A new class of mechanism-based inhibitors for *Trypanosoma cruzi* trans-sialidase and their influence on parasite virulence


1 Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Bloco G, 2 Fundação de Farmácia, Centro de Ciências da Saúde, Bloco A; Universidade Federal do Rio de Janeiro, Cidade Universitária, Ilha do Fundão, Rio de Janeiro-RJ, Brasil; and 3 Graduate School of Life Science, Hokkaido University, N21W11, Kita-ku, Sapporo 011-0021, Japan

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One of the most interesting aspects of *Trypanosoma cruzi* is its adaptation to obtain sialic acid from its host, fulfilling this need exclusively through the reaction catalyzed by enzymatically active trans-sialidase (aTS), thought to play an important role in the pathogenesis of Chagas’ disease. Herein, we report that 2-difluoromethyl-4-nitrophenyl-3,5-dideoxy-D-glycero-α-D-manno-2-nonulopyranosid acid (NeuNAcFNP) inactivates aTS time- and dose-dependently, and this inhibition was not relieved removing the inhibitor. Also, NeuNAcFNP causes a decrease in infection of mammalian cells. Characterization of labeled aTS by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry revealed that inactivation of the enzyme occurs through formation of a covalent bond between Arg 245 and Asp 247 and the inhibitor aglycone. Participation of Asp 247 in the catalytic mechanism was proved by constructing a D247A mutant, which presents only residual activity. Molecular dynamic simulations indicate that the D247A mutation results in a more open catalytic cleft. In summary, NeuNAcFNP is the first reported mechanism-based inhibitor of aTS, representing a new template for drug design and opening new possibilities for chemotherapy of Chagas’ disease, as well as for the elucidation of aTS function in *T. cruzi* pathogenesis and biology.

**Keywords:** dynamic simulation/irreversible inhibitor/mass spectrometry/trans-sialidase/ *Trypanosoma cruzi*

**Introduction**

Cardiopathy caused by the protozoan *Trypanosoma cruzi*, the etiologic agent of Chagas’ disease, is the main cause of death in endemic regions of Latin America. Debilitating forms of the infection account for 670,000 inept lives per annum and keep affected populations from improving their own living conditions due to its reduction in worker productivity as well as early deaths. Once restricted to Latin America, Chagas’ disease is becoming a significant health problem in developed countries (Hotez 2008). The United States and Spain, where specific health services for Chagas’ disease diagnosis and treatment are largely absent, house an unknown number of infected individuals (Briceño-León 2009). In addition, the presence of a sylvatic and domestic cycle of *T. cruzi* transmission in United States (Beard et al. 2003; Kjos et al. 2008) is reflected in the increasing numbers of native infection cases reported (Dorn et al. 2007).

Chemotherapy of this disease is still very unsatisfactory, being based on nitrofurans and nitroimidazoles. These compounds are inadequate due to frequent toxic side effects and present a limited efficacy. There is therefore urgent need for new drugs against Chagas’ disease, designed on the basis of the known differences between human metabolism and that of the causative parasite.

*T. cruzi* is evolutionarily adapted to the incorporation of sialic acid from exogenous sialoglycoconjugates by means of a glucoside hydrolase trans-sialidase (aTS) (Previato et al. 1985), rather than by the well-known route in which cytidine monophosphosialic acid is an intermediate. Much evidence suggests that aTS activity plays a key role in host-cell invasion (Schenkman et al. 1991; Ming et al. 1993) as well as in the regulation of host immunological responses triggered by the infection (DosReis et al. 2005) and dampening Ag-specific CD8+ T-cell response favoring the persistence of *T. cruzi* in the mammalian host (Freire-de-Lima et al. 2010). Soluble aTS is a powerful virulence factor, increasing parasitemia and mortality in *T. cruzi*-infected mice (Chuenkova and Pereira 1995). aTS is a T cell and B mitogen and an inducer of non-specific Ig secretion (Gao et al. 2002; Todeschini et al. 2002). Therefore, aTS is the pathogen-associated molecule most likely
to be responsible for the polyclonal lymphocyte activation seen during T. cruzi infection, a condition underlying the induction of immunopathology and hampering effective vaccination (Minoprio et al. 1986). These findings highlight aTS as a promising target for chemotherapy of Chagas’ disease.

aTS is an exo-alpha sialidase (Achyuthan and Achyuthan 2001) member of the glycoside hydrolase (GH) family number GH-33 (http://www.cazy.org) that 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-Galp) containing acceptors (Vandekerckhove et al. 1992). Otherwise, in the absence of a proper acceptor, trans-sialidase (TS) catalyzes sialoside hydrolysis with retention of configuration (Todeschini et al. 2000).

There are no effective inhibitors of aTS yet reported. The potent inhibitor of the related Trypanosoma rangeli sialidase (70% sequence homology to aTS in the catalytic domain) 2-deoxy-2,3-didehydro-D-α-acetilneuraminic acid, inhibits aTS with a Ki of 12.3 mM (Paris et al. 2005). A variety of sialic acid mimetic compounds have been tested without successful enzymatic inhibition (Neres et al. 2008). Recently, the α2,3-difluoro-D-α-acetylneuraminic acid, which covalently modifies the enzyme’s Tyr-342 residue, has been proposed as a lead compound for the synthesis of new aTS inhibitors (Buchini et al. 2008). However, inhibition is transitory, and the enzyme spontaneously recovers its activity.

Herein, we apply a strategy employed by Hinou et al. (2005), who reported the labeling of Vibrio cholerae neuraminidase by a suicide-type substrate. We report that the 2-difluoromethyl-4-nitrophenyl-3,5-dideoxy-D-gluco-α-D-galacto-2-nonulopyranoside acid (NeuAcFNP) (Figure 1A) irreversibly inactivates aTS time- and dose-dependently, and incubation of trypomastigotes with NeuAcFNP decreases parasite invasion of mammalian cells. Characterization of inactivated aTS using a 5-acetamido-2-(4-N-5-dimethylamino-naphthalene-1-sulfonyl-2-difluoromethylphenyl)-3,5-dideoxy-D-gluco-α-D-galacto-2-nonulopyranosonic acid (dansyl-NeuAcFP) (Figure 1B) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF mass spectrometry (MS) analysis revealed that inactivation of enzyme occurs through a covalent bond formation between the Arg245 and Asp247 residues with the reactive aglycone generated by the hydrolysis of dansyl-NeuAcFP. The relevance of Asp247 during the catalytic activity of aTS was proved by the construction of the TSD247A mutant and by molecular dynamic simulation. Together, these results open up an elegant approach for the irreversible inhibition of aTS.

Results and discussion

Inhibition of aTS by NeuAcFNP

Kinetic parameters for the inhibition of T. cruzi aTS by NeuAcFNP were evaluated assaying both activities of the enzyme: sialidase and trans-sialidase. Figure 2A shows that both activities present similar patterns of activation by the substrate 4-methyl-umbelliferyl-N-acetylneuraminic acid (4-Me-Umb-Neu5Ac), presenting $K_{0.5}$ of $0.21 \pm 0.02$ mM and $0.19 \pm 0.02$ mM for sialidase and trans-sialidase activities, respectively. When aTS was incubated in the presence of 0.6 mM of the NeuAcFNP, a slight inhibition was observed (Figure 2A: compare filled triangles and squares for trans-sialidase activity and empty triangles and squares for sialidase activity). Moreover, the $K_{0.5}$ for the substrate increased to $0.35 \pm 0.02$ mM for sialidase activity ($P < 0.05$ compared to control, Student’s t-test) and to $0.37 \pm 0.03$ mM for trans-sialidase activity ($P < 0.05$ compared to control, Student’s t-test). However, the compound did not alter the maximal velocity of both activities, showing that high concentrations of the substrate are able to prevent the enzyme from the inhibitory effects observed at lower concentrations of the substrate. These results suggest that the inhibitor and the substrate compete for the same site at the enzyme.

Pre-incubation of aTS for 15 min in the presence of 0.6 mM NeuAcFNP resulted in 50% inhibition for both activities assayed when compared to their respective controls (Figure 2A: compare filled triangles and circles for trans-sialidase activity and empty triangles and circles for sialidase activity). Furthermore, the $K_{0.5}$ values for the substrate measured after pre-incubation were not different from those assessed for controls ($0.20 \pm 0.02$ mM for sialidase and $0.19 \pm 0.02$ mM for trans-sialidase), which is compatible with an irreversible mechanism. These results also corroborate that the differences between the $K_{0.5}$ for the substrate observed when the inhibitor was present in the reaction medium were due to competition between the substrate and the inhibitor, since when competition was terminated by dilution of the inhibitor (as in the pre-treatment experiments), the $K_{0.5}$ values were no longer affected by the presence of the inhibitor. Indeed, inhibition of aTS by NeuAcFNP is a time-dependent process as shown in Figure 2B. It can be seen that upon incubation of the enzyme with 0.6 mM NeuAcFNP its activities (trans-sialidase and sialidase) progressively decay, reaching the maximal inhibition after 10 min.

In order to definitely prove the irreversible effects of the inhibitor on T. cruzi aTS, a set of experiments was performed in which aTS was pre-incubated in the presence of 0.6 mM, 3 mM or 6 mM NeuAcFNP and sialidase and trans-sialidase activities assessed in a medium containing the same concentrations of the inhibitor (before dilution), in a medium containing inhibitor after 100-fold dilution or after removing the inhibitor through desalting micro spin G-25 columns. From these results, shown in Figure 2C, it is clear that the two activities were affected in the same way after every treatment. Hence,
after pre-incubation, it does not matter whether the inhibitor is still present, is diluted or is removed: the inhibition remains, and aTS does not recover its catalytic activity, confirming that the inhibition is not reversible.

The inhibition constant for the NeuNAcFNP ($I_{0.5}$) was evaluated for both activities. Figure 2D shows the trans-sialidase activity using N-acetyl-lactosamine as acceptor upon pre-incubation of the enzyme with different concentrations of NeuNAcFNP (filled circles). The $I_{0.5}$ calculated for these experiments was $0.57 \pm 0.06$ mM. Similar results were obtained assessing the sialidase activity (Figure 2D, empty circles), where the $I_{0.5}$ was $0.57 \pm 0.06$ mM when the enzyme was pre-incubated with NeuNAcFNP.

These results support the three hypotheses formerly presented, namely: (a) the inhibitor and the substrate (4-Me-Umb-Neu5Ac) compete for the same site at the enzyme; (b) the inhibitor is metabolized by the enzyme; and (c) the inhibitor acts on the enzyme irreversibly.
NeuNAcFNP inhibits host cell invasion by T. cruzi

To determine whether aTS inhibition has any direct impact on susceptibility of mammalian cells to T. cruzi infection, trypanosomes (2 × 10⁵) from Y strain were incubated with NeuNAcFNP (0, 0.1, 1.0 and 10.0 mM) and p-nitrophenol (p-NP) used as negative control (0, 0.1, 1.0 and 10.0 mM) for 30 min and further co-cultured with LLC-MK2 cells (2 × 10⁶) for 2 h. Cells were then washed three times, and the number of infected cells was assessed 3 days later.

Characterization of labeled aTS

According to the general method and conditions described previously (Kurogochi et al. 2004), aTS was inactivated with the dansyl-labeled inhibitor and subjected to peptidase treatment so as to give rise to the complex peptide fragments observable in MALDI-TOF MS (Figure 4A). TOF/TOF MS analysis in the range m/z 900 to m/z 2500 followed by MASCOT search allowed every major fragment to be assigned to aTS, with 34.6% sequence coverage. Purification of the mixture by anti-dansyl antibody column yielded material giving a clear signal of m/z 2163 and corresponding to the [M + H]⁺ ion of a labeled peptide fragment, as indicated in Figure 4B. The isolated ion was subsequently subjected to LIFT-TOF/TOF fragmentation analysis, yielding a spectrum with a series of b-ions and y-ions, as shown in Figure 5. This allowed us to identify the peptide as LIINTRVDYRR in which the Arg245 and Asp247 were dansylated (calculated molecular mass for [M + H]⁺ = 2163.01) (Figure 5). Interestingly, a similar pair of amino acid residues, Asp576 and Arg577, was identified by our previous labeling study on V. cholerae neuraminidase using the same suicide substrate (Hinou et al. 2005). The Arg245 residue is widely recognized as being among the conserved amino acids in all known sialidases. However, this is the first time that Asp247 is shown to be present in the catalytic site. Our data suggest that the Asp residue co-labeled by the probe together with the Arg residue may be involved in the catalytic function of sialidases. To test the hypothesis that Asp247 is involved in the catalytic function of aTS, we undertook a mutation study of this residue.

Site-directed mutagenesis of Asp247 residue and molecular dynamic simulation

The relevance of Asp247 to the catalytic activity of aTS was studied by the construction of a TSD247A mutant by site-directed mutagenesis. No conformational changes in the tertiary structure could be detected by measurement of the intrinsic fluorescence of mutant TSD247A (Figure 6, left panel). In addition, the center of spectral mass values was the same for both proteins (28931 cm⁻¹), suggesting that no exposition of Trp residues had occurred in the absence of denaturant. Likewise, in the presence of 8 M urea, the center of spectral mass decreased ~1000 cm⁻¹, indicating total unfolding of both proteins (Figure 6, right panel). However, TSD247A presented only residual sialidase and trans-sialidase activities compared to aTS, indicating a critical function for Asp247 in the catalysis, which has not been previously revealed by crystal structures (Amaya et al. 2004). Therefore, we went on to compare the architecture of the catalytic cleft of the two enzymes through molecular dynamic simulations.

The structure of the T. cruzi aTS was retrieved from PDB 1MS3, and the TSD247A mutant was generated by Swiss Pdb-Viewer. After an equilibration step, a 7-ns run simulation was carried out that showed significant alterations in the scaffold of the active site: the Asp247→Ala mutation induced modifications in the hydrogen bonding network of the binding pocket (Figure 7). In aTS, this network connects the residues Asp247, Arg245 (one of the Arg triad residues that interact with sialic acid for catalysis), Glu230, Tyr248, Gln282 and Tyr342 (Figure 7B). The Asp247→Ala mutation disrupts the hydrogen bond between Asp247 and Tyr248, which thus presents an increased mobility (Figure 8A), standing away from Glu282, as evidenced by the increased distance between its carbon alpha (Ca) and the Ca of Glu282 (Figure 8B).

The alterations in Tyr248 position in the TSD247A mutant affects the arrangement of Trp312 throughout the dynamics, as described by the Trp312 dihedral angles shown in Figure 8C. The increased mobility of Glu282 and Trp312 residues results in a widely open binding pocket, suggesting an increase in exposure to the solvent. The Trp312 position is important as it provides a binding site for lactose during enzymatic catalysis (Buschiazzo et al. 2002). In aTS, the Trp312 is, during most of the simulation time, parallel to the aromatic Tyr119 (Figure 7A) and ready to interact with the lactose part of the sialoside, as previously described (Buschiazzo et al. 2002).

Our results reveal that the D247A mutation in T. cruzi TS mainly alters the conformation of the catalytic residues, changing the overall architecture of the catalytic cleft and resulting in loss of the “gate” function of Trp312, which as proposed by Demir and Roitberg (2009) opens and closes the aTS catalytic
The binding pocket of TSD247A is more open than that of the wild-type enzyme and consequently more accessible to the water. This might influence the enzymatic reaction by decreasing the probability of encounter between the correct conformation of the substrate and the enzyme.

The molecular dynamic simulations (as well as the MALDI-TOF/TOF mass spectrometry results revealing that two molecules of inhibitor react with one of aTS) are in agreement with previous studies suggesting a flexible aTS catalytic site rather than the static one suggested by the crystal structure. The results of the proteomic analysis of labeled aTS imply that, once a covalent bond has been formed between the inhibitor aglycone and either Arg245 or Asp247, the entrance of a second molecule of inhibitor is still allowed, indicating considerable elasticity of the catalytic cleft. Molecular dynamic simulation shows that catalytic site of aTS prefers a more open conformation than that observed in the crystal structure, in agreement with recent results by Demir and Roitberg (2009).

Early studies demonstrated that an inactive TS bearing a Tyr342→His substitution (TSY342H) binds either α2,3-sialyllactose or lacto-N-tetraose and methyl β-lactose in a ternary complex (Todeschini et al. 2004). In the same manner, as we have shown for inactive TS, Buschiazzo et al. (2002) demonstrated that sialic acid binding induces a conformational modification in the crystal structure of aTS, allowing the acceptor substrate (lactose) to bind, and presented surface plasmon resonance results showing that lactose binds to an inactive TS mutant (Asp59→Asn) in the presence of α2,3-sialyllactose. In addition, nuclear magnetic resonance spectroscopy studies indicated simultaneous interaction of the sialic acid donor and the lactose acceptor with the enzyme’s active site (Haselhorst et al. 2004). Together, these results suggest that aTS has a flexible catalytic site.

**Fig. 4.** MALDI-TOF mass spectra of fluorescence-labeled peptides. Peptide fragments produced by digestion with trypsin were analyzed: (A) mixtures of peptides derived from the labeled enzyme and (B) peptide isolated using anti-dansyl antibody column chromatography.

**Fig. 5.** MALDI-TOF/TOF mass spectrum of the dansylated peptide fragment m/z 2163 and assignment of the identified sequence 241LIINTRVDYRR251 with dansylated Arg245 and Asp247.
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property that must be considered in future studies on the catalytic mechanism and in the design of new selective inhibitors of TS.

Further structural work to elucidate reaction mechanism and structural features of \textit{T. cruzi} TS that support its efficient sugar transfer activity, as opposed to simple hydrolytic activity, are still needed. Given the importance of TS and sialidases as virulence factors in several infections (Mendonça-Previo et al. 2010), structural and mechanistic works must be relentless, and it must be kept in mind that mutations in key amino acids may produce important modifications in the mechanism of catalysis, as shown here for TSD247A mutant. For instance, studies on the \textit{Micromonospora viridifacies} sialidase demonstrated that some mutants in the nucleophilic Tyr residue remain active and still perform catalysis (Watson et al. 2005), while substitution of this Tyr by smaller amino acid produces inverting sialidase. In contrast, inclusion of a larger amino acid (Asp versus Glu) changes the mechanism back to retention of configuration (Watson et al. 2005).

To our knowledge, this is the only irreversible inhibitor for aTS so far reported. Lately, several compounds have been tested as potential inhibitors of aTS with no success (Neres et al. 2008). Among them, 2,3-difluorosialic acid inactivates temporarily the TS through a covalent binding with the hydroxyl group of Tyr342. However, complete inactivation requires very high concentrations of the inhibitor (20 mM), and the enzyme spontaneously recovers its full catalytic activity (Watts et al. 2003). Incorporation of an aromatic substituent to C9 of the 2,3-difluorosialic acid molecule increased inhibition (Buchini et al. 2008), but enzymatic reactivation remained a problem.

In this work, we apply a previously successful strategy (Hinou et al. 2005) achieving irreversible inhibition of the \textit{T. cruzi} aTS. The corresponding structures provide an excellent template for designing novel aTS-specific drugs. Our current central interest is to improve the inhibition affinity and specificity of this inhibitor. Substitution of sialic acid C9 by aryl groups (Buchini et al. 2008) appears as a good strategy. In addition, dansyl-labeled sialosides allied to MALDI-TOF/TOF mass spectrometry have potential for application in proteomics analysis of active trans-sialidases expressed by different \textit{T. cruzi} strains. Association of dansylated mechanism-based inhibitors with fluorescence resonance energy transfer (FRET) is also documented as a novel tool for the real-time monitoring of glycosyltransferase-catalyzed reactions. It was demonstrated that FRET method, in combination with click chemistry, greatly facilitates the discovery of selective inhibitors (Hinou and Nishimura 2009).

Furthermore, our experiments demonstrating that NeuNAcFNP decreases infection of mammalian cells support the hypothesis that \textit{T. cruzi} aTS is involved in host cell invasion. These studies suggest aTS as a drug target against Chagas’ disease, surpassing technical difficulties in obtaining a \textit{T. cruzi} lacking TS by gene knockout or interference RNA. Finally, this work brings into light a new strategy for chemotherapy in Chagas’ disease and for disclosure of aTS function in \textit{T. cruzi} pathogenesis and biology.

Materials and methods

General methods and material

Trypsin, ammonium bicarbonate (NH$_4$HCO$_3$), dithiothreitol, iodoacetamide and trifluoroacetic acid were obtained from Wako Pure Chemical. NeuNAcFNP and dansyl-NeuNAcFP were obtained as described previously by Hinou and et al. (2005).

Inhibition of aTS by NeuNAcFNP

For analysis of aTS sialidase activity, 100 ng of enzyme were incubated in bicarbonate buffer (pH 7.5) in the presence of 4-Me-Umb-Neu5Ac at the concentrations indicated for each experiment. Formation of 4-methylumbelliferone (4-Me-Uone) was followed in a spectrofluorimeter (Jasco FP-6300) at 25°C.
exciting the sample at 367 nm and measuring the fluorescence emission at 450 nm. Enzyme activity was calculated from the angular coefficient of the linear phase of reaction (initial velocity). *trans*-Sialidase activity was assessed by incubation of aTS (100 ng) in bicarbonate buffer (pH 7.5) in the presence of 1 mM N-acetyl-lactosamine (acceptor) and 4-Me-Umb-Neu5Ac (donor) at the concentrations indicated for each experiment. Formation of 4-Me-Uone was followed as above, and enzyme activity was calculated also as above, with subtraction of values obtained for blanks performed in the absence of an acceptor.

**Labeling of aTS**

aTS (500 µg) was incubated with dansyl-NeuNAcFP (final concentration 5 mM) at 28°C for 30 min in 300 µl of 100 mM Tris–HCl buffer at pH 7.5. The reaction mixture was directly applied into a fast desalting column HR 10/10 gel filtration column (Pharmacia Biotech) using 100 mM ammonium bicarbonate buffer (pH 8.0) to remove the excess suicide substrate. The protein-containing fractions were lyophilized.

**Preparation of anti-dansyl antibody column**

Anti-dansyl rabbit IgG (0.5 ml, 1 mg/ml) was dialyzed at 4°C against a solution of 0.1 M sodium bicarbonate containing 0.1 M NaCl using Centricon YM-30 (Milliprep). The solution was suspended with 0.5 ml of Affi-Gel 10 (Bio-Rad) according to the manufacturer’s instructions, and the mixture was packed into a 0.5 × 2 cm column.

**Analysis of labeled aTS**

Labeled aTS (100 µg) was dissolved in 100 µl of 100 mM NH₄HCO₃ and digested with 1 µg of trypsin at 37°C overnight. The mixture was applied to the anti-dansyl antibody column (0.5 ml) using phosphate-buffered saline (PBS) as loading buffer, and after a wash with 3 ml of PBS, elution was carried out with 30% acetic acid (1.5 ml). This fraction, containing labeled peptide, was concentrated by centrifugal evaporation, desalted with C18 ZipTip™ (Millipore) according to the manufacturer’s instructions and analyzed by MALDI-TOF MS.

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Fig. 7. Seven-nanosecond frame of molecular dynamic simulation of the aTS (A) and TSD247A (B) with distances and dihedral angles featured. 

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Fig. 8. (A) Hydrogen bonds formed between amino acid residues Tyr282 and Asp247 for aTS (black line) and between Tyr248 and Ala247 for TSD247A (gray line), as a function of time. (B) Distance between alpha carbon (Ca) of Gln282 and Ca of Tyr248 as a function of time, for aTS (black line) and TSD247A (gray line). (C) Dihedral angle (Ca–Cb–Cg–Cd) variation of the Trp312 residue between 6 and 7 ns. Black line represents aTS, and gray line TSD247A.
MALDI-TOF MS and TOF/TOF MS

Samples were dissolved in water, applied on the target spot, mixed with 1 µl of matrix solution (10 mg/ml DHB in water) and dried at room temperature. Measurements were obtained in an Ultraflex TOF/TOF mass spectrometer equipped with a reflector and controlled by the Flexcontrol 2.1 software package (Bruker Daltonics GmBH, Bremen, Germany). In the MALDI-TOF MS reflector mode, ions generated by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm, 5 Hz) were accelerated to a kinetic energy of 23.5 kV. Metastable ions generated by laser-induced decomposition of the selected precursor ions were analyzed without any additional collision gas. In MALDI-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage. Masses were automatically annotated by using the FlexAnalysis 2.1 software package. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II ($m/z$ 1046.542), bombesin ($m/z$ 1619.823), ACTH (18–39) ($m/z$ 2465.199) and somatostatin 28 ($m/z$ 3147.472) and utilizing a customized macro command of the FlexControl 2.1 software package. TOF/TOF spectra were annotated with the BioTools 2.2 software package.

Parasite culture and host cell invasion

Trypomastigotes of *T. cruzi* Y strain were maintained in LLMCK2 cells in Dulbecco’s modified Eagle’s medium (DMEM) medium containing 2.0% fetal bovine serum, 2 g/l sodium bicarbonate, 100 µg/ml streptomycin and 100 µg/ml penicillin (Dias et al. 2008). Trypomastigotes ($2 \times 10^5$) were incubated with NeuNAcFNP or NeuNAcFNP (0, 0.1, 1.0 and 10.0 mM) for 30 min and further incubated with LCC-MK2 cells ($2 \times 10^4$) for 2 h. Cells were washed three times, and the number of infected cells was accessed 3 days post-infection.

Site-directed mutagenesis

Site-directed mutagenesis of the aTS gene was performed using the double-stranded pTrcHisA-aTS plasmid (Buschiazzo et al. 1996) as template. Complementary mutagenesis oligonucleotide pairs incorporating single amino acid substitutions were as follows. Sense oligonucleotide (Sense-aTS-D247A) was 5′AACACTCGAGTTGCTATCGCCG 3′, and antisense oligonucleotide (Antisense-aTS-D247A) was 5′GCGGCGA-TAGGCAAATCGAGTTGT 3′. Mutated plasmid containing staggered nicks was generated by extension of primers annealed to opposite strands of the denatured plasmid by temperature cycling (one cycle of 95°C for 30 s, 16 cycles of 95°C for 30 s, 60°C for 60 s, 72°C for 13 min and a final cycle of 72°C for 10 min) in the presence of the high-fidelity *Pfu* DNA polymerase (Fermentas Life Sciences). Synthesized DNA containing the desired mutation was selected from the original DNA template by incubation with *DpnI* (New England Biolabs) at 37°C for 1 h. Nicks in the plasmid were repaired following transformation of the synthesized products into competent *Escherichia coli* XL1-Blue cells. Ampicillin-resistant transformants were randomly selected and inoculated to overnight Luria–Bertani (LB) broth (Invitrogen) cultures for plasmid preparation with QIAprep™ Miniprep (QIA-GEN). Correct incorporation of the mutation was monitored by DNA sequencing using a Perkin Elmer ABI/Prism 377 automated DNA sequencer according to manufacturer’s instructions. On the basis of emerging DNA sequences, additional walking primers were synthesized in the forward and reverse orientations for the sequencing of both DNA strands. DNA sequence analysis was performed using DNA Star. Mutated plasmid was transformed to competent *E. coli* BL21 (DE3) cells for subsequent protein expression.

Fluorescence spectroscopy measurements

Fluorescence spectroscopy was carried out using Hitachi F-4500 spectrofluorometer (Hitachi, Tokyo, Japan). Excitation wavelength was fixed at 278 nm, and the emission spectrum was recorded from 300 to 400 nm. The slits used both in the excitation and emission were of 5 nm. Unless otherwise stated, the experiments were performed at 25°C. Fluorescence spectra were quantified by specifying the center of spectral mass $<\nu>$:

$$<\nu> = \sum \nu_i \cdot F_i / \sum F_i$$ (1)

Where $F_i$ is the fluorescence emitted at wave number $\nu_i$. All experiments were performed at 25°C (Sousa et al. 2006).

Expression and purification of His-aTS and His-aTS-D247A fusion proteins

Recombinant active TS containing the C-terminal repeats was obtained from *E. coli* MC1061 electro-transformed with plasmid pTrcHisA containing the wild-type TS insert, TSREP.C (Buschiazzo et al. 1996), while His-aTS-D247 was obtained as described above. A freshly transformed bacterial colony was picked up to inoculate 50 ml of LB and grown at 37°C overnight. The next day, the inoculated LB was transferred to 1 L of TB and grown at 37°C. The culture medium reached an $A_{600}$ of 0.9, 30 mg/ml of isopropyl-1-thio-β-D-galactopyranoside was added, and incubation continued overnight at 28°C. The bacteria were harvested by centrifugation, washed with cold PBS and lysed at 4°C in 20 mM Tris, pH 7.8 supplemented with 2.0 mg/ml lysozyme, 25% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5.0 µg/ml leupeptin, 1.0 µg/ml trypsin inhibitor and 0.1 µM iodoacetamide. The bacterial DNA was sheared by sonication, and the lysate was cleared by centrifugation (25,000 × g, 30 min). The supernatant was passed through a Ni$^{2+}$-immobilized acid sepharose column equilibrated with (20 mM Tris, pH 7.8, 0.5 M NaCl) buffer. The column was washed with six column volumes of equilibration buffer, six column volumes of wash buffer 1 (5 mM imidazole, pH 7.8, 20 mM Tris, pH 7.8, 0.5 M NaCl) and six column volumes of wash buffer 2 (50 mM imidazole, pH 8.0, 20 mM Tris, pH 8.0, 0.5 M NaCl). Recombinant TS was eluted with two column volumes of elution buffer (500 mM imidazole, pH 7.8; 20 mM Tris, pH 7.8, 0.5 M NaCl). Recombinant TS was dialyzed into (20 mM Tris, pH 7.8, 0.05 M NaCl) buffer at 4°C and further purified by ion-exchange chromatography on Mono Q and Mono S columns, applying linear NaCl gradients (0.005–1 M) (Todeschini et al. 2000) and stored in
20 mM Tris, pH 7.8, 0.05 M NaCl at 4°C until used. The homogeneity of purified recombinant proteins was evaluated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Molecular dynamic simulations

The crystallographic structure of the T. cruzi aTS solved by X-ray diffraction (resolution of 1.65 Å) was retrieved from Protein Data Bank (PDB) server (Dutta et al. 2009) under the code 1MS3 (Buschiazzo et al. 2002). Since nine residues (DPASSSSSER) between the amino acids Ala399 and Gly409 were missing in the 1MS3 structure, the final aTS model was solved by comparative modeling of 1MS3 and the complete sequence in FASTA format, acquired from the PDB server, using the automatic module of the SWISS-Model workspace (Bim pikis et al. 2003; Bordoli et al. 2009). In order to generate the D247A mutant of aTS, the Asp247 was replaced with Ala residue using Swiss Pdb-Viewer (Schwede et al. 2003). Two systems were prepared: aTS and TSD247A.

Our model was validated by Ramachandran plot analysis (Sheik et al. 2002; Ho and Brasseur 2005; Gopalakrishnan et al. 2007) through the PROCKECK server (http://deposit.pdb.org/validate/). After validation, the input files were prepared to run in GROMOS 96 force field (Daura et al. 1998; Bonvin et al. 2000; Baran and Mazerski 2002). Each of the two models (aTS and TSD247A) was immersed in a hydrated dodecahedral box with periodic boundary conditions containing 33,161 SPC water molecules (Tironi et al. 1996) and five chloride anions, which were used as counter-ions to neutralize the total charge of the systems.

The systems were then energy minimized with 7111 steps of Steepest Descent with position restrain of the heavy atoms, 8314 steps of Steepest Descent without position restrain, followed by 3004 steps of Conjugate Gradient and 16 steps of Newton method. Particle Mesh Ewald (Darden et al. 1995; Essmann et al. 1995) (PME, Fourier spacing 1.2 Å, fi=pdb.org/validate/). After validation, the input files were prepared to run in GROMOS 96 force field (Daura et al. 1998; Bonvin et al. 2000; Baran and Mazerski 2002). Each of the two models (aTS and TSD247A) was immersed in a hydrated dodecahedral box with periodic boundary conditions containing 33,161 SPC water molecules (Tironi et al. 1996) and five chloride anions, which were used as counter-ions to neutralize the total charge of the systems.

The systems were then energy minimized with 7111 steps of Steepest Descent with position restrain of the heavy atoms, 8314 steps of Steepest Descent without position restrain, followed by 3004 steps of Conjugate Gradient and 16 steps of Newton method. Particle Mesh Ewald (Darden et al. 1995; Essmann et al. 1995) (PME, Fourier spacing 1.2 Å, fourth order and tolerance $10^{-5}$), and 6–12 Lennard-Jones potentials were applied to account for Coulomb and van der Waals interactions, using a cutoff of 1.0 nm for both interactions. The LINCS algorithm (Hess et al. 1997) was applied over all covalent bonds intending to preserve the covalent character of linkages between atoms. The systems were equilibrated after 500 ps of molecular dynamic simulation, i.e. temperature reached 310 K and pressure, 1 bar. After equilibration, molecular dynamic simulations without position restrain of 7000 ps were carried out for the two systems using the GROMACS package (Berendsen et al. 1995; Van der Spoel et al. 2005; Hess et al. 2008) version 4.0.

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Abbreviations

aTS, enzymatically active trans-sialidase; DMEM, Dulbecco’s modified Eagle’s medium; FRET, fluorescence resonance energy transfer; GH, glycoside hydrolase; 4-Me-Uone, 4-methylumbelliferonyl-α-acetyleuraminic acid; 4-Me-Uone, 4-methylumbelliferonyl-α-acetylenameric acid; NeuNAcFNP, 2-di-4-methyl-umbelliferyl-α-D-galacto-2-nonulopyranosid acid; PDB, Protein Data Bank; p-NP, p-nitrophenol.

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