Enzyme Inhibitors

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A New Generation of Specific *Trypanosoma cruzi trans*-Sialidase Inhibitors**

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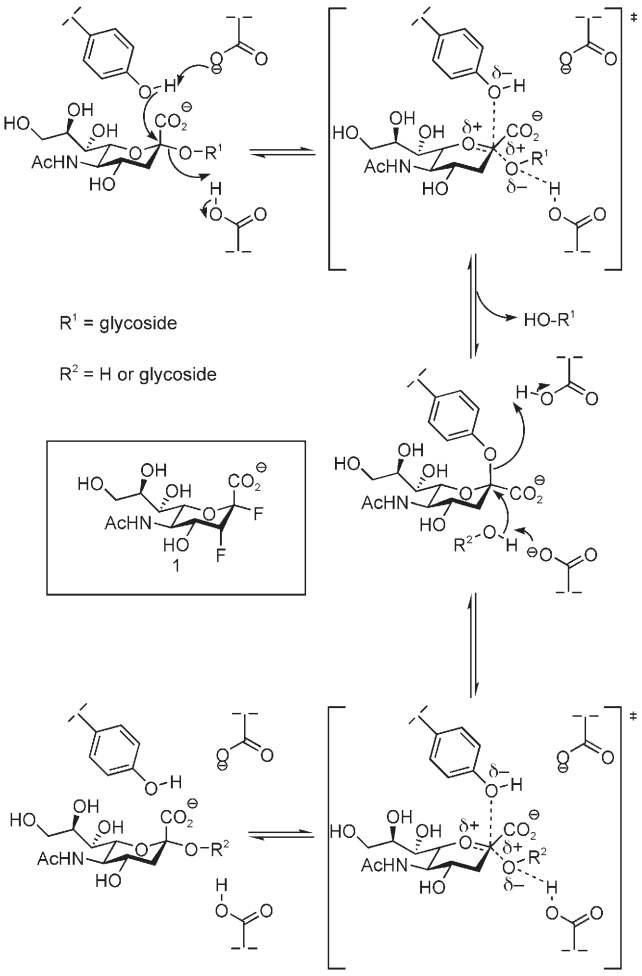
The protozoan parasite *Trypanosoma cruzi* is the causative agent of human American trypanosomiasis, also known as Chagas' disease, which in its chronic form can lead to severe debilitation and ultimately death.[1] According to World Health Organization (WHO) estimates, 16 to 18 million people were infected in Latin America in 2005. Only two approved drugs (nifurtimox and benznidazole) are currently used for the treatment of the infection, but both display low efficacy and are associated with severe undesired side effects.[2] The whole genome sequence of *Trypanosoma cruzi*, completed in 2005, contains genes predicted to encode almost 23000 proteins.[3] The identification of essential proteins specific to the parasite will hopefully provide attractive new drug targets. Among such targets, the *Trypanosoma cruzi trans*-sialidase (TcTS) has been extensively studied. TcTS is a glycosylphosphatidylinositol-anchored surface protein that is differentially expressed during the infective developmental stage of the parasite.[4] It belongs to the glycoside hydrolase family 33 (http://afmb.cnrs-mrs.fr/CAZY) and catalyzes the α-(2,3) transfer of sialic acid residues from host glycoconjugates to the terminal galactosyl units of mucin-like glycoproteins on the surface of the parasite.[5] Extensive sialylation of the *T. cruzi* surface is pivotal for the establishment of a chronic infection. The crystal structure of TcTS was recently solved, and a two-step, double-displacement mechanism involving a covalent sialyl–enzyme intermediate was demonstrated (Scheme 1).[6] Tyr342 was identified as the catalytic nucleophile by the use of 3-fluorosialyl fluoride **1** as a substrate analogue, which forms a trapped intermediate species (Scheme 1).[7] The fluorine atom at C3 inductively destabilizes the positively charged oxocarbenium ion like transition states, thereby slowing both the formation and the hydrolysis of the covalent intermediate. However, the presence of a good leaving group at C2 allows glycosylation to proceed rapidly and results in the trapping of the sialyl–enzyme intermediate. Compounds of this class therefore have the potential to act as anti-trypanosomal agents if they can be made selective for TcTS over human sialidases and if they form long-lived intermediates.



Scheme 1. Mechanism of action of *T. cruzi trans*-sialidase.

3-Fluorosialyl fluoride **1** was shown previously to inactivate wild-type TcTS in a time-dependent manner according to the kinetic model illustrated in Scheme 2.[8] However, after removal of excess inactivator, the enzyme rapidly recovered full activity by transglycosylation when incubated with the natural acceptor lactose. Such rapid transglycosylation would render these compounds useless as anti-trypanosomal agents.

Inspection of the three-dimensional structures of TcTS and the only human sialidase as to yet be characterized crystallographically, Neu2,[9] revealed that the TcTS site is more spacious and hydrophobic in the region around C9 of

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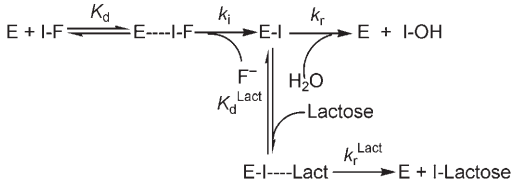


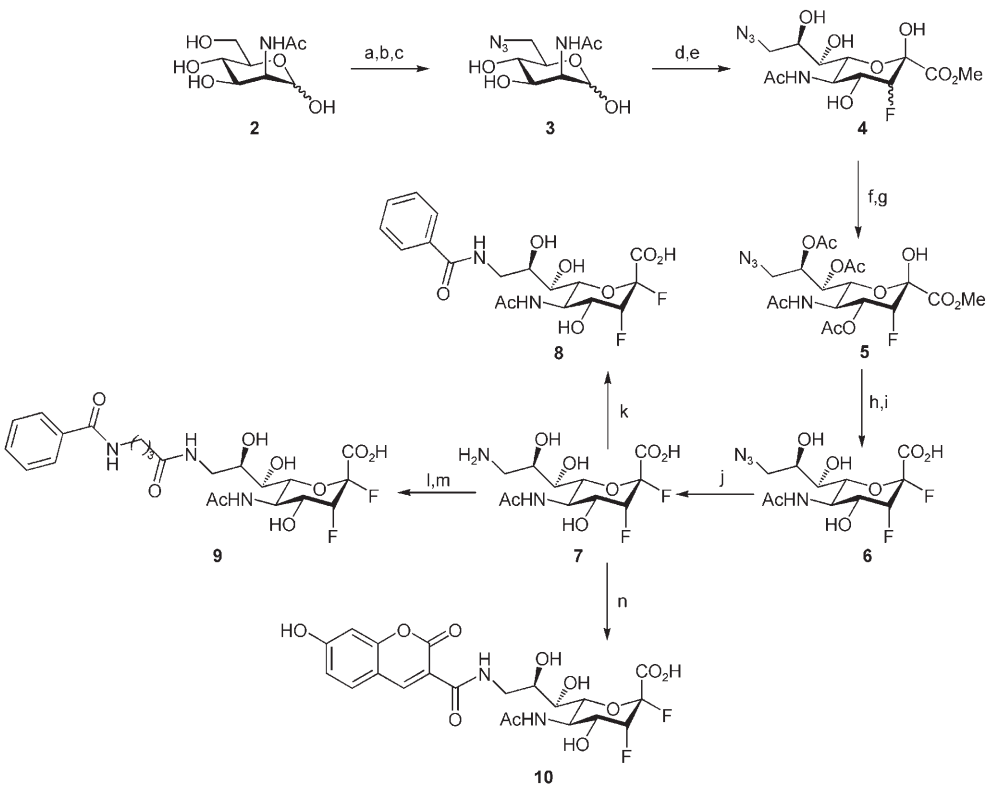
Scheme 2. Kinetic scheme for inhibition. I-F: inhibitor fluoride, E: enzyme, E-I: sialyl–enzyme intermediate.

the sialic acid than is Neu2. Incorporation of a larger substituent, such as an aromatic group, at C9 might therefore confer specificity. In addition, such a substitution might well affect the positioning of the nearby Tyr119 group, which moves upon binding of sialic acid and appears to be important in the binding of lactose.[6,7] This could alter transglycosylation rate constants.

A second generation of 3-fluorosialyl fluoride derivatives bearing various modifications at C9 was thus developed by following a similar synthetic pathway to that used previously for **1** (Scheme 3).[10] 6-Azido-6-deoxy-*N*-acetylmannosamine (**3**)[11] was coupled with 3-fluoropyruvic acid using the commercially available *N*-acetylneuraminic acid aldolase (Neu5Ac aldolase, EC: 4.1.3.3) to yield a mixture of 9-azido-3-fluorosialic acids (axial/equatorial 5:1 to 10:1), which were separated after protection. Selective deprotection of the anomeric site with hydrazine acetate yielded the hemiacetal **5** which, upon treatment with DAST followed by Zemplén deacetylation and saponification, was converted into **6**.

Catalytic hydrogenation afforded the corresponding 9-amino derivative **7**, which was coupled to several activated esters to give **8**, **9**, and **10**. The configurations at C2 and C3 were assigned on the basis of ^{1}H and ^{19}F NMR coupling constants.

Incubation of TcTS with different concentrations of each compound resulted in time-dependent decreases in the enzyme activity, as expected for mechanism-based inhibitors acting according to Scheme 2, and as shown for **8** in Figure 1 A. Saturation behavior was not observed, as inactivation of the enzyme at high concentrations of the inactivators was too fast to allow reliable rate measurements. Individual values of k_i and K_d could not therefore be determined. However, the second-order rate constant k_i/K_d was calculated from the slope of the plot of the apparent first-order rate constants versus the concentration of **8** (Figure 1 B).



Scheme 3. Synthesis of the 3-fluorosialyl fluorides. a) TsCl, Py, 4 h, 0 °C, then Ac_2O, 4 h, RT, 69%; b) TMS-N_3, TBAF, CH_3CN, 9 h, 90 °C, 82%; c) NaOMe, MeOH, 2 h, 0 °C, 85%; d) F-pyruvate sodium salt, Neu5-aldolase (23 U mg^{-1}), H_2O, 10–20 h, RT, 93%–quantitative; e) Amberlite IR-120 H, MeOH, overnight, RT, 80–88%; f) Ac_2O, Py, 20 h, 0 °C→RT, 62%; g) hydrazine acetate, CH_2Cl_2/MeOH, 6 h, 0 °C, 60–80%; h) DAST, CH_2Cl_2, 1 h, −30 °C, 60–80%; i) NaOMe, MeOH, 3 h, RT, then NaOH, 40 min, RT, quantitative; j) cat. Pd/C, MeOH, H_2, 7 h, RT, quantitative; k) *N*-(benzoyloxy)succinimide, Et_3N, DMF, 5 h, RT, 68%; l) pentafluorophenyl-4-(*tert*-butoxycarbonylamino)butanoate, Et_3N, DMF, 4 h, RT, 83%; m) *N*-(benzoyloxy)succinimide, Et_3N, DMF, 6 h, RT, 52%; n) *N*-succimidyl-7-hydroxycoumarin-3-carboxylate, sodium carbonate buffer pH 8.5, DMF, 14 h, RT, 42%. Ts = toluene-4-sulfonyl, Py = pyridine, TMS = trimethylsilyl, TBAF = tetrabutylammonium fluoride, DAST = diethylaminosulfur trifluoride.

As summarized in Table 1, with the exception of **7**, the k_i/K_d values for the compounds tested are all of the same order of magnitude, with **6** and **10** being the most efficient in the series. The presence of aryl substituents (**8, 9, 10**) had no deleterious effect on the inactivation, and in one case improved it, while clearly the presence of the charged ammonium substituent at C9 (**7**) did.

Samples of TcTS that had been inactivated with each reagent and from which excess inactivator had been removed by gel filtration were incubated in the presence of different concentrations of lactose and the reactivation of TcTS monitored. No reactivation was observed in the absence of lactose; furthermore, much higher concentrations of lactose were necessary to promote significant reactivation by transglycosylation than was the case for **1** (Figure 1 C, Table 1). Saturation behavior was

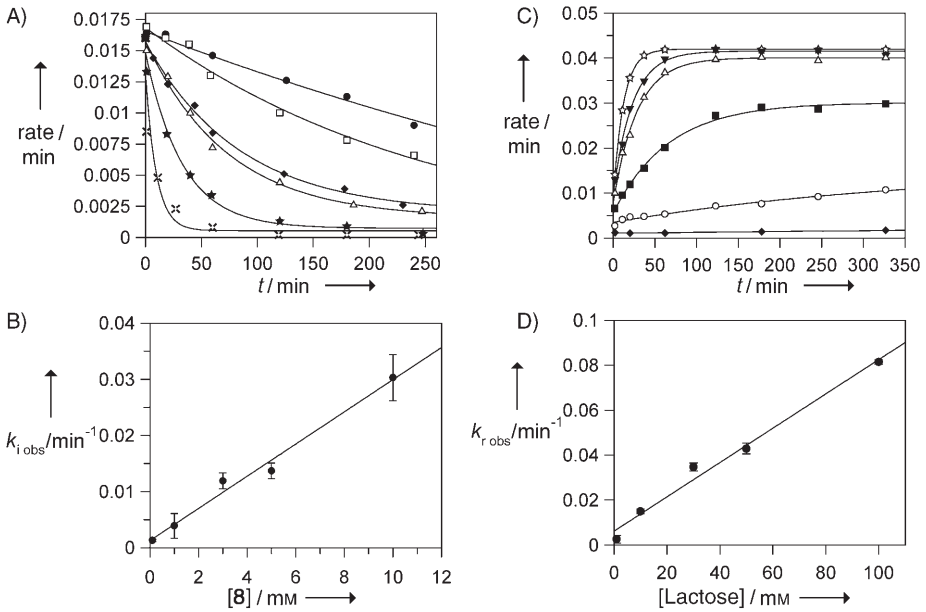


Figure 1. Time-dependent inactivation and reactivation of wild-type TcTS by **8**. The enzyme was incubated with the indicated concentrations of **8**, and aliquots assayed with 0.5 mM trifluoromethylumbelliferyl sialic acid substrate. A) Inactivation by **8** at 0.1 mM (●), 1 mM (□), 3 mM (◆), 5 mM (△), 10 mM (★), and 20 mM (×). B) Plot of pseudo-first-order inactivation kinetic constants ($k_{i\,obs}$) from (A) versus concentration of **8**. C) Reactivation of TcTS in the presence of lactose at 0 mM (◆), 1 mM (○), 10 mM (■), 30 mM (△), 50 mM (▼), and 100 mM (☆). D) Plot of the first order reactivation kinetic constants ($k_{r\,obs}$) from (C) versus concentration of lactose.

observed only for the 9-hydroxy and 9-azido (**1** and **6**) derivatives and in these cases individual values for the kinetic parameters for reactivation by lactose (k_r^{Lact}) and K_d^{Lact} could be measured. For the other sialic acids, in the absence of saturation, an accurate second-order rate constant k_r^{Lact}/K_d^{Lact} was derived from the plot of the apparent rate constants versus the lactose concentration (Figure 1D). Astonishingly the presence of an aromatic functionality at C9 (in **8**, **9**, and **10**) led to more than a 1000-fold decrease in the second-order rate constants for reactivation by lactose. Although it was not possible to accurately measure the individual kinetic parameters for reactivation (k_r^{Lact} and K_d^{Lact}) it seems probable that the major effect is on the binding of lactose (K_d^{Lact}), since the K_m value for lactose with wild-type TcTS is about 40 μM (data not shown) while the K_d^{Lact} value in this system is likely over 100 mM.

Insight into the structural basis for the effect of the aryl substituent at C9 was provided by X-ray crystallographic analysis of the trapped glycosyl–enzyme intermediate (for technical details, see the Supporting Information). The structure measured at a resolution of 1.7 Å reveals that the presence of the benzoyl substituent at C9 induces a reorientation of the whole glycerol side chain compared to the unsubstituted covalent complex (Figure 2A).[7] This effect is a direct consequence of the pocket (W120, T121, Q195, V203) being too small to accommodate the bulky benzoyl group. The final position of the glycerol group of the sialic acid closely resembles that found in the Michaelis complexes of TcTS with sialyl–lactose (SL) or 4-methylumbelliferyl α-D-sialoside (MU-NANA).[7] The benzoyl group occupies the space that would otherwise accommodate the glucose moiety of the lactose acceptor and interacts hydrophobically with the phenyl side chain of Tyr119, thereby locking it in the conformation seen when the lactose binding site is filled (Figure 2B). Interestingly a molecule of 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (hepes) is bound in the galactose site in this case. The 9-benzoyl substituent therefore seriously impairs the binding of lactoside acceptors, drastically reducing reactivation rates without any deleterious effect on the inactivation by the sialyl fluoride.

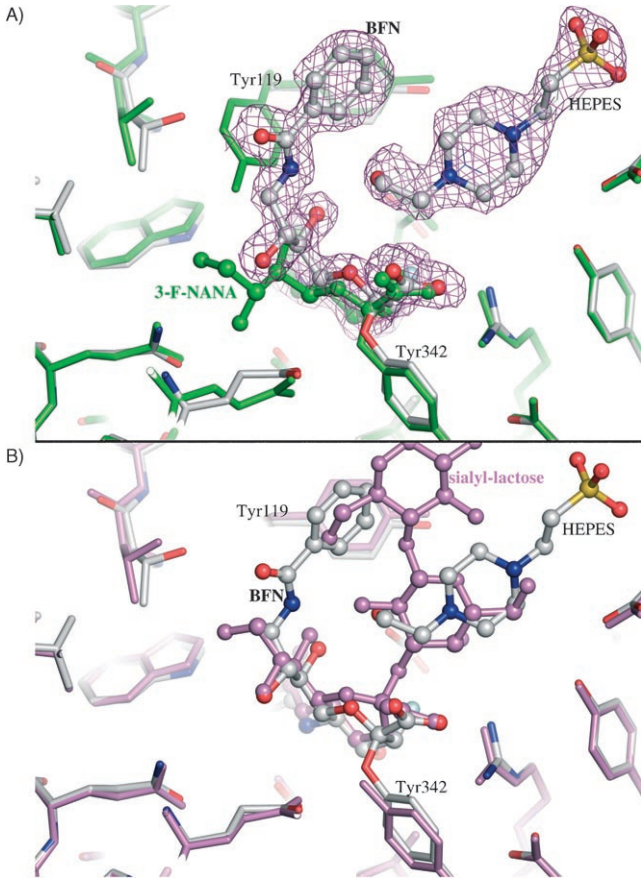


Figure 2. X-ray structure of the covalent intermediate formed between TcTS and BFN (**8**). 3D superpositions with related complexes are shown to highlight the induced structural rearrangements as described in the text. A) TcTS–BFN structure (colored according to atomic elements) superimposed onto TcTS-3F-NANA (2AH2, in green). Note the variation of the glycerol group of NANA. The final refined 2 mFobs-DFcalc Fourier map is contoured at 1.2σ, only drawn around the BFN and the hepes moieties for clarity. B) TcTS–BFN superimposed on to the TcTS–sialyl-2,3-lactose Michaelis complex (1SOI, in violet), in the same orientation as in (A). Note the single conformation of Tyr119, equivalent in the two structures. The benzoyl substituent in BFN blocks the binding of lactose. BFN = 9-benzoyl-3-fluoro-*N*-acetylneuraminic acid.

Table 1: Kinetic parameters for the reaction of wild-type TcTS with 3-fluorosialyl fluorides bearing substituents at C9.[a]

Compd	$k_i/K_d \times 10^{-3}$ [$min^{-1}\,mM^{-1}$]	$k_r^{Lact} \times 10^{-3}$ [min^{-1}]	K_d^{Lact} [mM]	$(k_i/K_d)^{Lact} \times 10^{-3}$ [$min^{-1}\,mM^{-1}$]
1	8.0 ± 0.4	120 ± 16	0.09 ± 0.02	1330 ± 345
6	14 ± 0.8	23 ± 5	0.05 ± 0.02	460 ± 210
7	N.D.	N.D.	–	–
8	2.9 ± 0.2	N.D.	> 200[b]	0.8 ± 0.05
9	3.2 ± 0.5	N.D.	> 500[b]	0.3 ± 0.02
10	14 ± 1.8	N.D.	> 150[b]	0.3 ± 0.02

[a] All the experiments were carried out in 20 mM tris(hydroxymethyl)-aminomethane (Tris)/30 mM NaCl buffer, pH 7.6 containing 5% bovine serum albumin (BSA). [b] Extrapolated from the highest lactose concentration. N.D. = not detectable.

This is an ideal characteristic for *trans*-sialidase inactivators. Furthermore, the ability to also incorporate a fluorescent moiety may allow the monitoring of labeled trypanosomes in vivo. Encouragingly, preliminary studies show that these C9-substituted compounds inactivate the human sialidase Neu2 some 150 times more slowly than do the parent inactivators, as desired. With this proof of principle having been established, current efforts are focused upon improving inherent affinities (K_d) of the reagents to allow their use at lower concentrations as potential anti-trypanosomals.

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