the TcTS reaction. (**A**) Compound **1** and SL, without enzyme; (**B**) **1** was incubated with SL and TcTS for 15 min at 25°C and the reaction mixture analyzed by HPAEC-PAD; (**C**) the same as above but TcTS was preincubated with a neutralizing antibody. A CarboPac PA-100 ion exchange analytical column was eluted with 50 mM NaAcO in 100 mM NaOH at 1 mL/min. L, lactose; SA, sialic acid; SL, sialyllactose; S-**1**, sialylated disaccharide **1**.

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HRP (data not shown). According to the previous results, the fluorometric determination was performed at room temperature (Figure 3B). In order to evaluate the sialidase activity in the same experiment, the enzyme was incubated with all the reagents except for the acceptor **1** which was then added and incubation continued to evaluate the sialyl transferase activity. In the absence of a suitable sialyl unit acceptor such as **1**, the TcTS displayed a poor sialidase activity.

It is important to point out that these assays rendered measurable colorimetric/fluorometric data that allow continuously measuring the TS activity through time because no stopping step is required and the exposed galactose residues are oxidized by galactose oxidase being therefore not suitable for resialylation.

Detection of TS activity inhibition

To assay whether the test might be useful to determine TS inhibition, purified neutralizing antibodies from mouse (mAb 13G9) or from human patients (purified AbsNt) or high concentrations of DANA (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid) were included in the test. Inhibitors were allowed to react with TS for 15 min and then the substrates fetuin and **1** were incorporated to each well. The activity was measured by the adapted galactose oxidase method after incubation at 37°C. A significant decrease ($P < 0.01$) in the optical density was observed in comparison with the activity for TS in the absence of inhibitors (Figure 4). To evaluate if the decrease of the optical density could be due to interference in color development, two positive controls were included adding asialofetuin to each well contain-

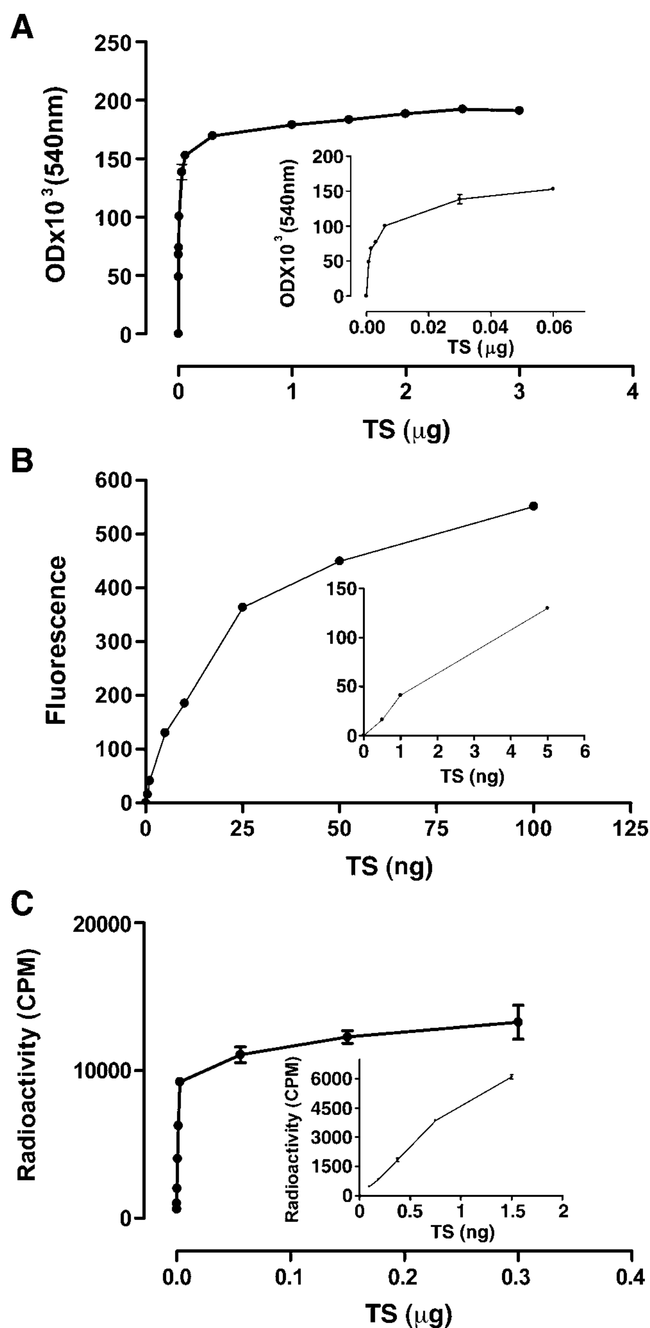

Fig. 2. Comparison of sensitivities for the nonradioactive and radioactive assays. (**A**) Colorimetric assay: different amounts of TcTS were incubated with fetuin and **1** at room temperature for 30 min. Then, galactose oxidase, HRP and Amplex Red Reagent were added and incubation continued at 37°C. The absorbance at 540 nm was measured for each TcTS concentration. An expansion of the more diluted area is shown in the inset. (**B**) Fluorometric assay: different amounts of TcTS were mixed with SL, disaccharide **1**, galactose oxidase, HRP and Amplex Red Reagent. Fluorescence emission at 590 nm was read after 30 min of incubation at room temperature. Fluorescence readings are expressed as arbitrary units. Inset: expansion of values obtained at the lower TcTS amounts tested. (**C**) Radioactive assay: different amounts of TcTS were incubated with SL and [D-glucose-1-^{14}C]-lactose. After 30 min of incubation at room temperature, a slurry of quaternary aminoethyl-Sephadex was added to bind the ^{14}C-sialyllactose product, washed with water and the counts per minute retained were quantified. An expansion of the more diluted area is shown in the inset.

ing TS inhibitors. Galactose residues from asialofetuin were quickly oxidized by galactose oxidase, increasing the optical density without any interference from the inhibitors tested. In addition, *Clostridium perfringes* neuraminidase was also included as a positive control (C+). Unfortunately, the straightforward detection of neutralizing antibodies in human serum samples was hampered by a violet interference developed during the reaction that could not be prevented by preincubation of sera at 56°C for 30 min (data not shown).

Discussion

Trans-sialidases are crucial in the biology of African and American pathogenic trypanosomes. Their notable transferase activity is of interest also for biotechnology applications. Because it is very effective and specific for the sialylation of oligosaccharides, the TS from *T. cruzi* has been used with preparative purposes (de Lederkremer and Agusti 2009). Therefore, the development of a one-step assay for TS activity screening/measurement is of great interest to perform high throughput screening of substrates and inhibitors. Due to its relevance in Chagas disease diagnosis, the possible use of the TS inhibition assay (TIA) (Leguizamón et al. 1997; Blejer et al. 2008), where neutralizing antibodies present in mammalian sera are detected, also requires a user-friendly and nonradioactive detection system.

In the last years other methods which avoid the use of radioactive substrates have been described. For instance, HPAEC has been used to define TS substrate specificity and for screening of inhibitors (Agusti et al. 2004). Moreover, two fluorimetric assays have been reported based on the use of a 4-methylumbelliferyl glycoside. Schrader et al. (2003) used 4-methylumbelliferyl-β-D-galactoside as SA acceptor and SL as donor and adapted this method to a 96-well-plate fluorescence test. In this test, a further step of acid hydrolysis to release and measure 4-methyl umbelliferone is necessary for better sensitivity. Neres et al. (2006) have used 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid as SA donor; this method was described for the screening of inhibitors of TS from *T. cruzi* (Neres et al. 2009); however, it does not discriminate sialidase from TS activities. Also, a spectrophotometric assay has been described by Lee et al. (2000) that used *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as SA acceptor and the remnant nonsialylated ONPG is detected by a colorimetric assay.

The test described in the present work avoids radioactively labeled compounds, requires small amounts of substrate and enzyme, uses equipment available for routine test in all diagnosis laboratories, requires low manipulation without washing or stopping steps and is easy to escalate since multiwell plates can be used. From the biochemist point of view, it allows the continuous quantification of the product, may discriminate between sialidase and TS activities and, because the reaction is unidirectional, it allows the formal determination of enzymatic parameters. The D-fucose disaccharide substrate is easily prepared from the monosaccharide constituents. The benzyl glycoside of the disaccharide is used instead of the free sugar because it is as good as acceptor, is more stable and is obtained in a previous step in the synthesis of the free sugar. The replacement of D-galactose by D-fucose as the acceptor unit does not impair the TS reaction as shown by HPAEC-PAD

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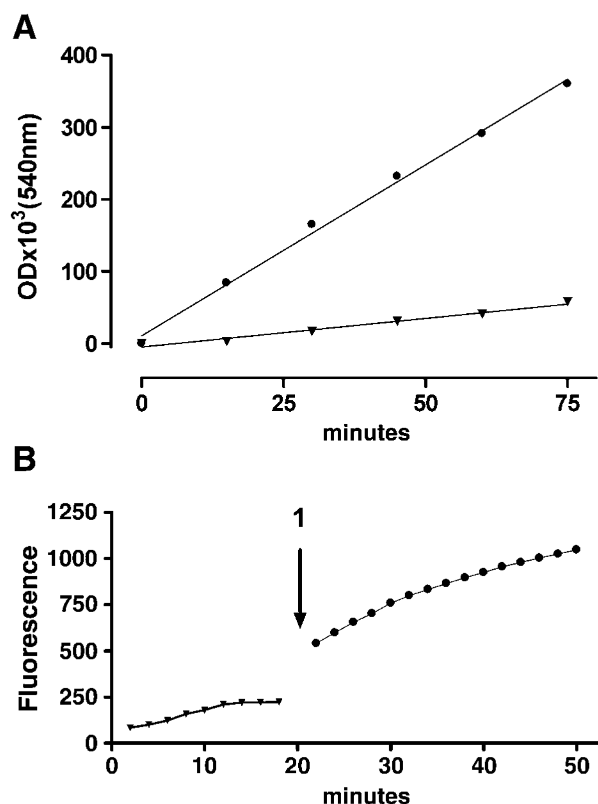

Fig. 3. Assay of sialidase and *trans*-sialidase activities. TcTS was assayed in the presence (dots) or absence (triangles) of disaccharide **1**. (**A**) TcTS (0.1 µg) was incubated at 37°C with SL as sialyl donor, galactose oxidase, HRP and Amplex Red Reagent and OD_{540nm} measured at different times. (**B**) The same reaction as in A was performed at room temperature in the absence of **1** that was incorporated after 20 min (arrow) and incubation continued. Fluorescence was recorded at different incubation times and expressed as arbitrary units.

analysis (Figure 1). It proves that the hydrogen bond of the OH-6 of the acceptor substrate with the Glu_{362} of the enzyme through a water molecule, shown by the crystal structure of the enzyme (Buschiazzo et al. 2002), is not crucial for the action of the enzyme. Once established that the D-fucose disaccharide was a good substrate in the TS reaction, it could be coupled to the galactose oxidase assay for determination of TcTS activity and the study of inhibitors for this enzyme. A galactose oxidase kit (Molecular Probes, Carlsbad, CA) developed for the analysis of sialidases may be used.

By employing the assay proposed here, we may measure both sialidase and TS activities simply by running a control without the fucose disaccharide. The galactose oxidase assay will then measure terminal galactosyl residues in the donor, produced by SA hydrolysis. In this respect, enzymes with different TS and sialidase activities have been described (Tiralongo et al. 2003; Ratier et al. 2008). The test can be used for monitoring TS purification, elicitation of neutralizing antibodies and for the high throughput screening of potential inhibitors. Although throughout this work we used TcTS, the enzyme from the African trypanosomes might be also determined following this assay.

Materials and methods

General procedures

Thin layer chromatography was performed using 0.2 mm silica gel 60 F254 (Merck, Whitehouse Station, NJ) aluminum-supported plates. Detection was done by ultraviolet light or by spraying with 5% (v/v) sulfuric acid in EtOH and charring. Column chromatography was performed on silica gel 60 (230–400 mesh, Merck). Melting points were determined with a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 343 polarimeter. NMR spectra were recorded with a Bruker AC 200 spectrometer at 200 MHz (^{1}H) and 50.3 MHz (^{13}C) or with a Bruker AM 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). *N*-acetyllactosamine, *N*-acetylglucosamine, D-fucose and chemical

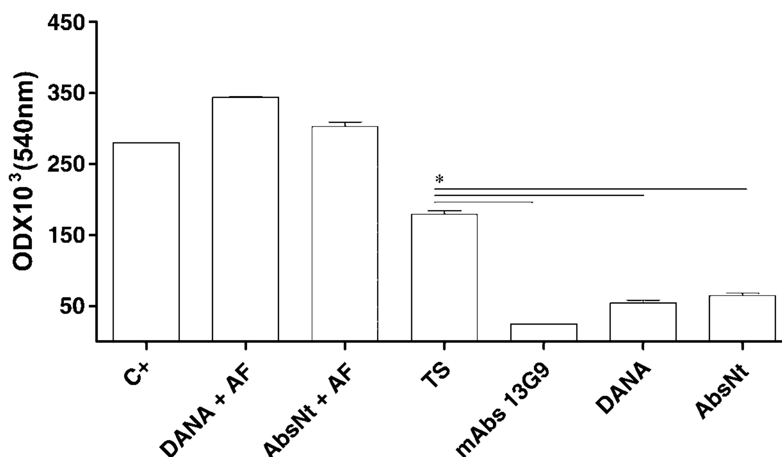

Fig. 4. Nonradioactive TcTS inhibition assay. Purified neutralizing antibodies from mouse (mAb 13G9) or from human patients (purified AbsNt, 2 µg) or 15 mM DANA (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid) were allowed to react with TcTS (0.3 µg) for 15 min and then fetuin and disaccharide **1** substrates were incorporated. Later, galactose oxidase, HRP and Amplex Red Reagent were added and incubated at 37°C. Two positive controls were included adding asialofetuin (AF) to each well containing TcTS inhibitors (DANA + AF and AbsNt + AF). *Clostridium perfringes* neuraminidase was also included as another positive control (C+). *$P < 0.01$.

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reagents were purchased from Sigma (St Louis, MO). SL was obtained from bovine colostrum by an adaptation of a reported method (Veh et al. 1981). Galactose oxidase (EC 1.1.3.9 from *Dactylium dendroides*), HRP (EC 1.11.1.7) and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red Reagent) were purchased from Molecular Probes/Invitrogen (Invitrogen, Carlsbad, CA). A recombinant TcTS expressed in *Escherichia coli* was used for sialylation (Buschiazzo et al. 1996). Absorbances were measured with a Multiscan EX-Labsystems Spectrophotometer. Analysis by HPAEC-PAD was performed using a Dionex ICS-3000 HPLC system equipped with a pulse amperometric detector. A CarboPac PA-100 ion exchange analytical column (4 × 250 mm) equipped with a guard column PA-100 (4 × 50 mm) was eluted with 50 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 mL/min at room temperature.

*1,2,3,4-tetra-O-benzoyl-α-D-fucopyranose (**2**)*

Benzoyl chloride (3 mL, 26 mmol) was slowly added to an ice-cold solution of D-fucose (514 mg, 3.13 mmol) in anhydrous pyridine (7 mL). The reaction was stirred overnight at room temperature and then quenched with MeOH and processed as usual. The product was obtained in 75% yield after recrystallization from ethanol. The ^{1}H and ^{13}C NMR spectroscopic data of **2** were in agreement with those already available in the literature (Ross et al. 2001; Piochon et al. 2009).

*Synthesis of benzyl 2,3,4-tri-O-benzoyl-β-D-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-α-D-glucopyranoside (**4**)*

To an externally cooled (0°C) solution of 1,2,3,4-tetra-*O*-benzoyl-α-D-fucopyranose (**2**; 0.300 g, 0.46 mmol) in dry CH_2Cl_2 (5 mL), tin(IV) chloride (0.065 mL, 0.56 mmol) was added. After 15 min of stirring at 0°C, benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside (Kuhn and Baer 1958) (**3**; 0.174 g, 0.56 mmol) and dry CH_3CN (0.3 mL) were added, and stirring was continued for 20 h at room temperature. The mixture was diluted with CH_2Cl_2 (30 mL) and poured into saturated aqueous $NaHCO_3$ with vigorous stirring. The aqueous layer was extracted with CH_2Cl_2 (2 × 50 mL), and the combined organic solutions were washed with water until pH 7, dried (MgSO4), filtered and concentrated. The resulting syrup was purified by column chromatography (1:30 toluene-EtOAc). A first fraction gave unreacted 1,2,3,4-tetra-*O*-benzoyl-α-D-fucopyranose (15 mg). The next fraction afforded 150 mg of **4** (42%) (R_f= 0.49; 1:9, methanol/CH_2Cl_2); m.p. (EtOH) 203–204°C; $[\alpha]_D$ +150° (c 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 8.11–7.23 (m, 20 H, aromatic), δ 5.80 (dd, 1H, *H*-2′, *J*=10.3, 7.8 Hz), δ 5.71 (d, 1H, H-4′, *J*=3.0 Hz), δ 5.57 (dd, 1H, H-3′, *J*=10.3, 3.0 Hz), δ 4.85 (d, 1H, H-1′, *J*=7.8 Hz), δ 4.69 (d, 1H, H-1, *J*=4.0 Hz), δ 4.57, 4.16 (2d, 2H, PhCH_2, *J*=11.8 Hz), δ 4.1–4.06 (1H), δ 3.91 (m, 1H, H-5′), δ 3.87–3.74 (m, 3H), δ 3.65–3.38 (m, 2H), δ 1.94 (s, 3H, CH_3CO), δ 1.35 (d, 3H, H-6′, *J*= 6.4 Hz). ^{13}C NMR ($CDCl_3$): δ 172.0 (CH_3*C*O), δ 166.02, 165.7, 165.43 (3xPh*C*O), δ 128–134 (aromatic), δ 102.1 (*C*-1′), δ 96.3 (*C*-1), δ 74.28, 72.0, 71.68, 71.07, 70.49, 69.95, 69.79, 69.39, δ 53.6 (C-2), δ 23.18 (CH3CO), δ 16.3 (C6′). Anal. calcd. for $C_{42}H_{43}NO_{13}$.1/2H_2O: C, 64.77; H, 5.69; N, 1.8. Found: C, 64.75; H, 5.50; N, 1.95. High-resolution electrospray ionization mass spectrometry *m/z* 770.2810 $[M+H]^+$ (calcd. for C42H44NO13, 770.2813); *m/z* 792.2622 $[M+Na]^+$ (calcd. for C42H43NO13Na, 792.2632).

*Synthesis of benzyl β-D-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-α-D-glucopyranoside (**1**)*

To a suspension of **4** (0.13 g, 0.17 mmol) in anhydrous MeOH at 0°C, 0.5 M NaOMe in MeOH (10 mL) was added. After stirring for 1.5 h at room temperature, water (2 mL) was added, and the solution passed through a column (1.5 cm × 2 cm) containing BioRad AG 50 W-X12 (H^+) resin (BioRad, Hercules, CA). The solvent was evaporated and the remaining methyl benzoate was removed by five successive coevaporations with water, to afford **1** as a white solid (0.073 g, 94%), R_f 0.65 (7:1:2 nPrOH–EtOH–H_2O). Crystallization from EtOH gave m.p. 229–230°C; $[\alpha]_D$ +95° (c 1, H_2O); ^{1}H NMR ($CDCl_3$): δ 7.27 (m, 5H, Bn*H*), δ 4.76 (d, 1H, *H*-1, *J*=3.4 Hz), δ 4.62, 4.40 (dd, 2H, PhCH_2, *J*=12 Hz), δ 4.24 (d, 1H, N*H*, *J*=5.6 Hz), δ 3.94 (d, 1H, H-1′, *J*=9.6 Hz), δ 1.79 (s, 3H, CH_3CO), δ 1.10 (d, 3H, H-6′, *J*=6.6 Hz). ^{13}C NMR ($CDCl_3$): δ 175.1 (CH_3*C*O), δ 129.4 (*Ph*CH_2), δ 104.2 (*C*-1′), δ 96.6 (*C*-1), δ 69.2 (*C*-6), δ 54.4 (*C*-2), δ 22.6 (CH_3CO), δ 16.2 (*C*-6′). Anal. calcd. for $C_{21}H_{31}NO_{10}$.H_2O: C, 53.05; H, 7.00; N, 2.95. Found: C, 52.97; H, 6.76; N, 2.94.

Radioactive assay for TS activity

The recombinant TcTS was incubated with 1 mM SL and 12 μM [D-glucose-1-^{14}C]-lactose (GE Healthcare, Waukesha, WI; 54.3 mCi/mmol) in a final volume of 30 μL of 20 mM Tris buffer pH 7.6. After 30 min of incubation at room temperature, 1 mL of bidistilled water was added to stop the reaction. A dense slurry of quaternary aminoethyl-Sephadex A-25 (Sigma) was added to bind the ^{14}C-labeled sialyllactose reaction product. After being vortexed briefly, beads were washed three times with bidistilled water, the bound material was eluted with 800 μL of 0.5 M NaCl and the counts per minute were quantified.

Enzyme kinetics

Reaction mixtures of 20 μL containing 20 mM Tris buffer, pH 7, 30 mM NaCl, 1 mM SL as donor and 1 mM disaccharide **1** as acceptor substrate were incubated with 300 ng purified TcTS. After incubation, reaction mixtures were diluted with 40 μL deionized water and analyzed by HPAEC-PAD. Sialylation of **1** was calculated as the percentage of the sialylated product obtained over the total amount of SA (free or linked to a saccharide).

For K_m calculations, 1 mM SL and different concentrations of disaccharide **1** were incubated with 300 ng purified TcTS in 20 μL of 20 mM Tris buffer, pH 7, 30 mM NaCl for 15 min at 25°C as before. Samples were then diluted five times, and 20 μL of each was analyzed by HPAEC. The extent of sialylation of **1** was calculated from the decrease in the concentration of the donor substrate using galacturonic acid as internal standard, in comparison with the corresponding control without enzyme. The K_m values were obtained graphically by the Lineweaver–Burk method (Lineweaver and Burk 1934).

Colorimetric/fluorometric assay for TcTS activity

The indicated amounts of TcTS were incubated with 5 μL of fetuin (10 mg/mL, Molecular Probes) or 10 μL SL (10 mg/mL, Sigma) and **1** (2 mg/mL) at room temperature for 30 min in 30 μL of 20 mM Tris–HCl buffer, pH 7, 30 mM NaCl, con-

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taining 0.1% of bovine albumin. Then, 70 µL of a solution containing 3 µL of galactose oxidase (2 U/mL), 0.25 µL of HRP (1 U/mL) and 1 µL of Amplex Red Reagent (1 mM) in buffer Tris–HCl 50 mM pH 7.2 with 1 mM $CaCl_2$ was added and incubated at 37°C. $OD_{540\,nm}$ was measured at different incubation times. In the fluorometric assays, reactions were performed incubating all the reagents at room temperature in buffer Tris–HCl 50 mM pH 7.2 with 1 mM $CaCl_2$ containing 0.1% of bovine albumin in a final volume of 200 µL and measuring fluorescence at excitation/emission 530/590 nm in an Aminco-Bowman Series 2 Luminescence Spectrometer (Thermo Spectronic, Walthman, MA).

Time course of TS and sialidase activities

The colorimetric assay was performed incubating TcTS (0.1 µg), the substrates and all the reagents at 37°C. The $OD_{540\,nm}$ was measured at different times. Sialidase activity was measured in the absence of **1**.

For the fluorometric assay, a similar reaction was performed at room temperature in the absence of **1** that was incorporated after 20 min and incubation continued. Fluorescence was recorded at different incubation times and expressed as arbitrary units.

Production and purification of TcTS-neutralizing antibodies

Monoclonal antibodies were obtained by growing the IgG_{2a}-secreting 13G9-34 hybridoma (Risso et al. 2007). Human antibodies were obtained from serum samples pooled from eight chronically *T. cruzi*-infected patients. Both monoclonal and human antibodies were purified using HiTrap Protein A HP columns (GE, Healthcare). Human sera were gently supplied by the Argentine National Reference Center for Chagas Disease Diagnosis (Instituto Nacional de Parasitologia "Dr Mario Fatala Chaben", Buenos Aires, Argentina).

TIA

Purified antibodies or DANA (15 mM, Sigma) were incubated with recombinant TcTS for 15 min at room temperature in a final volume of 20 µL. A mixture (10 µL) containing 5 µL of fetuin (10 mg/mL) and 5 µL of disaccharide **1** (2 mg/mL) was later added and TS activity measured as described above.

Funding

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Universidad de Buenos Aires and National Institutes of Health (Grant R01AI075589 to O.C.). The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. R.A., M.S.L., O.C. and R.M.L. are researchers and P.A.S. is a fellow from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina.

Conflict of interest statement

None declared.

Acknowledgements

We thank A.C.C. Frasch from Universidad Nacional General San Martín, Argentina for a kind gift of TcTS.

Abbreviations

DANA, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid; HPAEC-PAD, high pH anion exchange chromatography with pulse amperometric detection; HRP, horseradish peroxidase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; SA, sialic acid; SL, α-(2,3)sialyllactose; TcTS, *T. cruzi trans*-sialidase; TIA, TS inhibition assay; TS, *trans*-sialidase.

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