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α-Selective glycosylation affords mucin-related GalNAc amino acids and diketopiperazines active on *Trypanosoma cruzi*

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ABSTRACT

This work addresses the synthesis and biological evaluation of glycosyl diketopiperazines (DKPs) cyclo[Asp-(αGalNAc)Ser] **3** and cyclo[Asp-(αGalNAc)Thr] **4** for the development of novel anti-trypanosomal agents and *Trypanosoma cruzi trans*-sialidase (TcTS) inhibitors. The target compounds were synthetized by coupling reactions between glycosyl amino acids αGalNAc-Ser **7** or αGalNAc-Thr **8** and the amino acid (*O*-*t*Bu)-Asp **17**, followed by one-pot deprotection-cyclisation reaction in the presence of 20% piperidine in DMF. The protected glycosyl amino acid intermediates **7** and **8** were, in turn, obtained by α-selective, $HgBr_2$-catalysed glycosylation reactions of Fmoc-Ser/Thr benzyl esters **12**/**14** with $\alpha GalN_3Cl$ **11**, being, subsequently, fully deprotected for comparative biological assays. The DKPs **3** and **4** showed relevant anti-trypanosomal effects (IC_{50} 282–124 μM), whereas glycosyl amino acids **1** and **2** showed better TcTS inhibition (57–79%) than the corresponding DKPs (13–25%).

1. Introduction

Among parasitic illnesses, Chagas' disease, caused by the protozoa *Trypanosoma cruzi*, accounts for 10,000 deaths annually, 30% of the patients in chronic condition develop severe cardiac disorders and 10% suffer from intestinal illnesses, both involving clinical manifestations that can lead to death after ten years. Thus, the long-term disability and mortality induced by Chagas' disease have been resulting in significant economic and social impacts in many South and Central American countries.[1–3] Despite the attempts to develop vaccines, the current treatment and control of Chagas' disease depend on chemotherapeutic agents such as benznidazol (Rochagan®) and nifurtimox (Lampit®), which are almost 100% effective in the acute infection treatment, however, they have limited efficacy in the chronic infection phase and produce severe side effects due to their high toxicity.[4,5] In this context, posaconazole, an antifungal triazole interfering with sterols biosynthesis, has been poised as a new effective treatment for the acute and chronic Chagas' disease patients and is entering a phase II clinical trial in the near future.[5–7]

Trypanosoma cruzi mucins found in insect epimastigote stage and in the infective trypomastigotes are GPI (glycosylphosphatidylinositol)-anchored plasma membrane glycoproteins that play a role in the host cell invasion and in the ability to induce secretion of pro-inflammatory cytokines IL-12, TNF-α and nitric oxide in activated macrophages.[8,9] Comparative studies involving *O*-glycans of *T. cruzi* mucins and those found in mammalian systems have revealed major differences between both species, such as the peptide backbone linked through an α-*N*-acetylglucosamine (αGlcNAc) residue in *T. cruzi*, which are further substituted by galactose at O-4 and O-6 positions, rather than the α-*N*-acetylgalactosamine (αGalNAc) substituted at O-3 and O-6 positions in mammalian mucins (Fig. 1).[10,11]

The interaction between parasite mucins (TcMUC) and a unique surface *trans*-sialidase enzyme (TcTS) is the most important mechanism by which parasite invades cells and causes infections since TcMUC must be sialylated by the action of TcTS before entering the cells. This process involves the transfer of sialic acid from host glycoconjugates to β-galactopyranosyl acceptor groups on *T. cruzi* mucins, forming an α-2,3 linkage catalysed by TcTS, that does not require CMP-sialic acid as the monosaccharide donor (Fig. 1). As a result, the parasite acquires about 1×10^7 sialic acid residues, increasing the parasite virulence and protecting it against complement-independent lyses induced by human anti-α-galactosyl antibodies because of the strong negative charge on the surface.[8,9,11–13] However, although it is primarily a transferase, TcTS does have some residual hydrolase activity.[14]

All these features contribute to consider TcTS as a potential target, especially for searching a drug that could display potent and selective activity against the parasite during the infection process.

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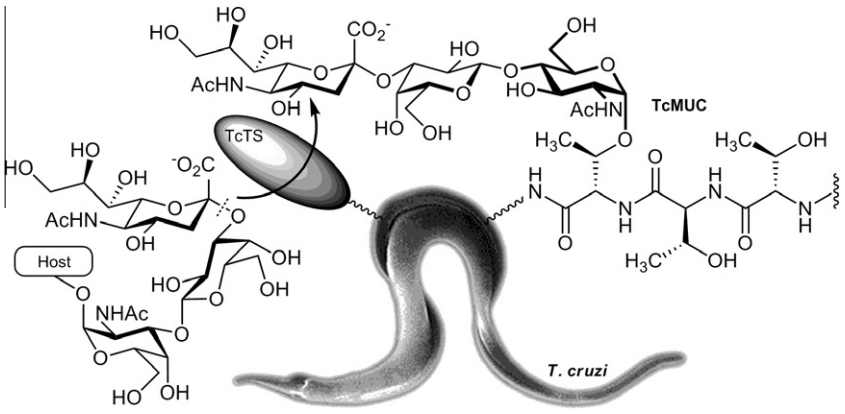


Figure 1. Model of recognition and sialylation of parasite surface mucins by *T. cruzi trans*-sialidase.

In this regard, a panel of TcTS inhibitors described so far are able to occupy both donor (sialic acid) and acceptor (β-galactose) binding sites, so that the sialic acid transfer reaction is hindered or even blocked.[15] Thus, a remarkable activity against TcTS was achieved by assaying sulfonamide chalcones (IC_{50} 0.9–2.5 μM) and quinolinones (IC_{50} 0.5–0.6 μM), obtained by chemical synthesis.[16] Alternatively, the identification of potential inhibitors by molecular docking studies on TcTS active site, such as the recently described benzothiazole derivatives (IC_{50} 0.12–0.15 μM), were accomplished by in silico screening,[17] exploring the crystal structure of TcTS in complex with different substrates (donor/acceptor) or inhibitors,[18–21] and more recently, with Fab fragment of a neutralizing monoclonal IgG antibody.[22] At last, the biological screening using a wide natural product library led to the identification of a series of potent flavonoids (IC_{50} 17 μM) and anthraquinones (IC_{50} 0.58 μM).[23]

Accordingly, we assumed that hybrid molecules comprising an αGalNAc residue linked by a rigid diketopiperazine bridge to a carboxylic function, mimicking the charged sialic acid that interacts with the enzyme, could at same time occupy and block, respectively, both acceptor and donor TcTS binding sites, impairing the enzyme activity and then parasite cell invasion. Thus, the purpose of using αGalNAc in the place of βGal TcMUC core is supported by the fact that it does not act as a sialic acid acceptor due to the α-configuration, but it still has C-3 and C-4 required configurations to interact with the acceptor site, besides the resemblance with the natural αGlcNAc core. In addition, the 2,5-diketopiperazine (DKP) linker represents a peculiar rigid heterocyclic system with defined substituents around a proteolysis-resistant peptide-mimicking scaffold, which can be straightforwardly obtained by a conventional synthetic method, such as the cross-link between two amino acid residues via a pair of complementary amide bonds.[24,25] To investigate the influence of αGalNAc on the DKP-containing carboxylic group in the TcTS activity, we accomplished the synthesis of glycosyl amino acids αGalNAcSer (**1**) and αGalNAcThr (**2**), as well as their protected forms as building blocks to obtain target glycosyl diketopiperazines containing Ser/Thr and Asp amino acids, represented by *cyclo*[Asp-(αGalNAc)Ser] (**3**) and *cyclo*[Asp-(αGalNAc)Thr] (**4**) (Fig. 2). Therefore, this work focuses on the synthesis of compounds **1**–**4** and their biological evaluation as TcTS inhibitors and anti-trypanosomal agents.

2. Results and discussion

2.1. Synthesis

The target glycosyl diketopiperazines are represented by *cyclo*[Asp-(αGalNAc)Ser] **3** and *cyclo*[Asp-(αGalNAc)Thr] **4**, and originated from the head-to-tail cyclisation of dipeptides

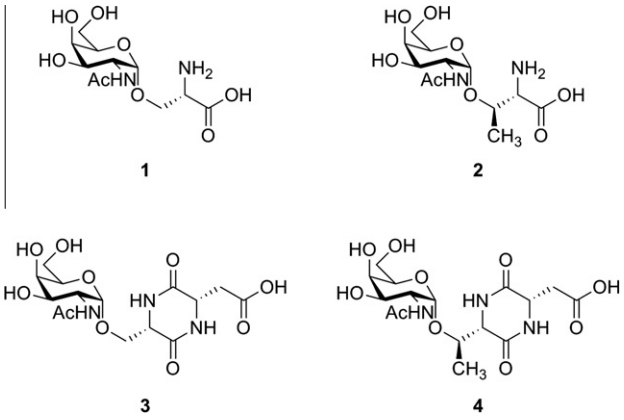


Figure 2. Structures of the target glycosyl amino acids **1** and **2**, and corresponding glycosyl diketopiperazines **3** and **4**.

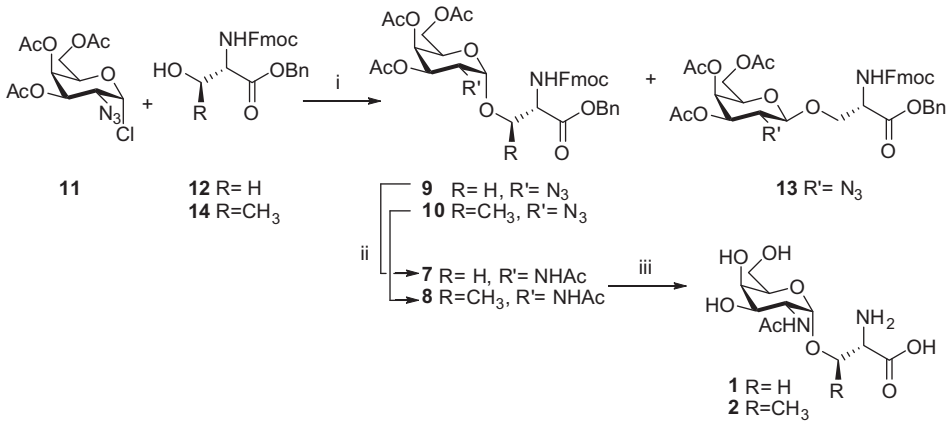
FmocAsp-(αGalNAc)SerOBn (**5**) and FmocAsp-(αGalNAc)ThrOBn (**6**). Their synthesis involved the previous preparation of protected glycosyl amino acids αGalNAc-FmocSerOBn (**7**) and αGalNAc-FmocThrOBn (**8**) as source of the mucin fundamental scaffold, which is supposed to be critical for the intended biological activity.

2.1.1. Synthesis of the glycosyl amino acids

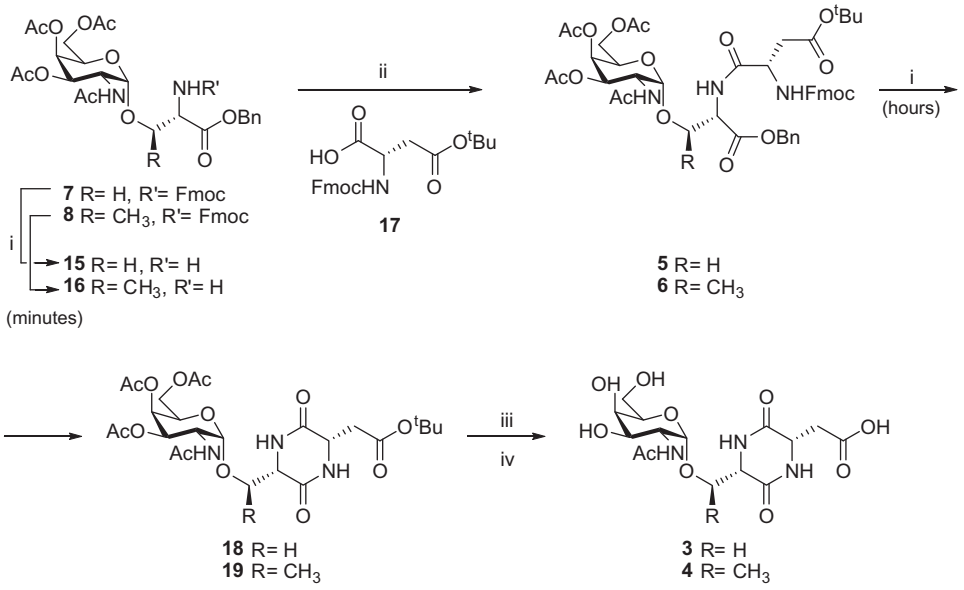
In the context of the preparation of glycosyl diketopiperazines **3** and **4** containing α-linked glycoside, the proposed synthetic routes to obtain the key precursors αGalN_3-FmocSerOBn (**9**) and αGalN_3-FmocThrOBn (**10**) were designed to favour selectively the formation of the α-anomer during glycosylation. For this purpose, the azide-derived glycosyl donor αGalN_3Cl (**11**),[26] unable to exhibit neighbouring group effect and straightforwardly converted to acetamide group, was employed in glycosylation reactions with suitably protected serine and threonine amino acids.

Anomeric fluorides, bromides and iodides have also been used in similar synthetic approaches, as well as several glycosyl donors with leaving groups other than halides, such as trichloroacetimidate, pentenyl ether, phenylthiol or phenylseleno derivatives. Glycosylation catalysts are commonly Lewis acids, notably silver perchlorate or triflate, in the presence of inorganic or alkylpyridine bases.[27–31] Nonetheless, the most employed glycosylation method to obtain building blocks such as **9** and **10** relies on Köenigs–Knorr strategy, particularly using silver salts as catalysts, since they are effective to assist leaving groups displacement by complexation with anomeric halides.[32]

In this regard, the glycosylation of FmocSer benzyl ester (**12**) with αGalN_3Cl **11** was firstly attempted utilizing Ag_2CO_3 and



Scheme 1. Synthesis of GalNAc-derived glycosyl amino acids. Reagents and conditions: (i) $HgBr_2$/1,2-DCE; (ii) Zn, $CuSO_4$, Ac_2O/AcOH/THF; (iii) H_2/Pd-C 10%; NaOMe/MeOH.



Scheme 2. Synthesis of glycosyl diketopiperazines. Reagents and conditions: (i) 20% piperidine/DMF; (ii) PyBOP, HOBt, DIEA/DMF; (iii) NaOMe/MeOH; (iv) TFA/DCM (1:1).

$AgClO_4$ as catalytic system, in toluene/DCM mixture. Despite of the extended reaction times and catalyst excess additions, the expected α-glycoside **9** was isolated in a very low yield (10%), leading us to exploit the use of an alternative Lewis acid catalyst, specifically the mercuric bromide, as previously described by Carvalho et al.[33,34] Therefore, the glycosylation performed under $HgBr_2$ catalyst gave the α-glycoside **9** in 48% yield, besides the βGalN_3-FmocSerOBn (**13**), the unexpected corresponding β-linked isomer, in 4% yield (Scheme 1). Even though nonselective, this reaction represents a direct approach to obtain both α and β anomers, which can be isolated by chromatography column under gradient conditions (EtOAc/toluene). The configuration of the formed α and β anomers **9** and **13** was assigned by their distinct coupling constants for H-1 doublet in ^{1}H NMR spectra, respectively with $J_{1,2}$ 3.2 and 8.0 Hz.

Regarding the glycosylation of FmocThr benzyl ester (**14**) with **11**, the α anomer **10** was obtained in 54%, whereas only trace amounts of the β anomer might have been formed, as judged by TLC, and could not be isolated (Scheme 1). Similarly to compound **9**, the spectrum of the α-glycoside **10** showed characteristic signal of H-1 with $J_{1,2}$ 3.4 Hz. According to the different results observed for the glycosylation of serine and threonine amino acids, the latter bearing an additional chiral centre in β carbon, stereoselectivity under the described conditions appears to be dependent on the acceptor nature, as already verified by Lemieux[26] earliest studies with the azido chloride donor.[‡]

Subsequently, the sugar azido groups of the glycosyl amino acids **9** and **10** were reductively acetylated using zinc powder in THF–acetic anhydride–acetic acid,[34] which afforded the corresponding αGalNAc-FmocSerOBn **7** and αGalNAc-FmocThrOBn **8**, in yields varying from 62% to 67% (Scheme 1). ^{1}H NMR analysis of **7** and **8** showed deshielded H-2 signals in comparison to the precursors **9** and **10**, besides the presence of NH*Ac*. The α-anomers **7** and **8** were then further utilised in the synthesis of the glycodipeptides **5** and **6**.

In parallel, small amounts of compounds **7** and **8** were fully deprotected (removal of *O*Bn, *N*Fmoc and *O*Ac groups) in order to assess their biological activities, considering that in this form they represent an analog of the elemental *T. cruzi* mucin core αGlcNAc-Ser/Thr. Thus, standard hydrogenation reactions (10% Pd-C/H_2) followed by Zemplen deacetylations afforded the final deprotected glycosyl amino acids **1** (80%) and **2** (90%),[34] whose structures were confirmed by the ^{1}H NMR and ESI-MS analysis (Scheme 1).

[‡] Lemieux (1979) have described $Hg(CN)_2$ catalysed glycosylation with azido chloride donor and benzyl or *tert*-butyl alcohols as acceptors, obtaining glycoside products with stereoselectivity of around 5 and 10 α/β ratio, respectively.

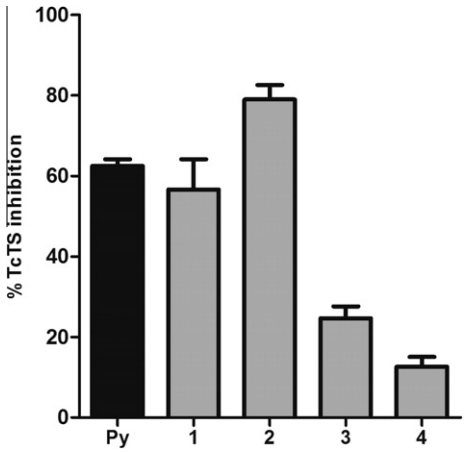


Figure 3. TcTS inhibition of glycosyl amino acids **1** and **2** and glycosyl diketopiperazines **3** and **4**. The results are based on three independent experiments, each one performed in triplicate.

2.1.2. Synthesis of glycosyl diketopiperazines

The synthesis of the glycosyl diketopiperazines *cyclo*[Asp-(αGalNAc)Ser] **3** and *cyclo*[Asp-(αGalNAc)Thr] **4** was accomplished in four steps, as outlined in Scheme 2. Firstly, the *N*-Fmoc deprotection of the glycosyl amino acids **7** and **8** with 20% piperidine in DMF resulted in the free amino products αGalNAc-SerOBn (**15**) (78%) and αGalNAc-ThrOBn (**16**) (69%), which were then condensed with the commercially available amino acid Fmoc-(*O*-*t*Bu)-Asp (**17**) to form a peptide bond, in the presence of the coupling reagents PyBOP, HOBt, and the basic catalyst DIEA in DMF. The glycodipeptides FmocAsp-(αGalNAc)SerOBn **5** and FmocAsp-(αGalNAc)ThrOBn **6** were isolated in 93% and 86% yields, respectively,[25] and their structures were confirmed by ^{1}H NMR spectra, with anomeric doublets ($J_{1,2}$ 3.4 and 3.5 Hz), and signals of characteristic methylenic hydrogens of the aspartic acid unit, together with ESI-MS confirmatory adducts. Subsequently, the *N*-Fmoc deprotection of the glycodipeptides **5** and **6** by treatment with 20% piperidine in DMF[25] led to the intramolecular nucleophilic attack of the free amino groups (in basic conditions) of **5** and **6** toward their terminal carboxylate function, with displacement of the benzyloxy group, giving the stable glycosyl diketopiperazines *cyclo*[Asp-(αGalNAc)Ser] (**18**) and *cyclo*[Asp-(αGalNAc)Thr] (**19**). Compounds **18** and **19** were obtained in the corresponding yields of 48% and 52% after purification by chromatography column, and their structures were confirmed by ^{1}H NMR spectroscopy, which showed the absence of aromatic and alkylic hydrogens of both *N*Fmoc and particularly *O*Bn protecting groups. Finally, full deprotection of glycosyl diketopiperazines **18** and **19** by means of Zemplen deacetylations, followed by treatment with TFA/DCM (1:1) for cleavage of *O*-*t*Bu group, afforded the final deprotected glycosyl diketopiperazines **3** (90%) and **4** (84%), confirmed by ^{1}H NMR and ESI-MS analysis.

2.2. Biological assays

2.2.1. *Trypanosoma cruzi trans*-sialidase inhibition assays involving compounds 1-4

The enzymatic inhibition assays involving glycosyl amino acids **1** and **2**, and glycosyl diketopiperazines **3** and **4** were performed using the continuous fluorimetric method, which is based on TcTS-catalyzed MuNANA hydrolysis.[35] Compounds were tested at 1.0 mM concentration, along with pyridoxal phosphate (Py) as control, which is reported to be a weak TcTS inhibitor (60%).[17]

As a result, compound **2** showed high TcTS inhibition (79%) at 1.0 mM, followed by compound **1**, for which satisfactory inhibitory activity (57%) was also verified (Fig. 3). Considering its significant TcTS inhibitory activity at 1.0 mM, compound **2** was further tested at 0.5 and 0.25 mM, resulting in an IC_{50} value of 0.32 mM. Conversely, compounds **3** (25%) and **4** (13%) presented very weak inhibitory activity at 1.0 mM (Fig. 3).

Therefore, this noteworthy difference of TcTS inhibition observed for glycosyl amino acids **1** and **2**, and glycosyl diketopiperazines **3** and **4** points out that the Asp-containing DKP heterocyclic system did not exert a relevant role in TcTS inhibition in comparison to the precursors **1** and **2** (see Fig. S14 in Supplementary data).

2.2.2. In vitro anti-trypanosomal activities of compounds 1–4 and cytotoxicity towards mammalian cells

The glycosyl amino acids **1** and **2** and glycosyl diketopiperazines **3** and **4** were evaluated against trypomastigote and amastigote forms of *T. cruzi* Tulahuen strain cultured with LLC-MK2 cells using benznidazole (*N*-benzyl-2-nitro-1-imidazolacetamide, Bz), the current frontline drug used to treat Chagas' disease, as control.[36,37]

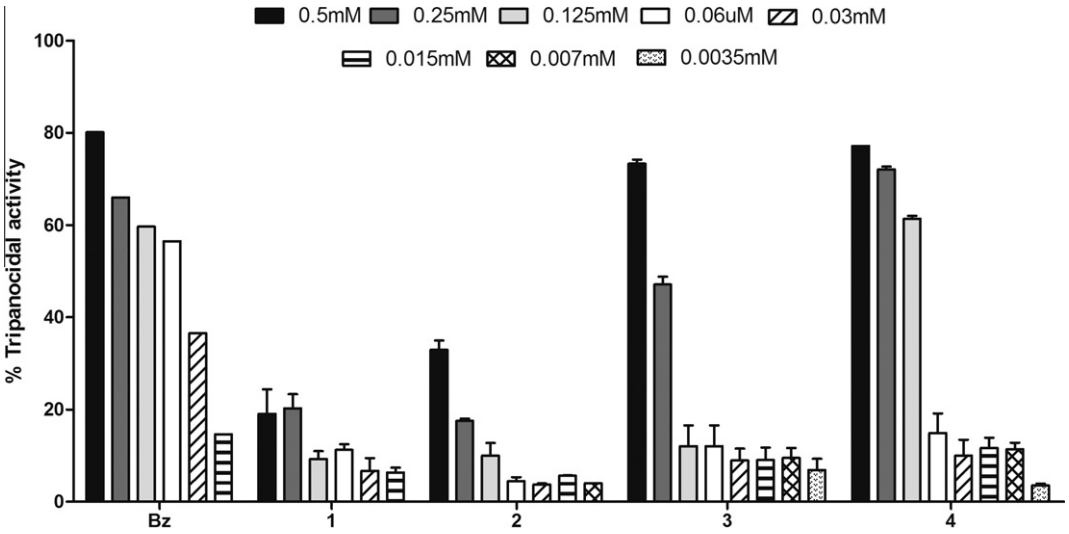


Figure 4. Percentage of anti-trypanosomal activities of compounds **1**–**4** and reference compound benznidazole (Bz) against *Trypanosoma cruzi* Tulahuen strain cultured with LLC-MK2 cells. The results are based on three independent experiments, each one performed in duplicate.

Table 1
Anti-trypanosomal activities of compounds **1**–**4** and benznidazole (Bz) expressed as IC_{50}

Compound	IC_{50} (mM)
1	>0.5
2	>0.5
3	0.282 ± 0.008
4	0.124 ± 0.007
Bz	0.030 ± 0.001

The values were calculated based on results from Figure 4 that were obtained from three independent experiments, each one performed in duplicate.

Results of parasite viability, measured based on a colorimetric reaction with Chlorophenol red-β-D-galactoside (CPRG) as substrate for *T. cruzi* β-galactosidase, are summarised in Figure 4. The concentrations of compounds corresponding to 50% anti-trypanosomal activity are expressed as IC_{50} (Table 1).

As shown in Figure 4 and Table 1, amongst the tested compounds, **4** displayed higher anti-trypanosomal activity against *T. cruzi* Tulahuen strain, presenting an IC_{50} value of 124 μM. In fact, in spite of presenting lower activity than Bz in the concentrations range from 0.125 to 0.015 mM, it displayed anti-trypanosomal activity in the lower concentrations of 7.00 and 3.50 μM, which was not observed for Bz. Regarding the remaining compounds, the glycosyl diketopiperazine **3** showed IC_{50} of 282 μM, while very low activity was observed for compounds **1** and **2**. Thus, these considerable distinct anti-trypanosomal activities observed for glycosyl amino acids **1** and **2**, and the glycosyl diketopiperazines **3** and **4** indicate that, differently from what was observed for TcTS inhibition, the additional 2,5-DKPs heterocyclic system containing Asp had a relevant anti-trypanosomal effect if compared to the single αGalNAc-Ser/Thr **1** and **2**.

In order to verify the ability of compound **4**, which displayed higher tripanocidal activity, to enter the cell and kill the intracellular parasite, Vero cells were infected with *T. cruzi* and after 12 h of incubation, parasites on the supernatant were removed and the cells were treated with compound **4** to additional 24 h. Survival and replication of intracellular amastigotes were evaluated. As shown in the Figure 5, compound **4** was able to enter the cell and was very efficient in eliminating the intracellular parasite in a dose-dependent manner, comparable to benznidazole. Hence, the significant capability to kill *T. cruzi* trypomastigotes and amastigotes forms highlights compound **4** as a prototype for further design of new anti-trypanosomal agents based on DKP scaffold.

To verify whether the toxicity of the compounds was either specific to the parasite or extended to mammalian cell, compounds **1**–**4** were screened against cultured mouse spleen cells. According to Figure 6, none of the compounds were cytotoxic in the range of 500–7.8 μM, the tested concentrations for which anti-trypanosomal activity was observed, showing, thus, the specific role of the compounds **3** and **4** on the parasites.

The fact that TcTS inhibition and anti-trypanosomal activities verified for compounds **1**–**4** were not directly correlated suggest that these compounds may act against *T. cruzi* by a mechanism of action different from the inhibition of the TcTS enzyme, which enables the adhesion and invasion of parasites to host cells, but it is not supposed to have a role in parasite survival in culture.[38] Moreover, considering the absence of cytotoxicity of compounds **1**–**4** against mouse spleen cells, a specific mode of anti-parasite action rather than a generic cytotoxic effect may be proposed.

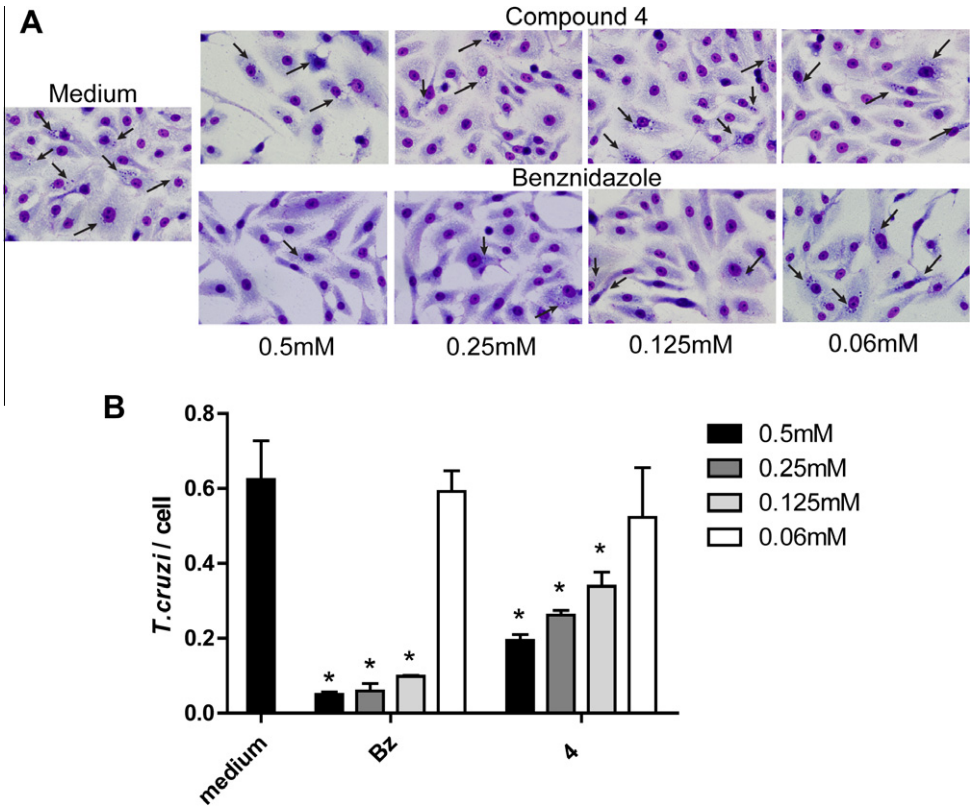


Figure 5. Anti-trypanosomal activity of compounds **4** and Bz against amastigote forms of *T. cruzi*. (A) Representative images of Vero cells infected with *T. cruzi* and treated with Bz, compound **4** or Medium. *The arrows indicate parasites inside the cells. (B) Quantitative analysis of parasites per cell after treatment. Values represent the results from two independent experiments, each one performed in triplicate.

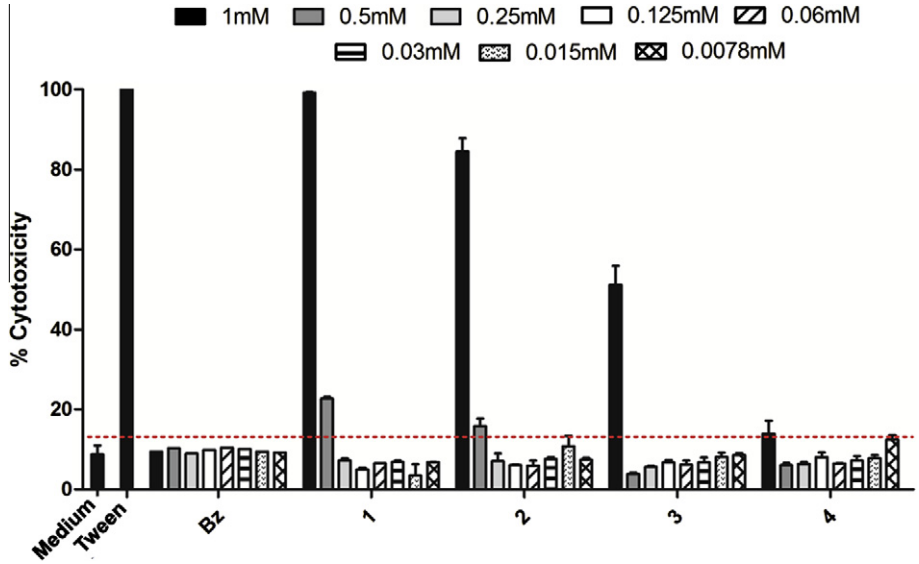


Figure 6. Percentage of cell death caused by compounds **1**–**4** and Bz, evaluated against cultured mouse spleen cells. The data are representative of three independent experiments, each one performed in duplicate.

3. Conclusion

In summary, the αGalNAc-Ser/Thr have been successfully enclosed into diketopiperazine scaffolds containing a carboxylic side chain, by means of amino acid coupling/intramolecular cyclisation approach, providing twofold functionalised compounds **3** and **4**. In addition, we have broadened the application of $HgBr_2$ as a catalyst for glycosylation reactions in distinct glycosyl donor and acceptor combinations, representing a comprehensive and effective method for synthesising glycosyl amino acids. In particular, the synthesis of αGalNAc core **1** and **2**, as analogues of *T. cruzi* mucins, was accomplished, as well as of $\beta GalN_3$ serine glycoside **13** as an unpredicted result.

Mucin-related αGalNAc amino acids and diketopiperazines showed different profiles in the biological assays. In the TcTS inhibition assay, **1** and **2** displayed higher activity (57% and 79%) compared to cyclic analogues, whilst **3** and **4** were much more active in anti-trypanosomal experiments (IC_{50} 282 and 124 μM) than single glycosyl amino acids. Although anti-trypanosomal activity appears to be independent of TcTS inhibition mechanism, it is also not related to a generic cytotoxic effect, so that these findings point out the αGalNAc diketopiperazines as prototypes for designing new anti-trypanosomal agents.

4. Experimental

4.1. General

All chemicals were purchased as reagent grade and used without further purification. Solvents were dried according to standard methods.[39] MuNANA (2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt) was acquired from Toronto Research Chemicals Inc. The *trans*-sialidase used in this study was a His-tagged 70 kDa recombinant material truncated to remove C-terminal repeats but retaining the catalytic N-terminal domain of the enzyme.[40] Reactions were monitored by thin layer chromatography (TLC) on 0.25 nm precoated silica gel plates (Whatman, AL SIL G/UV, aluminium backing) with the indicated eluents. Compounds were visualized under UV light (254 nm) and/or dipping in ethanol/sulfuric acid (95:5, v/v), followed by heating the plate for a few minutes. Column chromatography was performed on silica gel 60 (Fluorochem, 35–70 mesh) or on a Biotage Horizon High-Performance FLASH Chromatography system using 12 or 25 mm flash cartridges with the indicated eluents. Nuclear magnetic resonance spectra were recorded on Bruker Advance DRX 300 (300 MHz), DPX 400 (400 MHz) or DPX 500 (500 MHz) spectrometers. Chemical shifts (δ) are given in parts per million downfield from tetramethylsilane. Assignments were made with the aid of HMQC and COSY experiments. Accurate mass electrospray ionization mass spectra (ESI-HRMS) were obtained using positive or negative ionization modes on a Bruker Daltonics MicroOTOF II ESI-TOF mass spectrometer.

4.2. Synthesis of glycosyl amino acids

4.2.1. General procedure for glycosylation reactions

A mixture of 2-azido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl chloride **11** and *N*-(fluoren-9-ylmethoxycarbonyl)-serine/threonine benzyl ester **12**/**14** (prepared from commercially available amino acid Fmoc-serine/threonine by treatment with caesium carbonate and benzyl bromide in DMF)[41] in 1,2-dichloroethane (2.0 mL) was refluxed for 20 or 24 h with mercuric bromide (2.25 equiv) and the consumption of starting materials was followed by TLC (EtOAc/toluene 2:8, v/v; EtOAc/hexane 7:3, v/v). The resulting amber mixture was concentrated in vacuo and the residue was purified by a silica gel chromatography column (EtOAc/toluene 2:8, v/v; EtOAc/hexane 7:3, v/v).

4.2.1.1. *N*-(Fluoren-9-ylmethoxycarbonyl)-(2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-serine benzyl ester 9 and *N*-(fluoren-9-ylmethoxycarbonyl)-(2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-L-serine benzyl ester 13. According to the general procedure described in Section 4.2.1, the reaction mixture of **11** (204 mg, 0.58 mmol) and **12** (132 mg, 0.32 mmol), in 1,2-dichloroethane (2.0 mL) was refluxed for 20 h with mercuric bromide (258 mg, 0.71 mmol), affording both anomers (α/β 12:1) as amorphous solids (52%) after purification by silica gel chromatography column (EtOAc/toluene 2:8, v/v).

α **9** (112 mg, 0.153 mmol, 48%). δ_H ($CDCl_3$, 300 MHz) 7.69 (2H, d, *J* 7.4 Hz, C*H* Fmoc arom.), 7.55 (2H, d, *J* 7.2 Hz, C*H* Fmoc arom.), 7.35–7.22 (9H, m, C*H* Bn arom., C*H* Fmoc arom.), 5.91 (1H, d, *J* 8.0 Hz, N*H* Ser), 5.31 (1H, d, $J_{3,4}$ 2.6 Hz, H-4), 5.22 (1H, d, $J_{3,4}$ 2.7 Hz, H-3), 5.17 (2H, s, CH_2 Bn), 4.79 (1H, d, $J_{1,2}$ 3.2 Hz, H-1),

4.54 (1H, m, C*H* Ser), 4.33 (2H, d, *J* 7.1 Hz, CH_2 Fmoc), 4.17 (1H, t, *J* 7.1 Hz, C*H* Fmoc), 4.09 (1H, dd, *J* 2.6 Hz, 10.8 Hz, H-5), 4.02–3.85 (4H, m, H-6a, H-6b, CH_2 Ser), 3.51 (1H, dd, $J_{1,2}$ 3.3 Hz, $J_{2,3}$ 11.1 Hz, H-2), 2.07, 2.00, 1.89 (9H, 3s, COCH_3).

β **13** (9 mg, 0.012 mmol, 4%). δ_H ($CDCl_3$, 300 MHz) 7.77 (2H, d, *J* 7.5 Hz, C*H* Fmoc arom.), 7.61 (2H, m, C*H* Fmoc arom.), 7.43–7.28 (9H, m, C*H* Bn arom., C*H* Fmoc arom.), 5.85 (1H, d, *J* 8.4 Hz, N*H* Ser), 5.32 (1H, d, $J_{3,4}$ 3.3 Hz, H-4), 5.25 (2H, AB, J_{AB} 12.3 Hz, CH_2 Bn), 4.76 (1H, dd, $J_{3,4}$ 3.3 Hz, $J_{2,3}$ 10.7 Hz, H-3), 4.61 (1H, m, C*H* Ser), 4.46–4.37 (3H, m, CH_{2a} Ser, CH_2 Fmoc), 4.32 (1H, d, $J_{1,2}$ 8.0 Hz, H-1), 4.23–4.20 (1H, m, C*H* Fmoc), 4.12–4.07 (2H, m, H-6a, H-6b), 3.93 (1H, dd, *J* 3.3 Hz, 10.3 Hz, CH_{2b} Ser), 3.77 (1H, t, *J* 6.7 Hz, H-5), 3.67 (1H, dd, $J_{1,2}$ 8.0 Hz $J_{2,3}$ 10.7 Hz, H-2), 2.16, 2.06, 2.02 (9H, 3s, COCH_3). δ_C ($CDCl_3$, 75 MHz) 170.3, 170.0, 169.7, 169.4 (*C*OCH$_3$, *C*OCH$_2$Bn), 155.9 (*C*O Fmoc), 143.8, 143.7, 141.3, 141.3 (*C*quat. Fmoc), 135.2 (*C*quat. Bn), 128.6, 128.5, 127.7, 127.1, 125.1, 120.0 (*C*H Fmoc arom., *C*H Bn arom.), 102.6 (C-1), 70.9 (C-5), 70.8 (C-3), 70.1 (CH_2 Ser), 67.6 (*C*H$_2$Bn), 67.3 (*C*H$_2$ Fmoc), 66.2 (C-4), 61.1 (C-6), 60.8 (C-2), 54.3 (CH Ser), 47.1 (*C*H Fmoc), 20.6 (*C*OCH$_3$). ESI-HRMS: calcd for $C_{37}H_{39}N_4O_{12}$ $[M+H]^+$ 731.2559, found 731.2576.

4.2.1.2. *N*-(Fluoren-9-ylmethoxycarbonyl)-(2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-threonine benzyl ester 10.

According to the general procedure described in Section 4.2.1, the reaction mixture of **11** (100 mg, 0.286 mmol) and 1**4** (62 mg, 0.143 mmol), in 1,2-dichloroethane (2.0 mL) was refluxed for 24 h with mercuric bromide (115 mg, 0.319 mmol), affording the α anomer **10** as an amorphous solid (56 mg, 0.075 mmol, 54%) after purification by silica gel chromatography column (EtOAc/toluene 2:8, v/v). δ_H ($CDCl_3$, 500 MHz) 7.70 (2H, d, *J* 7.5 Hz, C*H* Fmoc arom.), 7.55 (2H, d, *J* 7.3 Hz, C*H* Fmoc arom.), 7.34–7.22 (9H, m, C*H* Bn arom, C*H* Fmoc arom.), 5.65 (1H, d, *J* 9.3 Hz, N*H* Thr), 5.37 (1H, d, $J_{3,4}$ 2.9 Hz, H-4), 5.22–5.10 (3H, m, $J_{2,3}$ 11.1 Hz, $J_{3,4}$ 3.0 Hz, $J_{A,B}$ 12.2 Hz, H-3 e CH_2 Bn), 4.83 (1H, d, $J_{1,2}$ 3.4 Hz, H-1), 4.40 (2H, t, *J* 7.0 Hz, CH_2 Fmoc), 4.35 (1H, dd, *J* 7.5 Hz, 10.4 Hz, α*CH* Thr), 4.27 (1H, dd, *J* 7.3 Hz, 10.6 Hz, β*CH* Thr), 4.21–4.13 (2H, m, C*H* Fmoc, H-5), 4.00 (2H, d, *J* 6.5 Hz, H-6a, H-6b), 3.52 (1H, dd, $J_{1,2}$ 3.4 Hz, $J_{2,3}$ 11.1 Hz, H-2), 2.08, 2.00, 1.96 (9H, 3s, COCH_3), 1.27 (3H, d, *J* 6.3 Hz, CH_3 Thr).

4.2.1.3. *N*-(Fluoren-9-ylmethoxycarbonyl)-(2-acetamido–deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-serine benzyl ester 7.

N-(Fluoren-9-ylmethoxycarbonyl)-(2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-serine benzyl ester **9** (92 mg, 0.126 mmol, 1 equiv), previously washed with aqueous 5% EDTA solution to remove mercuric remnants, was dissolved in a mixture of THF/Ac_2O/AcOH (5 mL, 3:2:1), followed by addition of zinc dust (102 mg, 1,55 mmol, 12 equiv). The reaction mixture was treated with saturated aqueous 10% $CuSO_4$ solution (100 μL) and stirred for 2 h at room temperature. The mixture was filtered through Celite and co-evaporated with toluene. Purification by chromatography column (EtOAc/hexane 3:2, v/v) afforded the product **7** as a pale viscous oil (63 mg, 0.084 mmol, 67%). δ_H ($CDCl_3$, 500 MHz) 7.71 (2H, d, *J* 7.5 Hz, C*H* Fmoc arom.), 7.55 (2H, d, *J* 7.0 Hz, C*H* Fmoc arom.), 7.35–7.24 (9H, m, C*H* Bn arom., C*H* Fmoc arom.), 5.86 (1H, d, *J* 7.8 Hz, N*H* Ser), 5.60 (1H, d, *J* 9.1 Hz, N*H*CO), 5.24 (1H, d, $J_{3,4}$ 2.1 Hz, H-4), 5.13 (2H, AB, J_{AB} 12.0 Hz, CH_2 Bn), 4.98 (1H, dd, $J_{2,3}$ 11.1 Hz, $J_{3,4}$ 2.1 Hz, H-3), 4.70 (1H, apparent s, H-1), 4.53 (1H, m, C*H* Ser), 4.46 (1H, apparent t, $J_{2,3}$ 11.2 Hz, H-2), 4.38 (2H, d, *J* 6.5 Hz, CH_2 Fmoc), 4.17 (1H, t, *J* 6.6 Hz, C*H* Fmoc), 4.07–3.85 (5H, m, H-5, H-6a, *H*-6b, CH_2 Ser), 2.09, 1.94, 1.91, 1.84 (12H, 4 s, COCH_3).

4.2.1.4. *N*-(Fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-threonine benzyl ester 8.

Following the same procedure described for compound **9**, the glycosyl amino acid **10** (67 mg, 0.090 mmol) was dissolved in a mixture of THF/Ac_2O/AcOH (4.5 mL, 3:2:1), followed by addition of zinc dust (76.5 mg, 1.17 mmol, 12 equiv) and saturated aqueous 10% $CuSO_4$ solution (100 μL). The reaction mixture was then stirred for 2 h at room temperature and was, subsequently, filtered through Celite and co-evaporated with toluene. Purification by chromatography column (EtOAc/hexane 3:2, v/v) afforded the product **8** as a pale viscous oil (42.5 mg, 0.056 mmol, 62%). δ_H ($CDCl_3$, 300 MHz) 7.71 (2H, d, *J* 7.5 Hz, C*H* Fmoc arom.), 7.57 (2H, d, *J* 7.3 Hz, C*H* Fmoc arom.), 7.37–7.24 (9H, m, C*H* Bn arom. e C*H* Fmoc arom.), 5.76 (1H, d, *J* 9.7 Hz, N*H* Thr), 5.62 (1H, d, *J* 9.4 Hz, N*H*CO), 5.31 (1H, d, $J_{3,4}$ 2.2 Hz, H-4), 5.12 (1H, d, *J* 11.9 Hz, CH_2a Bn), 5.03–4.98 (2H, m, CH_2b Bn, H-3), 4.73 (1H, d, *J* 3.6 Hz, H-1), 4.50–4.37 (4H, m, CH_2 Fmoc, α*CH* Thr, β*CH* Thr), 4.22–4.10 (3H, m, C*H* Fmoc, H-5, H-2), 4.02–3.95 (2H, m, H-6a, H-6b), 2.10 (3H, s, COCH_3), 1.96, 1.94, 1.91 (9H, 3s, COCH_3), 1.23 (3H, d, *J* 6.4 Hz, CH_3 Thr).

4.2.1.5. 2-Acetamido-2-deoxy-α-D-galactopyranosyl-L-serine 1.

A solution of *N*-(fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-serine benzyl ester **7** (25 mg, 0.033 mmol) in MeOH (1.0 mL) was treated with glacial AcOH (100 μL) and 10% Pd/C (11 mg) for removal of *O*-Bn and *N*-Fmoc groups. The reaction mixture was stirred and kept under H_2 (~1.5 atm) for 18 h and then, filtered through Celite, concentrated in vacuo and purified by chromatography column (DCM/MeOH 8:2 v/v). The obtained product (9.6 mg, 0.022 mmol, 66%) was dissolved in MeOH (0.5 mL) and the pH was raised by addition of 1 M NaOMe in MeOH. The reaction mixture was stirred for 3 h, and then neutralized with Dowex 50WX8–200 resin. Filtration and concentration of the reaction mixture gave the product **1** as a colourless amorphous solid (5.6 mg, 0.017 mmol, 80%). δ_H (D_2O, 300 MHz) 4.78 (1H, d, $J_{1,2}$ 3.8 Hz, H-1), 4.06–3.96 (3H, m, H-3, H-2, CH_2a Ser), 3.85–3.73 (4H, m, CH_2b Ser, H-4, H-5, C*H* Ser), 3.63–3.60 (2H, m, H-6a, H-6b), 1.88 (3H, s, NHCH_3CO). δ_C (D_2O, 75 MHz) 98.9 (C-1), 72.0 (C-5), 69.1, 68.2, 67.8, 66.0 (C-4, *C*H Ser, C-3, *C*H$_2$ Ser), 61.6 (C-6), 49.9 (C-2), 21.4 (NHCO*C*H$_3$). ESI-HRMS: calcd for $C_{11}H_{21}N_2O_8$ $[M+H]^+$ 309.1292, found 309.1293.

4.2.1.6. 2-Acetamido-2-deoxy-α-D-galactopyranosyl-L-threonine 2.

N-(Fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-threonine benzyl ester **8** (25 mg, 0.033 mmol) was dissolved in MeOH (1.0 mL), followed by treatment with glacial AcOH (100 μL) and 10% Pd/*C* (10 mg) for removal of *O*-Bn and *N*-Fmoc groups. The reaction mixture was stirred and kept under H_2 (~1.5 atm) for 15 h and then, filtered through Celite, concentrated in vacuo and purified by chromatography column (DCM/MeOH 8:2 v/v). The obtained product (5.0 mg, 0.011 mmol, 35.4%) was dissolved in MeOH (0.5 mL) and the pH was raised by addition of 1 M NaOMe in MeOH. The reaction mixture was stirred for 3 h, and then neutralized with Dowex 50WX8–200 resin. Filtration and concentration of the reaction mixture gave the product **2** as a colourless amorphous solid (3.4 mg, 0.010 mmol, 90%). δ_H (D_2O, 500 MHz) 4.82 (1H, m, H-1), 4.07–3.82 (4H, m, H-4, H-3, H-2, C*H*α Thr), 3.62–3.57 (3H, m, H-5, *H*-6a, *H*-6b), 3.50–3.48 (1H, m, C*H*β Thr), 1.90 (3H, s, N*H*CO), 1.25 (3H, d, *J* 6,8 Hz, CH_3 Thr). δ_C (D_2O, 75 MHz) 99.8 (C-1), 72.2 (βCH Thr), 71.6 (C-5), 69.2, 68.7 (C-4, C-3), 61.4 (C-6, αCH Thr), 50.0 (C-2), 21.8 (NCO*C*H$_3$), 17.9 (*C*H$_3$ Thr). ESI-HRMS: calcd for $C_{12}H_{23}N_2O_8$ $[M+H]^+$ 323.1449, found 323.1445.

4.3. Synthesis of glycodipeptides

4.3.1. *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-(*O*-*t*Bu)-L-aspartil-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine benzyl ester 5

N-(Fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-serine benzyl ester **7** (60 mg,

0.080 mmol) was treated with 20% piperidine in DMF (0.5 mL) for removal of the *N*-Fmoc group. The reaction mixture was stirred for 1 h at room temperature and then purified by chromatography column (EtOAc; MeOH/DCM 2:8 v/v) to afford the product **15** as a pale oil (33.5 mg, 0.063 mmol, 78%). Subsequently, a solution of **15** (33.5 mg, 0.063 mmol) in DMF (1.5 mL) was treated with *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-(*O*-*t*Bu)-L-aspartate **17** (32.7 mg, 0.079 mmol, 1.25 equiv), PyBOP (41.4 mg, 0.079 mmol, 1.25 equiv) and HOBt (10.7 mg, 0.079 mmol, 1.25 equiv), and then with the DIEA (25 μL, 20.16 mg, 0.16 mmol, 2.5 equiv) to initiate the reaction. The mixture was allowed to stir for 16 h at room temperature. Purification of the crude mixture by chromatography column (EtOAc/hexane, 7:3 v/v) gave the product **5** as an amorphous solid (54 mg, 0.058 mmol, 93%). δ_H ($CDCl_3$, 300 MHz) 7.69 (2H, d, *J* 7.5 Hz, C*H* Fmoc arom.), 7.53 (2H, d, *J* 6.9 Hz, C*H* Fmoc arom.), 7.36–7.22 (9H, m, C*H* Bn arom., C*H* Fmoc arom.), 6.03–5.97 (2H, m, N*H* Asp, N*H* Ser), 5.23 (1H, d, *J* 2.7 Hz, H-4), 5.10 (2H, s, CH_2 Bn), 5.02 (1H, dd, $J_{3,4}$ 3.0 Hz, $J_{2,3}$ 11.2 Hz, H-3), 4.75 (1H, d, $J_{1,2}$ 3.4 Hz, H-1), 4.73–4.67 (1H, m, C*H* Ser), 4.53–4.41 (2H, m, C*H* Asp, H-2), 4.36 (2H, d, *J* 7.3 Hz, CH_2 Fmoc), 4.16 (1H, t, *J* 7.0 Hz, C*H* Fmoc), 4.06–3.95 (3H, m, H-5, H-6a, H-6b), 3.87–3.83 (2H, m, CH_2 Ser), 2.80 (1H, dd, *J* 4.8 Hz, *J* 16.9 Hz, CH_2a Asp), 2.60 (1H, dd, *J* 6.1 Hz, 16.9 Hz, CH_2b Asp), 2.03, 1.91, 1.86, 1.83 (12H, 4s, COCH_3), 1.37 (9H, s, CH_3 *t*Bu). δ_C ($CDCl_3$, 75 MHz) 128.2, 127.8, 127.2, 126.5, 124.5, 119.4 (*C*H Fmoc arom., *C*H Bn arom.), 98.0 (C-1), 67.5 (CH_2 Ser), 67.4 (C-3), 67.1 (*C*H_2Bn), 66.8 (*C*H_2 Fmoc), 66.6 (C-5, C-4), 61.4 (C-6), 52.2 (CH Ser), 50.6 (C-2), 50.5 (CH Asp), 47.0 (*C*H Fmoc), 36.7 (CH_2 Asp), 27.4 (CH_3 *t*Bu), 22.4 (NCO*C*H_3), 20.0 (OCO*C*H_3). ESI-HRMS: calcd for $C_{47}H_{56}N_3O_{16}$ $[M+H]^+$ 918.3655, found 918.3655.

4.3.2. *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-(*O*-*t*Bu)-L-aspartil-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester 6

Following the same procedure described for compound **5**, the glycosyl amino acid **8** (53.2 mg, 0.070 mmol) was treated with 20% piperidine in DMF (0.5 mL) and the reaction mixture was stirred for 1 h at room temperature. Purification of the mixture by column chromatography (EtOAc; MeOH/DCM 2:8 v/v) afforded the product **16** as a pale oil (26.2 mg, 0.0486 mmol, 69%). Subsequently, a solution of **16** (26.2 mg, 0.0486 mmol) in DMF (1.0 mL) was treated with *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-(*O*-*t*Bu)-L-aspartate **17** (24.7 mg, 0.060 mmol, 1.25 equiv), PyBOP (31.2 mg, 0.060 mmol, 1.25 equiv) and HOBt (8.10 mg, 0.060 mmol, 1.25 equiv) and then, with the DIEA (22 μL, 15 mg, 0.12 mmol, 2.5 equiv) to initiate the reaction. The mixture was allowed to stir for 15 h at room temperature and the purification of the crude mixture by chromatography column (EtOAc/hexane, 7:3 v/v) gave the product **6** as an amorphous solid (38.9 mg, 0.0417 mmol, 86%). δ_H ($CDCl_3$, 300 MHz) 7.69 (2H, d, *J* 7.5 Hz, C*H* Fmoc arom.), 7.53 (2H, t, *J* 6.4 Hz, C*H* Fmoc arom.), 7.36–7.22 (9H, m, C*H* Bn arom., C*H* Fmoc arom.), 6.17 (1H, dd, *J* 1.4 Hz, 9.5 Hz, N*H* Thr), 6.06 (1H, d, *J* 7.5 Hz, N*H* Asp), 5.21 (1H, d, *J* 2.3 Hz, H-4), 5.10–4.96 (3H, m, J_{AB} 12.0 Hz, CH_2 Bn, H-3), 4.72 (1 H, d, $J_{1,2}$ 3.5 Hz, H-1), 4.56–4.53 (2H, m, C*H* Asp, α*CH* Thr), 4.47–4.37 (3H, m, H-2, CH_2 Fmoc), 4.23 (1H, dd, *J* 1.8 Hz, 6.6 Hz, β*CH* Thr), 4.18 (1H, t, *J* 7.2 Hz, C*H* Fmoc), 4.06–4.04 (1H, m, H-5), 3.92–3.89 (2H, m, H-6a, *H*-6b), 2.77 (1H, dd, *J* 4.7 Hz, 16.9 Hz, CH_2*a* Asp), 2.61 (1H, dd, *J* 6.4 Hz, 16.9 Hz, CH_2*b* Asp), 2.06, 1.89, 1.88, 1.85 (12H, 4s, COCH_3), 1.38 (9H, s, CH_3 *t*Bu), 1.17 (3H, d, *J* 6.4 Hz, CH_3 Thr). δ_C ($CDCl_3$, 75 MHz) 128.8, 128.6, 127.7, 127.1, 125.0, 120.0 (*C*H Fmoc arom., *C*H Bn arom.), 98.9 (C-1), 76.0 (β*C*H Thr), 68.2 (C-3), 68.0 (*C*H_2Bn), 67.7 (*C*H_2 Fmoc), 67.5 (C-4), 67.2 (C-5), 62.2 (C-6), 58.5 (α*C*H Thr), 51.5, 51.2 (C-2, CH Asp), 47.0 (*C*H Fmoc), 37.4 (CH_2 Asp), 28.1 (CH_3 *t*Bu), 23.3 (NCO*C*H_3), 20.7 (OCO*C*H_3), 18.5 (*C*H_3Thr). ESI-HRMS: calcd for $C_{48}H_{58}N_3O_{16}$ $[M+H]^+$ 932.3812, found 932.3831.

4.4. Synthesis of glycosyl diketopiperazines

4.4.1. c[(*O*-*t*Bu)-L-aspartil-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-seryl)] 18

Compound **5** (54 mg, 0.058 mmol) was treated with 20% piperidine in DMF (0.5 mL) and the reaction mixture was stirred for 14 h, being the consumption of the starting material followed by TLC (EtOAc/hexane, 7:3 v/v). The mixture was then concentrated in vacuo and the residue was purified by chromatography column (EtOAc; MeOH/CH_2Cl_2 1:9 v/v). The product **18** was obtained as a pale oil in 48% yield (16.5 mg, 0.028 mmol). δ_H ($CDCl_3$, 300 MHz) 6.95 (1H, d, *J* 9.5 Hz, N*H*CO), 6.60 (1H, apparent s, N*H* Asp), 6.50 (1H, d, *J* 8.9 Hz, N*H* Ser), 5.29 (1H, d, $J_{3,4}$ 3.1 Hz, H-4), 5.13 (1H, dd, $J_{3,4}$ 3.1 Hz, $J_{2,3}$ 11.3 Hz, H-3), 4.87 (1H, d, $J_{1,2}$ 3.8 Hz, H-1), 4.57 (1H, dd, $J_{1,2}$ 3.6 Hz, $J_{2,3}$ 11.4 Hz, H-2), 4.31 (1H, apparent d, *J* 9.1 Hz, C*H* Asp), 4.24–4.20 (1H, m, *J* 9.2 Hz, C*H* Ser), 4.16–3.99 (4H, m, CH_{2a} Ser, H-5, H-6a, H-6b), 3.61 (1H, t, *J* 9.2 Hz, CH_{2b} Ser), 2.89 (1H, dd, *J* 3.1 Hz, 17.3 Hz, CH_{2a} Asp), 2.59 (1H, dd, 9.2 Hz, 17.5 Hz, CH_{2b} Asp), 2.10, 2.01, 1.93, 1.85 (12H, 4s, COCH_3), 1.39 (9 H, s, CH_3 *t*Bu). δ_C ($CDCl_3$, 75 MHz) 98.9 (C-1), 69.9 (CH_2 Ser), 68.3 (C-3), 67.2, 66.5 (C-4, C-5), 62.4 (C-6), 51.0 (CH Ser, CH Asp), 46.8 (C-2), 39.7 (CH_2 Asp), 28.6 (CH_3 *t*Bu), 23.5 (NCO*C*H_3), 21.2 (OCO*C*H_3). ESI-HRMS: calcd for $C_{25}H_{38}N_3O_{13}$ $[M+H]^+$ 588.2399, found 588.2395.

4.4.2. c[(*O*-*t*Bu)-L-aspartil-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-treonyl)] 19

Compound **6** (35 mg, 0.038 mmol) was treated with 20% piperidine in DMF (0.5 mL) and the reaction mixture was stirred for 15 h, being the consumption of the starting material followed by TLC (EtOAc/hexane, 7:3 v/v). The mixture was then concentrated in vacuo and the residue was purified by chromatography column (EtOAc; MeOH/CH_2Cl_2 1:9 v/v). The product **19** was obtained as a pale oil in 52% yield (12 mg, 0.023 mmol). δ_H ($CDCl_3$, 300 MHz) 7.09 (1H, d, *J* 1.2 Hz, N*H* Thr), 6.97 (1H, d, *J* 1.7 Hz, N*H* Asp), 6.65 (1H, d, *J* 9.2 Hz, N*H*CO), 5.32 (1H, dd, $J_{4,5}$ 1.0 Hz, $J_{3,4}$ 3.1 Hz, H-4), 5.07 (1H, dd, $J_{3,4}$ 3.1 Hz, $J_{2,3}$ 11.5 Hz, H-3), 4.96 (1H, d, $J_{1,2}$ 3.6 Hz, H-1), 4.48 (1H, ddd, $J_{1,2}$ 3.6 Hz, $J_{2,3}$ 11.5 Hz, *J* 9.4 Hz, H-2), 4.29 (1H, apparent d, *J* 10.1 Hz, C*H* Asp), 4.22–4.15 (2H, m, H-5, β*CH* Thr), 4.02 (2H, d, $J_{5,6}$ 6.5 Hz, H-6a, H-6b), 3.94 (1H, m, α*CH* Thr), 3.00 (1H, dd, *J* 2.8 Hz, 17.2 Hz, CH_2*a* Asp), 2.62 (1H, dd, 10.1 Hz, 17.2 Hz, CH_2*b* Asp), 2.09, 1.98, 1.90, 1.88 (12H, 4s, CH_3CO), 1.40 (9H, s, CH_3 *t*Bu), 1.35 (3H, d, *J* 6.6 Hz, CH_3 Thr). δ_C ($CDCl_3$, 75 MHz) 170.9, 170.6, 170.5, 170.4 (*C*OCH$_3$, βCO Asp), 167.0, 165.3 (CO Thr, CO Asp), 99.5 (C-1), 82.6 (Cquat. *t*Bu), 73.9 (βCH Thr), 68.1 (C-3), 67.5 (C-4), 67.4 (C-5), 62.0 (C-6), 58.9 (α*C*H Thr), 51.5 (CH Asp), 48.0 (C-2), 39.1 (CH_2 Asp), 28.0 (CH_3 *t*Bu), 22.9 (NCO*C*H_3), 20.7 (OCO*C*H_3), 17.0 (CH_3 Thr). ESI-HRMS: calcd for $C_{26}H_{40}N_3O_{13}$ $[M+H]^+$ 602.2556, found 602.2558.

4.4.3. c(L-Aspartil-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl)] 3

Compound **18** (16 mg, 0.027 mmol) was dissolved in MeOH (0.5 mL) and the pH was raised by addition of 1 M NaOMe in MeOH. The reaction mixture was stirred for 3 h, and then neutralized with Dowex 50WX8–200 resin. Filtration and concentration of the reaction mixture gave an intermediate (11.5 mg, 0.025 mmol, 91.5%), which was subsequently treated with TFA/DCM (1:1 v/v) solution (0.5 mL) and stirred for 24 h. After neutralization with Dowex 50WX8–200 resin, the final product **3** was obtained as a pale amorphous solid (9 mg, 0.022 mmol, 90%). δ_H (D_2O, 300 MHz) 5.10 (1H, d, $J_{1,2}$ 3.6 Hz, H-1), 4.51 (1H, d, *J* 8.4 Hz, C*H* Ser), 4.43 (1H, dd, *J* 4.6 Hz, *J* 9.2 Hz, C*H* Asp), 3.98 (1H, dd, $J_{1,2}$ 3.7 Hz, $J_{2,3}$ 10.9 Hz, H-2), 3.85 (1H, d, $J_{3,4}$ 3.1 Hz, H-4), 3.79 (1H, dd, $J_{3,4}$ 3.4 Hz, $J_{2,3}$ 11.0 Hz, H-3), 3.74–3.54 (5H, m, CH_2 Ser, H-5, H-6a, H-6b), 2.98 (1H, dd, *J* 4.6 Hz, *J* 17.6 Hz, CH_{2a} Asp), 2.77 (1H,

dd, *J* 4.3 Hz, *J* 17.6 Hz, CH_{2b} Asp), 1.91 (3H, s, NHCOCH_3). δ_C (D_2O, 75 MHz) 91.0 (C-1), 68.6, 67.9 (C-4, C-3, CH_2 Ser), 61.2 (C-5, C-6) 52.0 (CH Ser, CH Asp), 50.0 (C-2), 36.5 (CH_2 Asp), 21.8 (NCOCH_3). ESI-HRMS: calcd for $C_{15}H_{21}N_3O_{10}Cl$ $[M-2H+Cl]^-$ 438.0921, found 438.1462.

4.4.4. c(L-Aspartil-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-treonyl)] 4

Compound **19** (12 mg, 0.023 mmol) was dissolved in MeOH (0.5 mL) and the pH was raised by addition of 1 M NaOMe in MeOH. The reaction mixture was stirred for 3 h, and then neutralized with Dowex 50WX8-200 resin. Filtration and concentration of the reaction mixture gave an intermediate (9.5 mg, 0.02 mmol, quantitative), which was subsequently treated with TFA/DCM (1:1 v/v) solution (0.5 mL) and stirred for 24 h. After neutralization with Dowex 50WX8-200 resin, the final product **4** was obtained as a pale amorphous solid (7 mg, 0.016 mmol, 84%). δ_H (D_2O, 300 MHz) 5.07 (1H, d, $J_{1,2}$ 3.6 Hz, H-1), 4.37 (1H, dd, *J* 4.3 Hz, *J* 8.8 Hz, C*H* Asp), 4.18 (1H, m, αC*H* Thr), 4.08 (1H, m, βC*H* Thr), 4.02 (1H, m, H-4), 3.93 (1H, dd, $J_{1,2}$ 3.7 Hz, $J_{2,3}$ 7.0 Hz, H-2), 3.78 (1H, dd, $J_{3,4}$ 3.7 Hz, $J_{2,3}$ 7.4 Hz, H-3), 3.66–3.53 (3H, m, H-5, H-6a, H-6b), 2.94 (1H, dd, *J* 4.6 Hz, *J* 17.4 Hz, CH_{2a} Asp), 2.75 (1H, dd, *J* 4.5 Hz, *J* 17.4 Hz, CH_{2b} Asp), 1.89 (3H, s, NHCOCH_3), 1.14 (3H, d, *J* 6.3 Hz, CH_3 Thr). δ_C (D_2O, 75 MHz) 90.9 (C-1), 76.1 (βCH Thr), 70.6 (C-5), 69.2 (C-4), 67.7 (C-3), 61.1 (C-6), 57.2 (αCH Thr), 50.6 (CH Asp), 49.7 (C-2), 36.4 (CH_2 Asp), 21.6 (NCOCH_3), 15.1 (CH_3 Thr). ESI-HRMS: calcd for $C_{16}H_{24}N_3O_{10}$ $[M-H]^-$ 418.1467, found 418.1497.

4.5. Biological assays

4.5.1. Fluorimetric TcTS inhibition assays

trans-Sialidase used in this study was a His-tagged 70 kDa recombinant material truncated to remove C-terminal repeats but retaining the catalytic N-terminal domain of the enzyme.[40] Inhibition was assessed using the continuous fluorimetric assay described by Douglas and co-workers.[35] Briefly, the assays were performed in triplicate in 96-well plates containing phosphate buffer solution at pH 7.4 (25 μL), recombinant enzyme solution (25 μL) and inhibitor solution (25 μL of 4.0 mM solution). This mixture was incubated for 10 min at 26 °C followed by addition of MuNANA (K_m = 0.68 mM[35] 25 μL of a 0.4 mM solution giving an assay concentration of 0.1 mM). The fluorescence of the released product (Mu) was measured after 10 min, with excitation and emission wavelengths of 360 and 460 nm, respectively, and the data were analysed with GraphPad Prism software version 4.0 (San Diego, CA, USA). Inhibition percentages were calculated by the equation: % $I = 100 \times [1 - (V_i/V_0)]$, where V_i is the velocity in the presence of inhibitor and V_0 is the velocity in absence of inhibitor.

4.5.2. Cytotoxicity assay

C57BL/6 mice were isolated by dissociation and incubated for 5 min with red blood cell lysis buffer (one part of 0.17 M Tris–HCl [pH 7.5] and nine parts of 0.16 M ammonium chloride). The cells were suspended in RPMI 1640 medium (Gibco-BRL Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Life Technologies Inc., Bethesda, MD) and antibiotics (Sigma Chemical Co., St. Louis) and cultured in flat-bottom 96-well plates at 5×10^5 cells/well with different concentrations of compounds **1**–**4** at 37 °C for 24 h.[42] Tween 20 at 0.5% was used as cell death positive control. Cells were harvested, incubated with 10 μg/mL propidium iodide (Sigma) and acquired using a FACSCantoII (Becton-Dickinson Immunocytometry System Inc., San Jose, CA, USA). Data analysis was performed using FlowJo software (Ashland, Oregon, USA).

4.5.3. In vitro anti-trypanosomal assays

To evaluate the anti-trypanosomal activity in both trypomastigote and amastigote forms of *T. cruzi*, LLC-MK2 cell strain (ATCC) were resuspended in RPMI medium without phenol red at 2×10^3 cells/well and were cultured in 96-well plates for 24 h. The cells were infected with 1×10^4 trypomastigotes forms of *T. cruzi* Tulahuen strain stably expressing the β-galactosidase gene from *Escherichia coli* (Tulahuen-lacZ), and after 24 h, compounds **1**–**4** or benznidazole were added at concentrations of 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.007 and 0.0035 mM. After 4 days of culture, 50 μl of PBS containing 0.5% of Triton X-100 and 100 μM Chlorophenol Red-β-D-galactoside (CPRG—Sigma) were added. Plates were incubated at 37 °C for 4 h and absorbance was read at 570 nm.[43] Results of parasite viability were measured based on the catalysis of CPRG by β-galactosidase.

Some of the same concentrations described above were also used to evaluate anti-trypanosomal activity of compound **4** on amastigote forms. To this purpose, Vero cells strains (ATCC) were cultured at 2×10^4/well on 8-well camber slides. After 12 h, the cells were infected with 2×10^5/well trypomastigote forms of *T. cruzi* Tulahuen strain for additional 12 h. The cells were washed with PBS to remove parasites on the supernatant and incubated with benznidazole (Bz) or compound **4** for 24 h at 37 °C. Cultures were stained by Giemsa dye and evaluated by optical microscopy. Anti-trypanosomal activity was determined by counting the amount of parasites/cell in at least 200 cells/well. The experiment was performed in triplicates.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.01.027. These data include MOL files and InChiKeys of the most important compounds described in this article.

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