Chemical and chemoenzymatic synthesis of glycosyl-amino acids and glycopeptides related to *Trypanosoma cruzi* mucins

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This study describes the synthesis of the α- and β-linked *N*-acetyllactosamine (Gal*p*-β-1,4-GlcNAc; LacNAc) glycosides of threonine (LacNAc-Thr). LacNAc-α-Thr was prepared by direct chemical coupling of a 2-azido-2-deoxy-lactose disaccharide donor to a suitable partially protected threonine unit. In contrast, stepwise chemical generation of β-linked *N*-acetylglucosamine followed by enzymatic galactosylation to give LacNAc-β-Thr proved effective, whereas use of a 2-azido-2-deoxy-lactose donor in acetonitrile failed to give the desired β-linked disaccharyl glycoside. This study illustrates that it is possible to overcome the inherent stereoselection for 1,2-*trans* chemical glycosylation with a GlcNAc donor, and that the well-established preference of bovine β-1,4-galactosyltransferase for β-linked acceptor substrates can also be overcome. Using this knowledge, short glycopeptide fragments based on *T. cruzi* mucin sequences, Thr-Thr-[LacNAcThr]-Thr-Thr-Gly, were synthesised. All LacNAc-based compounds outlined were shown to serve as acceptor substrates for sialylation by *T. cruzi trans*-sialidase.

Introduction

Parasitic infections remain a major cause of mortality in the developing world. However, commercial and socioeconomic factors mean that very few new drugs reach the clinic.[1] Identification of new leads and drug-able targets would enable the design of inhibitors of parasite-specific metabolic processes and help in the search for affordable new medicines.[2] Chagas′ disease, caused by the protozoan parasite *Trypanosoma cruzi*, is endemic in South and Central America, Mexico and the southern United States. It has been classified after malaria and schistosomiasis as one of the most serious parasite diseases in tropical regions: there are currently 18 million people infected and a further 100 million, mainly in Latin America, are at risk of infection. Typically 2–3 million individuals show clinical symptoms of the chronic phase of the disease at any one time, and 45000 die every year.[3] There is a clear need for new therapeutics.[4–6]

Recent sequencing shows that over 50% of the genome of *Trypanosoma cruzi* (strain CL Brener) is composed of repeated sequences.[7] These sequences included genes for large families of surface molecules, including *trans*-sialidases, mucins, gp63 proteases and novel mucin-associated surface proteins. As with many other pathogenic microorganisms, the nature and extent of cell surface glycosylation can have a profound impact on cell surface protein structure and biological function.[8] *T. cruzi* mucins are highly glycosylated (about 60% carbohydrate by weight), polyanionic glycoproteins that are rich in Thr, Ser and Pro residues, but contain few hydrophobic amino acids.[9,10] The tightly regulated expression of a large repertoire of mucin structures helps the parasite to interact with the infected host on one hand, whilst on the other it also serves to mask the presence of the parasite from the host immune response.

The *O*-glycans of *T. cruzi* mucins resemble those of mammalian mucins, although the oligosaccharides are linked to the threonine or serine of the parasite protein *via* an α-linked *N*-acetyl-glucosamine (GlcNAc) rather than an α-linked *N*-acetyl-galactosamine (GalNAc), as in vertebrate mucins.[9–11] The α-GlcNAc is substituted by galactose (Gal) on *O*-4 and *O*-6, with further galactosylation leading to complex branched structures. In the more infective Y and CL strains of *T. cruzi*, Gal residues are only present in the β-pyranose form (β-Gal*p*),[12–14] whereas in the less infective G strain the mucins also contain β-galactofuranose (β-Gal*f*).[15] The mucin glycans are also significant in being the major substrate for *trans*-sialidase which catalyzes transfer of sialic acid on the parasite cell surface.[16] Decoration of cell surface architecture with this negatively charged sugar is common practice for microbial pathogens.[17] In this case, *T. cruzi* is unable to synthesize sialic acid *de novo* and so uses a cell surface *trans*-sialidase enzyme (TcTS)[18] to scavenge this monosaccharide from host glycoconjugates in order to generate α-2,3-linked sialylated-β-galactopyranose units on the parasite mucins. The similarity[19] of the parasite *trans*-sialidase to the numerous microbial sialidases (neuraminidases) that have been investigated suggests potential for this enzyme as a therapeutic target. In addition, any of the other enzymes involved in mucin glycan biosynthesis might also be suitable targets for chemotherapeutic intervention: α-GlcNAc transferase activity has been characterised in *T. cruzi* microsomal membranes[20] and β-Gal*p* transferase activities have been detected in Golgi preparations.[21,22]

Given the heterogeneity of *T. cruzi* mucins, there are no suitable natural sources of homogeneous mucin glycopeptides with which to investigate mucin biosynthesis. The purpose of the study

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reported herein was to synthesise simple glycosylated amino acid and glycopeptide acceptor substrate analogues (Fig. 1) with which to study *T. cruzi* mucin glycosyltransferase and *trans*-sialidase activities.[20–22] In the latter case, we set out to complement the work of de Lederkremer and co-workers, who have synthesised and assessed various glycans from *T. cruzi* G strain mucins, with the preparation of fragments of the mucin glycopeptides found in the pathogenic *T. cruzi* Y strain.[23–25]

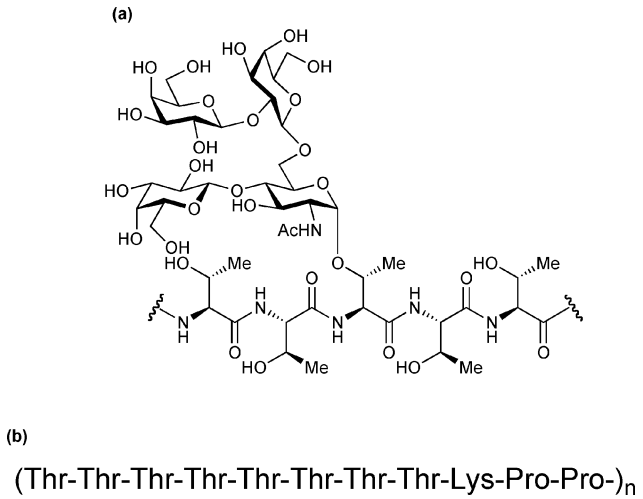

Fig. 1 (a) Structure of a *T. cruzi* CL/Y strain mucin glycopeptide fragment, and (b) the corresponding mucin tandem repeat peptide sequence.

We previously reported the α-selective iodine-promoted glycosylation of serine and threonine (Thr) with 2-azido-2-deoxy-glycosyl iodides, but sought a shorter route to α-linked GlcNAc-amino acids and galactosylated versions thereof.[26] Here we report chemical and chemoenzymatic syntheses of GlcNAc-α-Thr (**1**), GlcNAc-β-Thr (**3**) and the corresponding α- and β-linked *N*-acetyllactosamine (Gal*p*-β-1,4-GlcNAc; LacNAc) derivatives, (**2**) and (**4**) respectively, along with mucin-related *N*-acetyllactosamine-based glycopeptides, (**5**) and (**6**) (Fig. 2). Each of these structures was assessed for their ability to be glycosylated by recombinant *T. cruzi trans*-sialidase.

Results and discussion

In the context of the preparation of LacNAc-α-Thr (**2**) and diastereomeric LacNAc-β-Thr (**4**), linear glycosylation disconnections require the sequential glycosylation of suitably protected threonine with glucosamine and then galactose units, or a convergent approach would utilize direct introduction of an intact lactosamine disaccharide. Both strategies allow scope for enzymatic glycosylation steps, particularly the use of readily available, and well studied, bovine β-1,4-galactosyltransferase (β-1,4-GalT)[27] to effect the sequential galactosylation. Hence, this study exploited chemical methods for the synthesis of monosaccharyl-amino acids (**1**) and (**3**), and compared chemical and chemoenzymatic approaches to prepare disaccharyl-amino acids (**2**) and (**4**). Results from these studies informed the choice of methods for preparation of glycopeptides (**5**) and (**6**).

Chemical synthesis of glyco-amino acids GlcNAc-α-Thr (1) and GlcNAc-β-Thr (3)

Although it is counter intuitive to use a glycosyl donor possessing a C-2 participating group (in this case an acetamide) when attempting to synthesise a 1,2-*cis*-linked glycoside, 3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-glucopyranosyl chloride (**7**) is

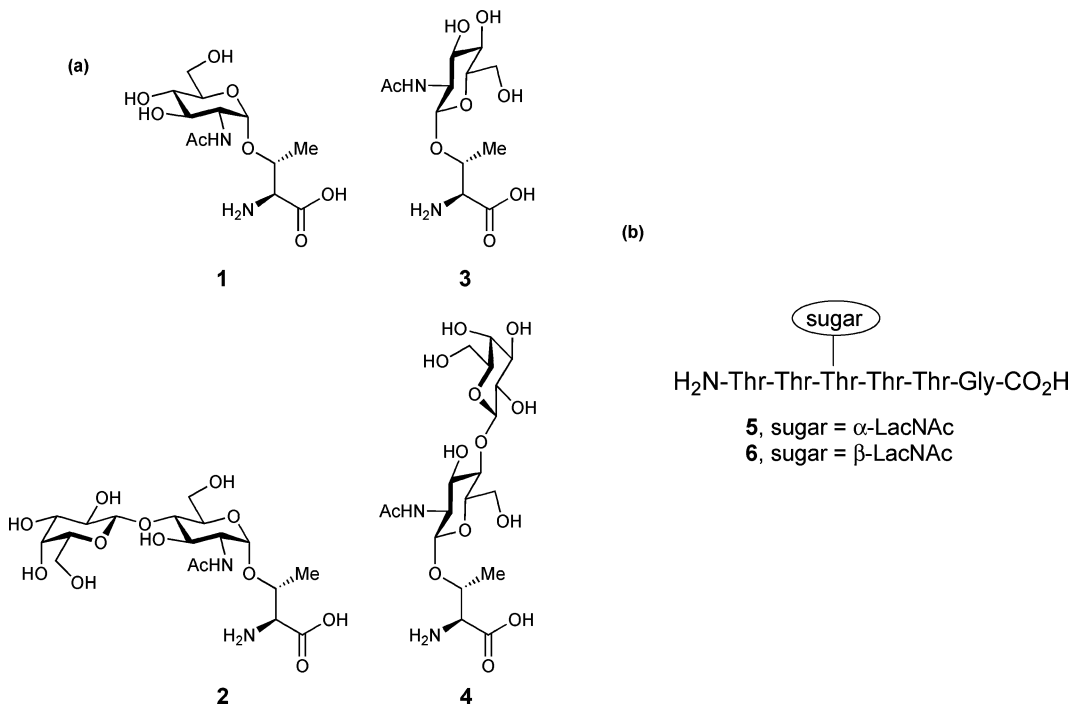

Fig. 2 (a) Structure of target α-linked glyco-amino acid fragments and the corresponding β-linked controls; (b) target mucin-related glycopeptides, prospective *trans*-sialidase substrates.

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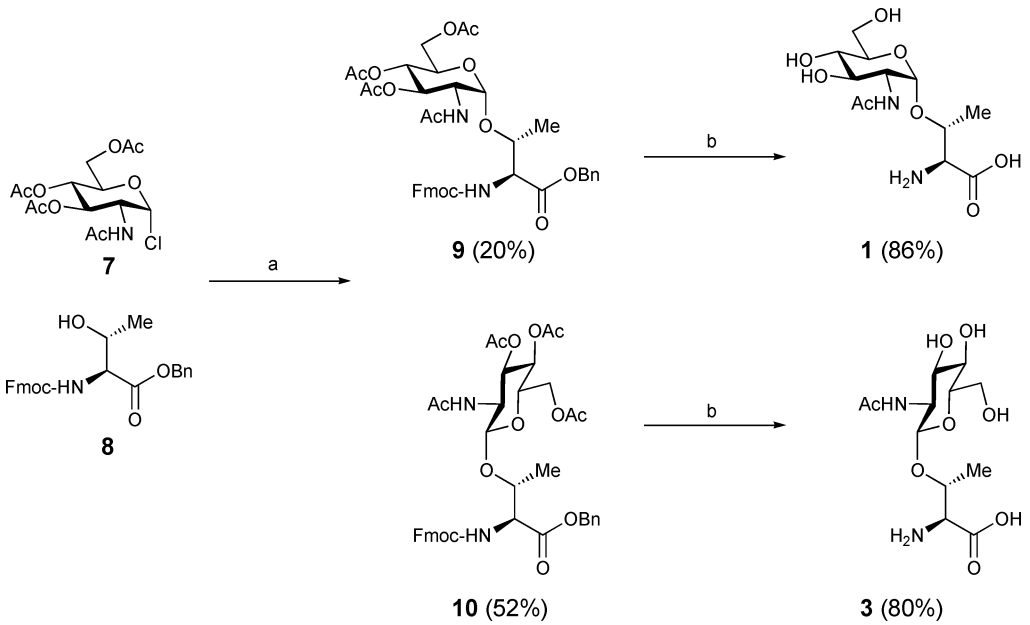
a shelf-stable crystalline solid that is readily accessible on a gram scale from cheap glucosamine.[28,29] We previously reported the glycosylation of suitably protected serine and threonine derivatives with glycosyl chloride (**7**) in the presence of mercuric bromide, isolating the expected β-linked glyco-amino acids in respectable yield (~60–65%).[29] Whilst these were practical yields for preparative purposes, it raised the question of mass balance: what happened to the remaining 35–40% of the material? On closer inspection, we were able to identify quantities of the unexpected α-linked glycoside from these reactions. On tweaking the reaction conditions (reaction at room temperature for 10 h, followed by reflux for 9 h, in contrast to just refluxing the reaction mixture for 9 h), glycosylation of *N*-Fmoc-Thr benzyl ester (**8**) with glycosyl chloride (**7**) in 1,2-dichloroethane in the presence of $HgBr_2$ gave the expected β-glycoside (**10**)[29] in 52% yield, accompanied by the corresponding α-linked isomer (**9**) in a low but reproducible 20% yield.† This method provided a straightforward and direct approach to the α-linked GlcNAc-Thr system that, given that there is no need to manipulate C-2 functionality, is competitive with other approaches to such α-linked 2-amino-2-deoxy-glucosides.[30,31] Removal of the benzyl ester and Fmoc groups from protected glyco-amino acids (**9**) and (**10**) was performed by standard hydrogenation (10% Pd-C/H_2); subsequent removal of the acetate groups was realised in the presence of 1 M NaOMe in MeOH. In this way, 20–30 mg quantities of the completely deprotected glyco-amino acids (**1**) and (**3**) were obtained (Scheme 1).

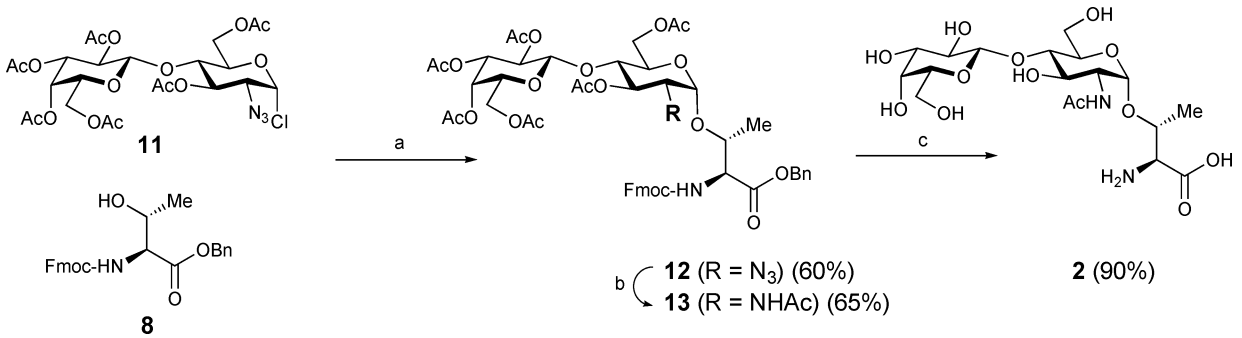
† The α-glycoside appears to be formed directly, not through equilibration of the β-glycoside. Addition of pure β-glycoside to the reaction mixture did not result in additional α-glycoside formation.

Preparation of disaccharide glycosides LacNAc-α-Thr (2) and LacNAc-β-Thr (4)

The chemical synthesis of the protected α-linked disaccharyl amino acid (**12**) was achieved by convergent glycosylation of *N*-Fmoc-Thr benzyl ester (**8**) with known α-linked glycosyl chloride (**11**)[32,33] under the same conditions described for synthesis of (**9**) and (**10**) (Scheme 2).[29] The only isolable glycosyl amino acid product obtained was the α-isomer, αLacN$_3$-FmocThrOBn (**12**), in



Scheme 1 *Reagents and conditions:* (a) $HgBr_2$, $ClCH_2CH_2Cl$, rt 10 h then reflux 9 h; (b) i. H_2-Pd/C, MeOH–AcOH (10 : 1), 10 h, ii. NaOMe, MeOH.



Scheme 2 *Reagents and conditions:* (a) $HgBr_2$, $ClCH_2CH_2Cl$, rt 10 h then reflux 9 h; (b) Zn powder, AcOH, Ac_2O, THF; (c) i. H_2-Pd/C, MeOH–AcOH (10 : 1), 10 h, ii. NaOMe, MeOH.

60% yield.‡ The configuration of the newly-formed sugar-amino acid glycosidic centre was confirmed by ^{1}H NMR spectroscopy (δ 4.50 ppm, d, $J_{1,2}$ 3.7 Hz, H-1). Subsequently, the sugar azido group in (**12**) was reductively acetylated using zinc powder in THF–acetic anhydride–acetic acid,[34] affording α-LacNAc-FmocThrOBn (**13**) in 65% yield. Complete deprotection, giving α-linked disaccharyl amino acid glycoside (**2**) in 90% yield, was realized by standard hydrogenation (to remove the *O*-Bn and *N*-Fmoc groups) followed by Zemplen deacetylation.

In an attempt to obtain the β-linked isomer of protected LacNAc-α-Thr (**13**) whilst limiting the number of building blocks in use, the glycosylation of (**8**) and (**11**) was performed in the presence of the participating solvent acetonitrile.[35–37],§ However, repeated attempts at this reaction gave, at best, only poor yields on the unwanted α-glycoside (**12**) (up to 10% yield), none of the desired β-glycoside and much hemiacetal. Hence we resorted to a chemo-enzymatic approach.

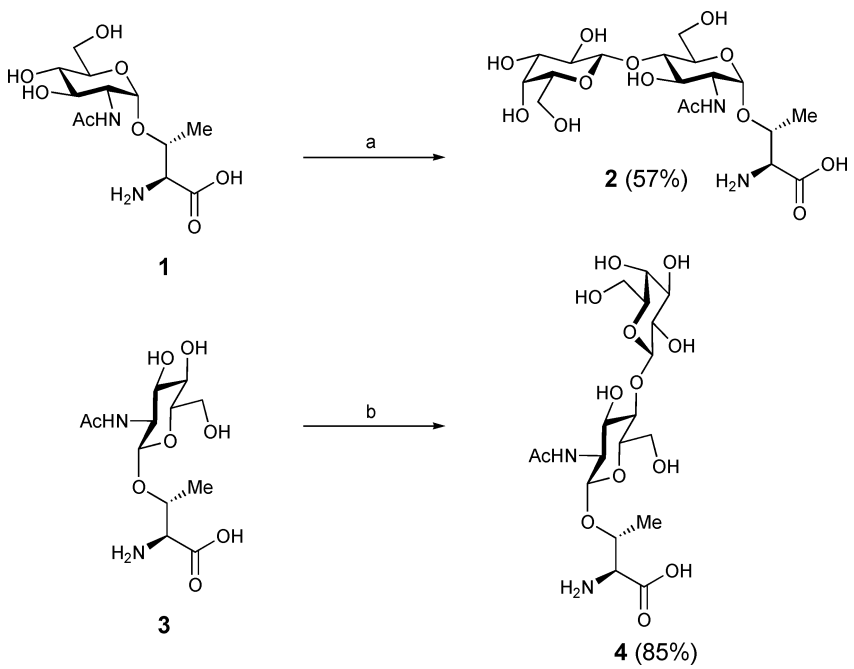
Whilst its natural preference is for glycosylation of β-linked acceptor substrates,[27] the relatively loose acceptor substrate tolerance of bovine β-1,4-galactosyltransferase (β-1,4-GalT) has previously been exploited in the 4-*O*-galactosylation of α-linked acceptors, such as maltose (Glc-α-1,4-Glc)[39] and uridine-diphospho-*N*-acetylglucosamine (GlcNAc-α-UDP).[40] We were therefore prompted to consider the enzymatic galactosylation of the glycosylated amino acids (**1**) and (**2**). These reactions was performed on a 5 mg acceptor scale using commercial UDP-galactose as the donor substrate in the presence of bovine β-1,4-GalT (0.5 U) and alkaline phosphatase, the latter to remove potentially inhibitory UDP (Scheme 3). In relation to the β-linked acceptor (**3**), complete conversion to the desired galactosylated product, LacNAc-β-Thr (**4**), was observed after 24 h reaction. In contrast, for α-linked acceptor (**1**), conversion to LacNAc-α-Thr (**2**) is much slower and product was not observable at this time point. This result shows the substantially lower reactivity of the β-1,4-GalT enzyme towards the α-linked acceptor. Hence, enzymatic galactosylation of (**1**) was repeated with high β-1,4-GalT concentration (2 U) for longer (96 h), at which point TLC indicated that conversion to galactoside (**2**) had progressed to >60%. The disaccharyl amino acids (**2**) and (**4**) were subsequently isolated by gel filtration chromatography in 57% and 85% yields, respectively, and their structures were confirmed by NMR spectroscopy and mass spectrometry. ^{1}H NMR spectra showed doublets at δ 4.77 ppm ($J_{1,2}$ 3.8 Hz) or 4.39 ppm ($J_{1,2}$ 8.1 Hz), corresponding to GlcNAc H-1 of (**2**) and (**4**), respectively. ESI-MS analysis showed characteristic adducts for the α-glycoside (**2**) (m/z [M + Na]$^+$ 507.17) and the corresponding β isomer (**4**) (m/z [M + H]$^+$ 485.19).

‡ Paulsen and Holck described the use of the corresponding β-linked glycosyl chloride donor, which gave excellent yields (>80%) of α-glycosyl amino acid as the sole glycoside product. Clearly the anomeric configuration of the glycosyl chloride plays little role in determining the stereochemical outcome of the glycosylation reaction with these particular sugar donors.[33]

§ Recent studies exploiting acetonitrile to influence glycosylation stereochemistry show that use in limited quantity can significantly outperform its use as a solvent.[38]

Solid-phase and chemo-enzymatic glycopeptide synthesis

Moving towards more complex mimetics of the mucin glycopeptides present on the *T. cruzi* parasite cell surface we next employed solid-phase glycopeptide synthesis, coupled with enzymatic glycosylation. Commercial Wang resin pre-loaded with Fmoc-Gly (0.65 mmol/g resin) was employed as the solid support for the synthesis of glycopeptides (**5**) and (**6**). Syntheses were performed manually in a fritted glass vessel fitted with a three-way tap so that argon purging could be used to effect mixing.[41]



Scheme 3 *Reagents and conditions:* (a) bovine β-1,4-GalT (2 U), UDP-galactose, alkaline phosphatase, 30 °C, 96 h; (b) bovine β-1,4-GalT (0.5 U), UDP-galactose, alkaline phosphatase, 30 °C, 24 h.

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After removal of *N*-Fmoc groups with 20% piperidine in DMF, the amino acid building blocks were coupled in DMF in the presence of the coupling reagents PyBOP–HOBt and *N*,*N*-diisopropylethylamine (DIPEA). The progress of amide coupling was monitored spectrophotometrically, based on quantitation of dibenzofulvene released (with piperidine) from a dried sample of resin-bound (glyco)peptide. Optimal coupling times ranged from 3 h to 24 h for the incorporation of Fmoc-Thr, depending on the preceding peptide sequence. For incorporation of the more sterically and electronically demanding glycosylated amino acids (**14**) (Scheme 4) and (**15**) (Scheme 5) the coupling times typically ranged from 24 h to 72 h.

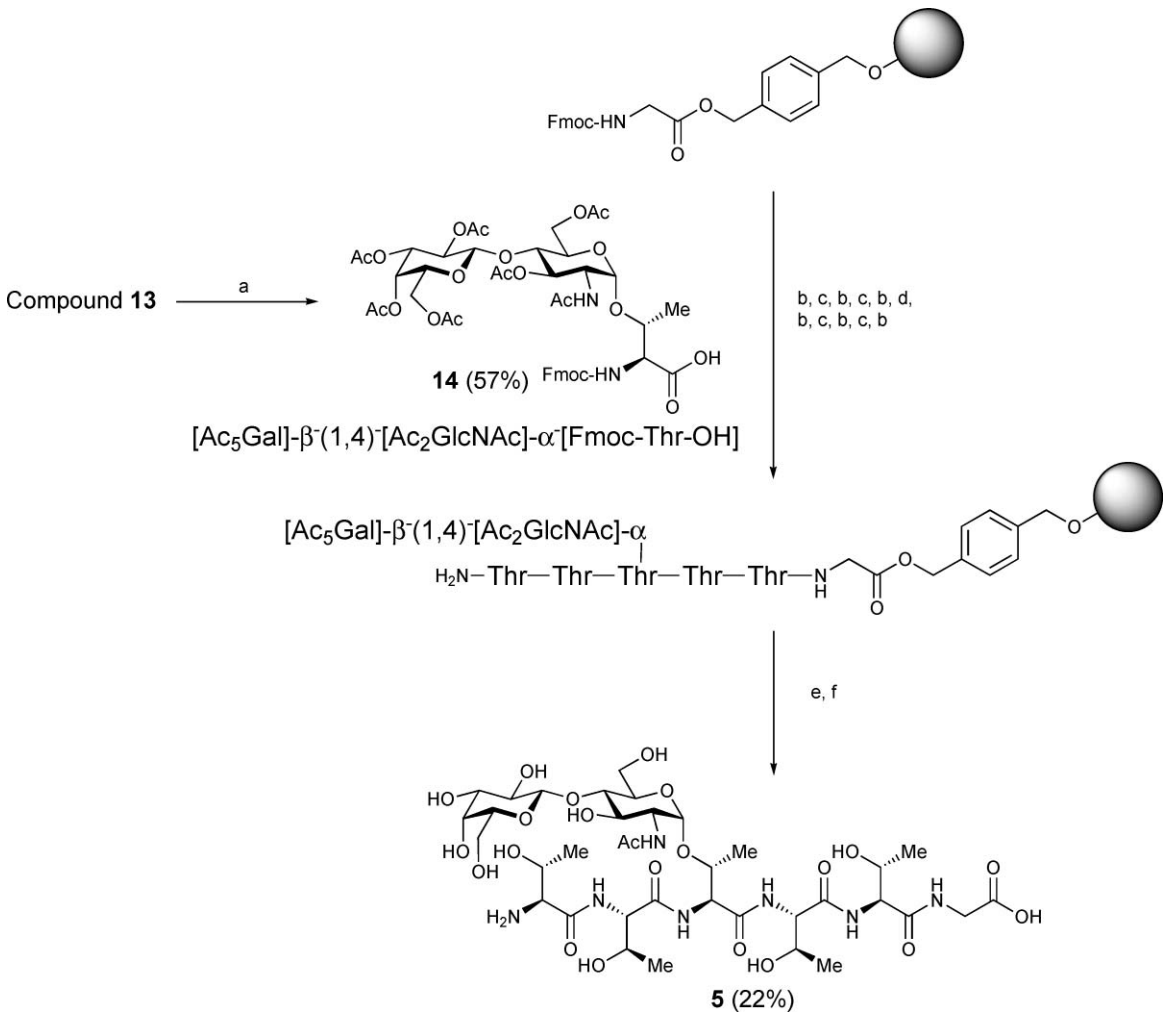
Synthesis of the glycopeptide H_2N-(Thr)$_2$-(LacNAc-α-Thr)-(Thr)$_2$-Gly-OH (5)

The synthesis of α-LacNAc-based glycopeptide (**5**) was initiated by cleavage of the Fmoc group from Fmoc-Gly-Wang resin, followed by two rounds of coupling–deprotection with Fmoc-Thr. Subsequent coupling employed glyco-amino acid building block αLacNAc-FmocThrOH (**14**), which was successfully prepared by careful hydrogenation of benzyl ester (**13**) without competing removal of the Fmoc group. Introduction of two further threonine residues completed preparation of the protected, resin-bound glycopeptide (Scheme 4). The peptide coupling efficiencies obtained, as judged by measuring released dibenzofulvene from the product, varied with the position in the peptide chain: Thr1 (90%), Thr2 (90%), LacNAc-α-Thr3 (54%), Thr4 (55%) and Thr5 (80%).

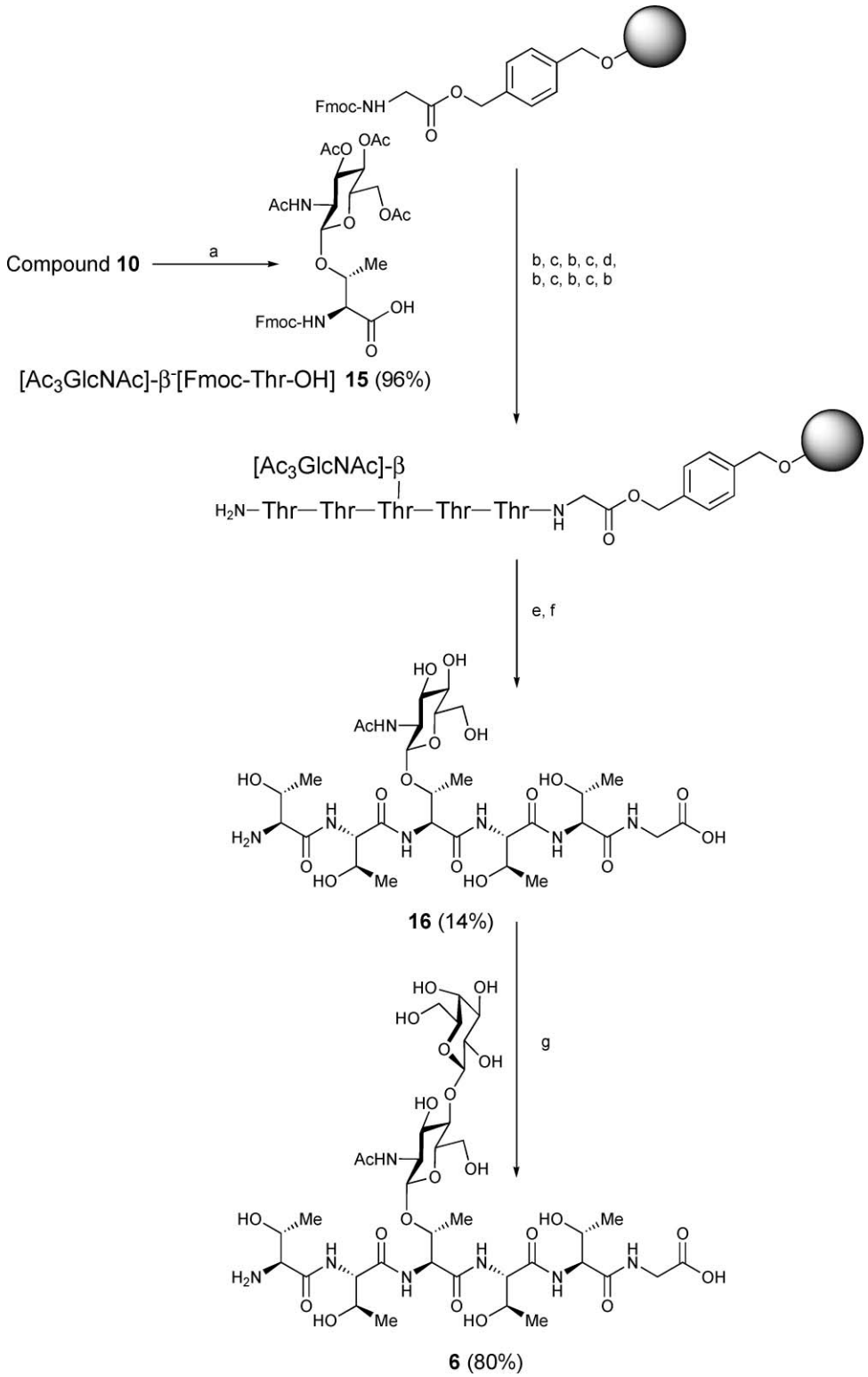
Cleavage from the resin with aqueous TFA, concentration *in vacuo* and trituration with cold ether gave crude glycopeptide. Finally, removal of the sugar acetate protecting groups with catalytic NaOMe in MeOH and purification by reverse-phase HPLC gave glycopeptide (**5**) in 22% overall yield. The structure of compound (**5**) was confirmed by NMR spectroscopy [characteristic ^{1}H signals for sugar H-1 at δ 4.71 ppm ($J_{1,2}$ 3.7 Hz), 1 × NH*Ac* and 5 × CH_3Thr units] and ESI-MS analysis (observed $[M + H]^+$ 946.4).

Chemoenzymatic synthesis of H_2N-(Thr)$_2$-(LacNAc-β-Thr)-(Thr)$_2$-Gly-OH (6)

Following on from our observations on chemical and chemoenzymatic approaches to LacNAc-β-Thr (**4**), a chemoenzymatic



Scheme 4 *Reagents and conditions:* (a) H_2–Pd/C, MeOH–AcOH (10 : 1), 1 h; (b) 20% piperidine in DMF; (c) Fmoc-Thr, PyBOP, HOBt, DIPEA; (d) Glyco-amino acid **14**, PyBOP, HOBt, DIPEA; (e) TFA; (f) NaOMe, MeOH.



Scheme 5 *Reagents and conditions:* (a) H_2–Pd/C, MeOH–AcOH (10 : 1), 1 h; (b) 20% piperidine in DMF; (c) Fmoc-Thr, PyBOP, HOBt, DIPEA; (d) Glyco-amino acid **15**, PyBOP, HOBt, DIPEA; (e) TFA; (f) NaOMe, MeOH; (g) bovine β-1,4-GalT, UDP-galactose, alkaline phosphatase, 30 °C, 24 h.

approach to glycopeptide (**6**) was explored. That is, GlcNAc-β-Thr-containing glycopeptide (**16**) was prepared first in a similar manner to glycopeptide (**2**). The peptide coupling efficiencies obtained, as judged by measuring released dibenzofulvene from the product, varied with the position in the peptide chain: Thr1 (90%), Thr2 (90%), GlcNAc-β-Thr3 (61%), Thr4 (58%) and Thr5 (85%). Cleavage from resin with aqueous TFA, concentration *in vacuo* and trituration with cold ether gave crude glycopeptide. Removal of the

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sugar acetate protecting groups with catalytic NaOMe in MeOH and purification by reverse-phase HPLC gave the glycopeptide (**16**) in 14% overall yield. The structure of compound (**16**) was confirmed by NMR spectroscopy [characteristic ^{1}H signals for sugar H-1 at δ 4.41 ppm ($J_{1,2}$ 8.1 Hz), 1 × NH*Ac* and 5 × CH_3Thr units] and ESI-MS analysis (observed $[M + H]^+$ 847.1).

Subsequent solution-phase enzymatic β-galactosylation of GlcNAc-glycopeptide (**16**) with bovine β-1,4-GalT and UDP-galactose gave the crude LacNAc-glycopeptide which, following purification by Toyopearl HW-40S gel filtration chromatography with 0.1% aqueous TFA, gave glycopeptide (**6**) in 80% yield from the corresponding β-GlcNAc-based glycopeptide (**16**). The structure of compound (**6**) was confirmed by NMR spectroscopy [characteristic ^{1}H signals for sugar H-1′ at δ 4.43 ppm ($J_{1,2}$ 7.5 Hz)] and ESI-MS analysis (observed $[M + Na]^+$ 968.4).

Enzymatic sialylation of glycopeptides with *Trypanosoma cruzi trans*-sialidase (TcTS)

Each of the LacNAc-containing glyco-amino acids (**2** and **4**) and glycopeptides (**5** and **6**) prepared in this study were subjected to the action of recombinant *Trypanosoma cruzi trans*-sialidase (TcTS) in the presence of a sialic acid donor substrate. In contrast to the use of sialidases, which require long incubation times and/or excess of donor substrate,[42] *trans*-glycosylation with TcTS takes place with equimolar amounts of substrates and much shorter reaction times.[18,43] It is therefore a very useful biocatalyst for this difficult glycosylation. Although a variety of TcTS sialyl donor substrates have been reported in the literature,[43–45] including sialic acid *para*-nitrophenyl and methylumbelliferyl glycosides and α-2,3-sialyl-lactose, amongst several others, the TcTS transfer activity depends strongly on reaction conditions, including pH, temperature and time. Each of the α-sialosides has its own problems, with cost, rate of reaction and complications with by-product separation limiting utility. Considering the disadvantages of the cited donors, and following the work of Kim *et al.*,[46] the glycoprotein fetuin from calf serum was chosen as a sialic acid donor for the experiments reported in this study. This glycoprotein contains ~8% α-2,3-linked sialic acid by weight and the glycoprotein by-product of the sialyl transfer reaction is easily removed by precipitation with ethanol or with the aid of a 10 kDa cut-off spin filter.[46] It therefore provides a highly competitive source of sialic acid in terms of availability and ease of use.

All of the LacNAc glycosides synthesized in this study (**2**, **4**, **5** and **6**) proved to be effective acceptor substrates for TcTS, giving complete conversion to the corresponding α-2,3-sialylated structures in the presence of a three fold excess of fetuin. Careful monitoring proved essential as in all cases prolonged incubation resulted in hydrolysis of the sialylated products, regenerating the LacNAc acceptors. From milligram scale reactions, the sialylated products (**17–20**) were readily isolated by removal of protein using a spin filter, followed by gel filtration (Toyopearl HW-40S column) in 5 mM NH_4HCO_3 buffer. Using this approach, the sialylated products were obtained in >80% isolated yield. NMR spectroscopy and mass spectrometry confirmed the product structures (Table 1).

Conclusion

In summary, we have developed chemical and chemoenzymatic routes to glyco-amino acids and glycopeptides related to *T. cruzi* mucin structures. The early phase of the study highlights the difficulty in predicting the outcome of both chemical and enzymatic glycosylation reactions, particularly in relation to stereochemistry. The latter part of the study shows that *T. cruzi trans*-sialidase has relaxed acceptor substrate specificity, acting efficiently on both its natural LacNAc-α-Thr-based substrates but also on the diastereomeric β-linked compounds.

Experimental

General

All chemicals were purchased as reagent grade and used without further purification. Solvents were dried according to standard methods.[47] Reagents for glycopeptide synthesis, including FmocGlyWang resin (loading 0.65 mmol/g), FmocThrOH and PyBOP, were purchased from Novabiochem. HOBt was purchased from Acros. Bovine β-1,4-galactosyltransferase (EC 2.4.1.90), UDP-galactose and calf intestinal alkaline phosphatase (EC 3.1.3.1) were purchased from Sigma Chemical Co. Fetuin (calf serum) was purchased from Sigma Chemical Co or Quest Biomedical.

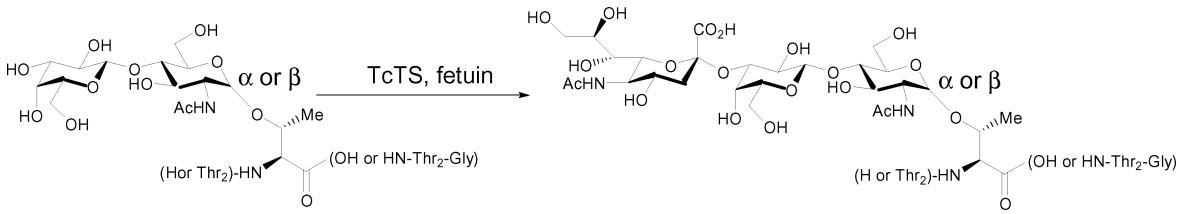
Reactions were monitored by thin layer chromatography (TLC) on 0.25 nm precoated silica gel plates (Whatman, AL SIL G/UV, aluminium backing) with the indicated eluents. Compounds were visualized under UV light ($\lambda = 254$ nm) and/or dipping in ethanol–sulfuric acid (95 : 5, v/v) or orcinol (2%) in aqueous sulfuric acid (10%), 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 mm or 25mm flash cartridges with the eluents indicated.

Nuclear magnetic resonance spectra were recorded on a Varian Gemini spectrometer. ^{1}H NMR spectra recorded at 400 MHz were referenced to δ_H 7.27 for $CDCl_3$, δ_H 3.35 for CD_3OD, δ_H 4.63 ppm for D_2O, and ^{13}C NMR spectra recorded at 100 MHz were referenced to δ_C 77.0 for $CDCl_3$ and δ_C 49.15 for CD_3OD. Chemical shifts of NMR signals recorded in D_2O are reported with respect to the methyl resonance of internal acetone at δ_H 2.22 ppm and δ_C 30.89 ppm. Assignments were made with the aid of HSQC and COSY experiments. Optical rotations were measured at ambient temperature on a Perkin-Elmer model 141 polarimeter using a sodium lamp. Accurate mass electrospray ionization mass spectra (ESI-MS) were obtained from the EPSRC National Mass Spectrometry Service Centre, Swansea using positive ionization mode on a Finningan MAT 900 XLT mass spectrometer or from the John Innes Centre metabolite analysis service on a Thermo Finningan DecaXP^{plus} mass spectrometer.

N-(Fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl)-L-threonine benzyl ester (9) and *N*-(fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-L-threonine benzyl ester (10)[29]

A mixture of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**7**)[29] (400 mg, 1.09 mmol) and *N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine benzyl ester (**8**)

Table 1 Data for TcTS-catalyzed sialylation of LacNAc-based acceptors



LacNAc acceptor	Glyco-amino acid linkage	Sialylated product	Isolated yield	Characteristic ^{1}H NMR data (D_2O)	ESI-HRMS data
(**2**)	α	Neu5Ac-α-2,3-LacNAc-α-Thr (**17**)	92%	δ 4.77 (1H, d, $J_{1,2}$ = 3.7 Hz, H-1 GlcNAc), 4.30 (1H, d, $J_{1,2}$ = 7.8 Hz, H-1 Gal), 4.28 (1H, m, βCHThr), 2.60 (1H, dd, $J_{3eq,3ax}$ = 12.0 Hz, $J_{3eq,4}$ = 4.4 Hz, H-3$_{eq}$ Neu5Ac), 1.92, 1.90 (6H, 2 × s, 2 × NH*Ac*), 1.60 (1H, t, $J_{3eq,3ax}$ = $J_{3ax,4}$ 12.0 Hz, H-3$_{ax}$ Neu5Ac), 1.24 (3H, d, J = 6.5 Hz, C*H*$_3$ Thr)	Calcd. for $C_{29}H_{48}N_3O_{20}Na^+$ [M + Na]$^+$: 798.2756, found: 798.2756.
(**4**)	β	Neu5Ac-α-2,3-LacNAc-β-Thr (**18**)	90%	δ 4.42 (1H, d, $J_{1,2}$ = 8.1 Hz, H-1 GlcNAc), 4.36 (1H, d, $J_{1,2}$ = 7.8 Hz, H-1 Gal), 4.20 (1H, m, βC*H*Thr), 2.60 (1H, dd, $J_{3eq,3ax}$ = 12.1 Hz, $J_{3eq,4}$ = 4.4 Hz, H-3$_{eq}$ Neu5Ac), 1.92, 1.90 (6H, 2s, NH*Ac*), 1.60 (1H, t, $J_{3eq,3ax}$ = $J_{3ax,4}$ 12.0 Hz, H-3$_{ax}$ Neu5Ac), 1.24 (3H, d, J = 6.5 Hz, C*H*$_3$ Thr).	Calcd. for $C_{29}H_{48}N_3O_{20}Na^+$ [M + Na]$^+$: 798.2756, found: 798.2756.
(**5**)	α	H_2N-(Thr)$_2$-[Neu5Ac-α-2,3-LacNAc-α-Thr]-(Thr)$_2$-Gly-OH (**19**)	85%	δ 4.68 (1H, d, $J_{1,2}$ = 3.7 Hz, H-1 GlcNAc), 4.36 (1H, d, $J_{1,2}$ = 8.1 Hz, H-1 Gal), 4.40–3.80 (10H, m, αCHThr, βCHThr), 2.62 (1H, dd, $J_{3eq,3ax}$ = 12.0 Hz, $J_{3eq,4}$ = 4.4 Hz, H-3$_{eq}$ Neu5Ac), 1.92, 1.90 (6H, 2 × s, 2 × NH*Ac*), 1.65 (1H, t, $J_{3eq,3ax}$ = $J_{3ax,4}$ = 12.0 Hz, H-3$_{ax}$ Neu5Ac), 1.20–1.00 (15H, m, 5 × C*H*$_3$ Thr)	Calcd. for $C_{47}H_{80}N_8O_{30}$ [M + H]$^+$: 1237.38, found: 1237.38.
(**6**)	β	H_2N-(Thr)$_2$-[Neu5Ac-α-2,3-LacNAc-β-Thr]-(Thr)$_2$-Gly-OH (**20**)	82%	δ 4.42 (1H, d, $J_{1,2}$ = 8.1 Hz, H-1 GlcNAc), 4.35 (1H, d, $J_{1,2}$ = 7.8 Hz, H-1 Gal), 4.40–3.90 (10H, m, αCHThr, βCHThr), 2.62 (1H, dd, $J_{3eq,3ax}$ = 12.1 Hz, $J_{3eq,4}$ = 4.4 Hz, H-3$_{eq}$ Neu5Ac), 1.93, 1.90 (6H, 2 × s, 2 × NH*Ac*), 1.65 (1H, t, $J_{3eq,3ax}$ = $J_{3ax,4}$ = 12.0 Hz, H-3$_{ax}$ Neu5Ac), 1.20–1.00 (15H, m, 5 × C*H*$_3$ Thr)	Calcd. for $C_{47}H_{80}N_8O_{30}$ [M + H]$^+$: 1237.39, found: 1237.39.

(235 mg, 0.54 mmol) (prepared from commercially available amino acid Fmoc-threonine by treatment with caesium carbonate and benzyl bromide in DMF)[48] in 1,2-dichloroethane (4 cm^3) was stirred for 10 h at room temperature with mercuric bromide (430 mg, 1.17 mmol) and then refluxed for 9 h,[29] at which point TLC (hexane–EtOAc, 3 : 7 v/v) showed the disappearance of the donor (**7**). The resulting amber mixture was concentrated *in vacuo* and the residue was purified by silica gel column chromatography (eluent: hexane–EtOAc, 3 : 7 v/v). The α-glycoside (**9**) eluted first, followed by the β-glycoside (**10**): both were obtained as amorphous solids.

α-Glycoside (9). (77 mg, 20%); R_f 0.31 [EtOAc–Hex (7 : 3)]; $[\alpha]_D^{25}$ +30.4 (*c* 1.0 $CHCl_3$); δ_H ($CDCl_3$) 7.77 (2 H, d, J = 7.5 Hz, C*H* Fmoc Ph), 7.64 (2 H, d, J = 6.0 Hz, C*H* Fmoc Ph), 7.49–7.26 (9H, m, Fmoc Ph, OC*H*$_2$Ph), 5.94 (1 H, d, J = 9.3 Hz, N*H*Ac), 5.73 (1 H, d, J = 9.5 Hz, N*H*Thr), 5.20–5.14 (1 H, m, H-3), 5.19, 5.09 (2 H, AB, J_{AB} = 12.2 Hz, OC*H*$_2$Ph), 5.08 (1 H, t, $J_{3,4}$ = 9.4 Hz, $J_{4,5}$ = 9.4 Hz, H-4), 4.70 (1 H, d, $J_{1,2}$ = 3.6 Hz, H-1), 4.55–4.44 (3 H, m, C*H*$_2$ Fmoc, αC*H*Thr), 4.31–4.07 (5 H, m, βC*H*Thr, C*H*Fmoc, H-2, H-6, H-6′), 4.01–3.97 (1 H, m, H-5), 2.06, 2.04, 2.03, 1.96 (12H, 4s, COC*H*$_3$), 1.28 (3 H, d, J = 6.2, C*H*$_3$ Thr); δ_C ($CDCl_3$) 171.5, 170.9, 170.8, 170.6 (*C*OCH$_3$), 169.5 (*C*OCH$_2$Ph), 156.7 (*C*O Fmoc), 144.0, 143.8, 141.5 (*C*quat Fmoc Ph), 134.5 (*C*quat. OCH$_2$Ph), 129.2, 129.1, 128.8, 128.0, 127.3, 125.3, 120.2 (*C*H Ph), 99.5 (C-1), 77.6 (β*C*HThr), 71.4 (C-3), 68.5, 68.4 (C-5, C-4), 68.0 (O*C*H$_2$Ph), 67.7 (*C*H$_2$ Fmoc), 62.2 (C-6), 58.7 (α*C*HThr), 51.8 (C-2), 47.3 (*C*H Fmoc), 23.3, 21.0, 20.9, 20.8 (CO*C*H$_3$), 18.5 (*C*H$_3$ Thr). ESI-HRMS: calcd. for $C_{40}H_{45}N_2O_{13}$ [M + H]$^+$: 761.2916, found 761.2918.

β-Glycoside (10). (200 mg, 52%); R_f 0.26 [EtOAc–Hex (7 : 3)]; $[\alpha]_D$ −15.6 (*c* 1.0, $CHCl_3$); δ_H ($CDCl_3$) 7.76 (2H, d, J = 7.6 Hz, C*H* Fmoc Ph), 7.64 (2H, d, J = 7.8 Hz, C*H* Fmoc Ph), 7.41–7.26 (9H, m, Fmoc Ph, OC*H*$_2$Ph), 5.83 (1H, d, J = 9.1 Hz, N*H*Thr), 5.54 (1H, d, J = 8.4 Hz, N*H*Ac), 5.25 (1H, t, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 9.9 Hz, H-3), 5.21, 5.14 (2H, AB, J_{AB} = 12.2 Hz, OC*H*$_2$Ph), 5.00 (1H, t, $J_{3,4}$, $J_{4,5}$ = 9.9 Hz, H-4), 4.65 (1H, d, $J_{1,2}$ = 8.1 Hz, H-1), 4.47–4.40 (3H, m, C*H*$_2$ Fmoc, βC*H*Thr), 4.35 (1H, dd, J = 7.3 Hz, J = 10.7 Hz, αC*H*Thr), 4.24 (1H, t, J = 7.3 Hz, C*H*Fmoc), 4.19 (1 H, dd, $J_{5,6}$ = 4.4 Hz, $J_{6,6'}$ = 12.3 Hz, H-6), 4.02 (1H, dd, $J_{6,6'}$, $J_{5,6'}$ = 2.2 Hz, H-6′), 3.67 (1H, dd, $J_{1,2}$, $J_{2,3}$, H-2), 3.51–3.47 (1H, m, H-5), 2.03, 2.02, 1.99, 1.93 (12H, s, COC*H*$_3$), 1.20 (3H, d, J = 6.3 Hz, C*H*$_3$ Thr); δ_C ($CDCl_3$) 170.9, 170.6, 170.4, 170.0 (*C*OCH$_3$), 169.4 (*C*OCH$_2$Ph), 156.8 (*C*O Fmoc), 144.0, 141.3 (*C*quat. Fmoc Ph), 135.5 (*C*quat. OCH$_2$Ph), 128.6, 128.5, 128.3, 127.7, 127.1, 125.5, 119.9 (*C*H Ph), 98.5 (C-1), 74.5 (β*C*HThr), 71.9, 71.7 (C-5, C-3), 68.5 (C-4), 67.3 (O*C*H$_2$Ph, *C*H$_2$ Fmoc), 61.9 (C-6), 58.7 (α*C*HThr), 55.3 (C-2), 47.3 (*C*H Fmoc), 23.3, 20.7, 20.5, 20.6 (CO*C*H$_3$), 17.0 (*C*H$_3$ Thr). ESI-HRMS: calcd.

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For $C_{40}H_{44}N_2O_{13}NH_4$ [M + NH_4^+]: 778.3187, found: 778.3187. The physical and analytical data for compound (**10**) were in accordance with the literature.[29]

2-Acetamido-2-deoxy-α-D-glucopyranosyl-L-threonine (1)

A solution of *N*-(fluoren-9-ylmethoxycarbonyl)-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-L-threonine benzyl ester (**9**) (76 mg, 0.09 mmol) in MeOH (3 cm^3) was treated with glacial AcOH (0.3 cm^3) and 10% Pd/C (40 mg) for removal of the *O*-Bn and *N*-Fmoc groups. The reaction mixture was stirred and kept under H_2 (~1.5 atm) for 8 h. The reaction mixture was then filtered through Celite, concentrated *in vacuo* and purified by column chromatography (DCM–MeOH 9 : 1 v/v). The product obtained was then dissolved in MeOH (1.5 cm^3) and made basic with 1 M NaOMe in MeOH. The reaction mixture was stirred for 2 h, and then neutralized with Dowex 50WX8–200 resin. Filtration and concentration of the reaction mixture gave the product (**1**) (28 mg, 86%) as colourless amorphous solid. $[\alpha]_D^{25}$ +61.0 (*c* 0.2, CH_3OH); δ_H (D_2O), 4.80 (1H, $J_{1,2}$ = 3.7 Hz, H-1), 4.40 (1H, m, βC*H* Thr), 3.82 (1H, m, αC*H* Thr), 3.72–3.65 (2H, m, H-2, H-6a), 3.62–3.48 (3H, m, H-3, H-5, H-6b), 3.33 (1 H, t, $J_{3,4}$ = 10.1 Hz, $J_{4,5}$ = 10.1 Hz, H-4), 1.85 (3H, s, NH*Ac*), 1.21 (3H, d, *J* 6.5 Hz, CH_3 Thr). δ_C (CD_3OD, 100 MHz) 99.3 (C-1), 73.2 (β*C*HThr), 73.0 (C-5), 70.8 (C-4), 69.0 (C-3), 61.0 (C-6), 56.8 (α*C*HThr), 53.0 (C-2), 21.3 (NCO*C*H_3), 17.3 (*C*H_3 Thr). ESI-HRMS: calcd. for $C_{12}H_{23}N_2O_8Na^+$ [M + Na]$^+$: 345.1269, found: 345.1270.

2-Acetamido-2-deoxy-β-D-glucopyranosyl-L-threonine (3)

The above procedure for the deprotection of compound (**9**) was applied to compound (**10**). Starting from compound (**10**) (60.0 mg, 0.078 mmol), the product (**3**) was obtained as a colourless amorphous solid (20.0 mg, 80%). $[\alpha]_D^{25}$ +81.1 (*c* 1.0, CH_3OH); δ_H (D_2O) 4.40 (1H, d, $J_{1,2}$ = 8.3 Hz, H-1), 4.18 (1H, m, βC*H* Thr), 3.82–3.78 (1H, m, H-6a), 3.65–3.62 (1H, m, H-2), 3.60 (1H, t, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 9.8 Hz, H-3), 3.52–3.50 (1H, m, 3.92, αC*H* Thr), 3.44 (1H, t, $J_{3,4}$, $J_{4,5}$ = 9.8 Hz, H-4), 3.37–3.30 (2H, m, H-5, H-6b), 1.95 (3H, s, NH*Ac*), 1.22 (3H, d, *J* = 6.5 Hz, CH_3 Thr); δ_C (CD_3OD) 101.9 (C-1), 77.6 (β*C*HThr, α*C*HThr), 76.7 (C-5), 74.7 (C-3), 70.9 (C-4), 61.5 (C-6), 56.5 (C-2), 22.8 (NCO*C*H_3), 18.9 (*C*H_3 Thr). ESI-HRMS: calcd. for $C_{12}H_{24}N_2O_8$ [M + H]$^+$: 323.1449, found: 323.1449.

3,6-Di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-azido-2-deoxy-α-D-glucopyranosyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine benzyl ester (12)

A mixture of 3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-azido-2-deoxy-α-D-glucopyranosyl chloride (**11**)[32,33] (200 mg, 0.31 mmol) and *N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine benzyl ester (**8**) (135.4 mg, 0.31 mmol) in 1,2-dichloroethane (2.5 cm^3) was refluxed with mercuric bromide[29] (112 mg, 0.31 mmol) for 8 h, when TLC (hexane–EtOAc, 3 : 7 v/v) showed the disappearance of the donor (**11**). The resulting amber mixture was concentrated *in vacuo* and the residue was purified by silica gel column chromatography (eluent: hexane–EtOAc, 1 : 1 v/v). The desired azido-sugar glycoside (**12**) was obtained as an amorphous solid (195 mg, 60%). $[\alpha]_D^{25}$ +32.2 (*c* 1.0, $CHCl_3$); δ_H ($CDCl_3$) 7.77 (2 H, d, *J* = 7.5 Hz, C*H* Fmoc Ph), 7.64 (2 H, d, *J* = 6.0 Hz, C*H* Fmoc Ph), 7.49–7.26 (9H, m, Fmoc Ph, OCH_2Ph), 5.79 (1 H, d, *J* 9.4 Hz, N*H*), 5.39 (1 H, dd, $J_{3',4'}$ = 3.4 Hz, $J_{4',5'}$ = 0.9 Hz, H-4′), 5.37 (1 H, dd, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 10.4 Hz, H-2′), 5.30 (2 H, AB, J_{AB} = 12.2 Hz, OCH_2Ph), 5.13 (1 H, dd, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 9.8 Hz, H-3), 4.96 (1 H, dd, $J_{2',3'}$, $J_{3',4'}$, H-3′), 4.80 (1 H, d, $J_{1,2}$ = 3.7 Hz, H-1), 4.47 (1 H, d, $J_{1',2'}$, H-1′), 4.40 (3 H, m, αC*H*Thr, βC*H*Thr, H-6b), 4.31 (1 H, t, *J* = 7.3 Hz, C*H*Fmoc), 4.26 (1 H, dd, $J_{6'a,6'b}$ = 11.0 Hz, $J_{5',6'b}$ = 5.9 Hz, H-6′b), 4.21 (1 H, dd, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 5.3 Hz, H-6a), 4.13 (1 H, $J_{6'a,6'b}$ = 11.0 Hz, $J_{5',6'a}$ = 7.4 Hz, H-6′a), 3.99 (1 H, ddd, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$, $J_{5,6b}$ = 1.9 Hz, H-5), 3.97 (2 H, m, CH_2 Fmoc), 3.85 (1 H, dd, $J_{5',6'a}$, $J_{5',6'b}$, H-5′), 3.66 (1 H, dd, $J_{3,4}$, $J_{4,5}$ = 9.5 Hz, H-4), 3.09 (1 H, dd, $J_{1,2}$, $J_{2,3}$, H-2), 2.16–1.96 (18 H, 6 s, 6 CH_3CO), 1.33 (3 H, d, *J* = 6.5 Hz, CH_3 Thr); δ_C ($CDCl_3$) 170.5–169.3 (*C*OCH_3, *C*OCH_2Ph), 157.1 (*C*O Fmoc), 144.0, 141.2 (*C*quat. Fmoc Ph), 135.1 (*C*quat. OCH_2Ph), 128.8, 127.9, 127.3, 125.5, 120.1 (*C*H Ph), 101.3 (C-1′), 99.2 (C-1), 76.6 (C-4), 71.2 (C-3′), 70.9 (C-5′), 70.1 (C-3), 69.3 (C-2′), 69.1 (C-5), 68.0 (C-4′), 66.7 (*C*H_2 Fmoc, O*C*H_2Ph), 62.1 (C-6), 61.6 (C-6′), 60.8 (α*C*HThr, β*C*HThr), 59.1 (C-2), 47.3 (*C*H Fmoc), 21.2–20.8 (CO*C*H_3), 18.9 (*C*H_3 Thr). ESI-HRMS: calcd. for $C_{50}H_{56}N_4O_{20}NH_4^+$ [M + NH_4^+]: 1050.3824, found: 1050.3831.

3,6-Di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranosyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine benzyl ester (13)

Azidosugar (**12**) (150 mg, 0.145 mmol) was added to a mixture of THF–acetic anhydride–acetic acid (7.0 cm^3) and zinc dust (247 mg, 3.8 mmol) was added.[33] Saturated aqueous $CuSO_4$ solution (0.4 cm^3) was added to the reaction mixture and it was stirred for 30 min at room temperature. The mixture was filtered through Celite and co evaporated with toluene. Purification by column chromatography (hexane–EtOAc 3 : 7 v/v) afforded the product (**13**) as an amorphous solid (99 mg, 65%). $[\alpha]_D^{25}$ + 28.8 (*c* 1.0, $CHCl_3$); δ_H ($CDCl_3$) 7.77 (2 H, d, *J* = 7.5 Hz, C*H* Fmoc Ph), 7.64 (2 H, d, *J* = 6.0 Hz, C*H* Fmoc Ph), 7.49–7.26 (9H, m, Fmoc Ph, OCH_2Ph), 5.85–5.62 (2 H, 2 d, *J* = 9.4 Hz, 2NH), 5.28 (1 H, dd, $J_{3',4'}$ = 3.4 Hz, $J_{4',5'}$ = 0.8 Hz, H-4′), 5.13 (1 H, dd, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 8.6 Hz, H-3), 5.10 (1 H, d, $J_{1',2'}$ 7.8 Hz, $J_{2',3'}$ = 10.4 Hz, H-2′), 5.02 (2 H, AB, J_{AB} = 12.2 Hz, OCH_2Ph), 4.90 (1 H, dd, $J_{2',3'}$, $J_{3',4'}$, H-3′), 4.57 (1 H, d, $J_{1,2}$ = 3.7 Hz, H-1), 4.48 (1 H, d, $J_{1',2'}$ 7.8 Hz, H-1′), 4.37 (3 H, m, αCHThr, CH_2 Fmoc, H-6b), 4.22 (1 H, t, *J* = 7.3 Hz, C*H*Fmoc), 4.14–4.09 (3H, m, βCHThr, H-2, H-6a′), 4.06 (1 H, dd, $J_{6'a,6'b}$ = 11.0 Hz, $J_{5',6'b}$ = 5.9 Hz, H-6b′), 4.01 (1 H, dd, $J_{6a,6b}$ = 11.0 Hz, $J_{5,6a}$ = 5.3 Hz, H-6a), 3.86 (1 H, ddd, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$, $J_{5,6b}$ = 1.9 Hz, H-5), 3.80 (1 H, dd, $J_{5',6'a}$ = 7.4 Hz, $J_{5',6'b}$, H-5′), 3.69 (1 H, dd, $J_{3,4}$, $J_{4,5}$, H-4), 2.15–1.93 (21 H, 7 s, 7 CH_3CO), 1.21 (3 H, d, *J* = 6.5 Hz, CH_3 Thr); δ_C ($CDCl_3$) 170.5–169.3 (*C*OCH_3, *C*OCH_2Ph), 157.1 (*C*O Fmoc), 144.0, 141.2 (*C*quat. Fmoc Ph), 135.1 (*C*quat. OCH_2Ph), 128.8, 127.9, 127.3, 125.5, 120.1 (*C*H Ph), 101.3 (C-1′), 99.3 (C-1), 76.6 (C-4), 71.2 (C-3′), 70.9 (C-3), 70.1 (C-5′), 69.3 (C-2′), 69.1 (C-5), 68.0 (*C*H_2 Fmoc, O*C*H_2Ph), 66.7 (C-4′), 62.1 (C-6), 61.6 (C-6′), 60.8 (α*C*HThr, β*C*HThr), 59.1 (C-2), 47.3 (*C*H Fmoc), 21.2–20.8 (CO*C*H_3), 18.9 (*C*H_3 Thr). ESI-HRMS: calcd. for $C_{52}H_{60}N_2O_{21}H^+$ [M + H]$^+$: 1049.3761, found: 1049.3768.

4-*O*-β-D-Galactopyranosyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-L-threonine (2)

A solution of 3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranosyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine benzyl ester (**13**) (48 mg, 0.045 mmol) in MeOH (3.6 mL) was treated with glacial AcOH (0.36 cm^3) and 10% Pd/C (51mg) for removal of the *O*-Bn and *N*-Fmoc groups. The reaction mixture was stirred under H_2 (~1.5 atm) for approx. 10 h, filtered in Celite, concentrated *in vacuo* and the crude product was purified by column chromatography (DCM–MeOH 9 : 1 v/v). The product obtained (43 mg, 0.058 mmol) was dissolved in MeOH (1.5 mL) and treated with NaOMe (1 M in MeOH) until the solution was basic. The reaction mixture was stirred for 8 h and then neutralized with Dowex 50WX8–200 ion exchange resin. Filtration and concentration of the mixture gave the deprotected α-linked disaccharyl threonine (**2**) as an amorphous solid (20 mg, 90%); $[\alpha]_D^{25}$ +10.0 (*c* 0.1, MeOH); δ_H (D_2O), 4.77 (1H, d, $J_{1,2}$ = 3.7 Hz, H-1), 4.30 (1H, d, $J_{1',2'}$ = 7.8 Hz, H-1′), 4.29 (1H, m, βCHThr), 3.80–3.65 (7H, m, H-2, H-3, H-6a, H-6b, H-3′, H-4′, αCHThr), 3.62–3.52 (4H, H-4, H-5, H-6a′, H-6b′,), 3.50 (1H, m, H-5′), 3.36 (1H, dd, $J_{1',2'}$, $J_{2',3'}$ = 10.1 Hz, H-2′), 1.87 (3H, s, NH*Ac*), 1.24 (3H, d, *J* = 6.5 Hz, CH_3 Thr). δ_C (D_2O) 103.7 (C-1′), 99.5 (C-1), 79.5 (C-4), 76.1 (C-5′), 75.6 (βCHThr), 73.3 (C-5), 71.92, 71.91 (C-3′, C-3), 71.7 (C-2′), 70.1 (α*C*HThr), 69.3 (C-4′), 61.8 (C-6′), 61.7 (C-6), 53.9 (C-2), 22.9 (NCOCH_3), 18.9 (CH_3 Thr). ESI-HRMS: calcd. for $C_{18}H_{32}N_2O_{13}.Na^+$ $[M + Na]^+$: 507.1797, found: 507.1798.

Enzymatic synthesis of 4-*O*-β-D-galactopyranosyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-L-threonine (2)

To a solution of 2-acetamido-2-deoxy-α-D-glucopyranosyl-L-threonine (**1**) (5 mg, 0.015 mmol), UDP-galactose (5.0 mg, 0.008 mmol), and alkaline phosphatase (1.2 mg) in HEPES buffer (0.4 cm^3, 50 mM, pH 7.0, containing 100mM $MnCl_2$) was added the enzyme bovine β-1,4-galactosyltransferase (2.0 U), and the mixture was incubated for 96 h at 30 °C. After completion of the reaction the enzyme was precipitated by the addition of cold ethanol, the mixture was centrifuged and the supernatant was concentrated to dryness. The resulting residue was dissolved in water and fractionated by gel filtration chromatography (15 × 850 mm Toyopearl HW-40S column, eluted with water containing 0.1% TFA, at a flow rate of 0.5 cm^3 min^{-1}). Lyophilization of relevant fractions, typically eluting in the 175–185 cm^3 range, gave deprotected α-linked disaccharyl threonine (**2**) as an amorphous solid (3.9 mg, 57%). Analytical data for this compound were identical to those reported for the same compound produced solely by chemical means.

Enzymatic synthesis of 4-*O*-β-D-galactopyranosyl-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-threonine (4)

The general procedure described for the preparation of compound (**2**) was applied to the galactosylation of β-linked glycosyl amino acid **3**. However, less β-1,4-galactosyltransferase (0.5 U) was employed and the reaction was incubated for a shorter time (24 h). Starting from compound (**3**) (5 mg, 0.015 mmol) the product (**4**) was obtained as an amorphous solid (6.35 mg, 85%). $[\alpha]_D^{25}$ −5.0 (*c* 0.1, MeOH). δ_H (D_2O), 4.39 (1H, d, $J_{1,2}$ = 7.5 Hz, H-1), 4.27 (1H, d, $J_{1',2'}$ = 7.8 Hz, H-1′), 4.23 (1H, m, βC*H*Thr), 3.78 (1H, d, $J_{\alpha CH, \beta CH}$ = 12.1 Hz, αC*H* Thr) 3.77–3.74 (2H, m, H-4′, H-4), 3.70–3.62 (2H, m, H-6a, H-6b), 3.60–3.50 (5H, m, H-6a′, H-6b′, H-5′, H-3, H-2), 3.47 (1H, dd, $J_{2',3'}$ = 9.9 Hz, $J_{3',4'}$ = 3.3 Hz, H-3′), 3.38–3.34 (2H, m, H-5, H-2′), 1.87 (3H, s, NH*Ac*), 1.24 (3H, d, *J* = 6.5 Hz, CH_3 Thr). δ_C (D_2O) 103.7 (C-1′), 100.5 (C-1), 79.0 (C-4), 76.1 (C-5′), 75.4 (C-5), 74.5 (β*C*HThr), 73.3 (C-3′), 72.9 (C-3), 71.7 (C-2′), 69.3 (C-4′), 61.7 (C-6′), 60.6 (C-6), 59.0 (α*C*HThr), 55.6 (C-2), 22.8 (NCOCH_3), 17.9 (CH_3Thr). ESI-HRMS: calcd. for $C_{18}H_{33}N_2O_{13}$ $[M + H]^+$: 485.1977, found: 485.1977.

General method for glycopeptide synthesis[41]

The glycopeptides (**5**) and (**6**) were assembled manually using a fritted glass reaction vessel with nitrogen purging for effective mixing. Pre-loaded FmocGly-Wang resin (typically 100 mg) was swollen in CH_2Cl_2 for 1 h and then washed with DMF (3×). Deprotection of the *N*-α-Fmoc group was carried out using 20% piperidine–DMF followed by filtration, washing with DMF (3×) and subsequently with DIPEA. Coupling reactions were performed with 2.0 mol equiv of Fmoc-amino acid and coupling agents (PyBOP and HOBt) dissolved in DMF. Coupling times were variable, ranging from 3 h to 24 h for the threonine amino acids and between 24 h and 72 h for the glycosylated amino acid (**13**) and the disaccharide glycoside (**15**). The reaction mixtures were filtered after each coupling and the resin washed three times with DMF, CH_2Cl_2 and MeOH. After drying *in vacuo*, small aliquots of resin (1 mg) were treated with 20% piperidine in DMF for removal of *N*-Fmoc group and consequent generation of dibenzofulvene product which absorb UV strongly (290 nm), offering potential for monitoring of coupling reactions by spectrophotometer. After removal of the *N*-Fmoc group of the last amino acid, the glycopeptides were cleaved from resin in the presence of 95% TFA in water and after cleavage was complete, the solutions were filtered to remove the cleaved resin and concentrated to yellowish oil. The crude glycopeptides were triturated with cold Et_2O, dried *in vacuo* and *O*-deprotected with 1 M sodium methoxide solution in MeOH. The purification of glycopeptides (**5**) and (**16**) was carried out on Perkin-Elmer HPLC equipment by reverse-phase chromatography using a Phenomenex Luna C18 column (250 × 10 mm) with a linear gradient of 0–30% CH_3CN in 0.1% aq TFA at 1.0 cm^3 min^{-1}. Under these conditions the retention times for glycopeptides (**5**) and (**16**) were 13.5 min and 13.0 min, respectively.

3,6-Di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranosyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine (14)

A solution of 3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranosyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L-threonine benzyl ester (**13**) (110 mg, 0.1 mmol) in MeOH (7.0 mL) was treated with glacial AcOH (0.7 cm^3) and Pd/C 10% (55 mg) for removal of the *O*-Bn group. The reaction mixture was stirred and kept under H_2 (~1.5 atm) for 1 h, with careful monitoring by TLC (longer reaction times resulted removal of the *N*-Fmoc group). The reaction mixture was then filtered through Celite, concentrated *in vacuo* and purified by column chromatography (DCM–MeOH 9 : 1 v/v). The product (**14**) was obtained as an amorphous solid

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(59.5 mg, 60%). $[\alpha]_D^{25}$ +47.7 (*c* 0.8, $CHCl_3$); δ_H ($CDCl_3$) 7.77 (2 H, d, $J = 7.5$ Hz, C*H* Fmoc Ph), 7.64 (2 H, d, $J = 6.0$ Hz, C*H* Fmoc Ph), 7.45–7.28 (9H, m, Fmoc Ph), 6.58 (1H, d, $J = 9.4$ Hz, N*H*), 6.12 (1H, d, $J = 9.4$ Hz, N*H*), 5.36 (1 H, m, H-4′), 5.16 (1 H, dd, $J_{2,3} = 10.5$ Hz, $J_{3,4} = 9.4$ Hz, H-3), 5.10 (1 H, d, $J_{1',2'}$ 7.8 Hz, $J_{2,3} = 10.4$ Hz, H-2′), 4.94 (1 H, dd, $J_{2',3'} = 10.4$ Hz, $J_{3',4'} = 3.4$ Hz, H-3′), 4.57 (1 H, d, $J_{1,2} = 3.7$ Hz, H-1), 4.48 (1 H, d, $J_{1',2'}$ 7.8 Hz, H-1′), 4.44–4.32 (3 H, m, αCHThr, CH_2 Fmoc, H-6b), 4.25 (1 H, t, $J = 7.3$ Hz, C*H*Fmoc), 4.16–4.08 (3H, m, βCHThr, H-2, H-6a′), 4.06–4.01 (2H, m, H-6b′, H-6a), 3.96–3.82 (2 H, m, H-5, H-5′), 3.72 (1 H, t, $J_{3,4} = 9.8$ Hz, H-4), 2.18–1.90 (21 H, 7 s, 7 CH_3CO), 1.26 (3 H, d, $J = 6.5$ Hz, CH_3 Thr); δ_C ($CDCl_3$) 171.0–169.5 (*C*OCH$_3$), 157.1 (*C*O Fmoc), 144.0, 141.2 (*C*quat. Fmoc Ph), 128.0–120.1 (*C*H Ph), 101.4 (C-1′), 99.0 (C-1), 76.1 (C-4), 71.2 (C-3′), 70.9 (C-3), 70.1 (C-5′), 69.3 (C-2′), 69.1 (C-5), 67.5 (CH_2 Fmoc), 66.8 (C-4′), 62.1 (C-6), 61.6 (C-6′), 60.8 (α*C*HThr, β*C*HThr), 58.5 (C-2), 47.4 (*C*H Fmoc), 21.4–20.8 (CO*C*H$_3$), 18.8 (CH_3 Thr). ESI-HSMS: calcd. for $C_{45}H_{55}N_2O_{21}H^+$ [M + H]$^+$: 959.3228, found: 959.3229.

H_2N-(Thr)$_2$-(LacNAc-α-Thr)-(Thr)$_2$-Gly-OH (5)

Using the standard peptide synthesis procedure outlined, followed by preparative reverse-phase chromatography, glycopeptide (**5**) was obtained as an amorphous solid (7.0 mg, 22%). $[\alpha]_D^{25}$ +3.0 (*c* 0.1, MeOH). δ_H (D_2O) 4.71 (1H, d, $J_{1,2} = 3.7$ Hz, H-1), 4.28 (1H, d, $J_{1,2} = 8.1$ Hz, H-1′), 4.50–4.30 (5H, m, αC*H*Thr), 4.10–3.95 (5H, m, βCHThr), 3.90–3.45 (12H, m, CH_2Gly, H-6a, H-6b, H-6′a, H-6′b, H-2, H-3, H-4, H-4′, H-5), 3.50 (1H, m, H-3′), 3.35 (1H, m, H-2′), 1.85 (3H, s, NHAc), 1.20–1.00 (15H, m, 5 × CH_3Thr). ESI-HSMS: calcd. for $C_{36}H_{63}N_7O_{22}$ [M + H]$^+$: 946.4099, found: 946.4103.

N-(Fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-L-threonine (15)

A solution of *N*-(fluoren-9-ylmethoxycarbonyl)-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-L-threonine benzyl ester (**10**) (119 mg, 0.157 mmol) in MeOH (5.0 mL) was treated with glacial AcOH (0.2 cm^3) and Pd/C 10% (27 mg) for removal of the *O*-Bn group. The reaction mixture was stirred and kept under H_2 (~1.5 atm) for 1 h. After this time, it was filtered in Celite, concentrated *in vacuo* and purified by column chromatography (DCM–MeOH 9 : 1 v/v). The product (**15**) was obtained as an amorphous solid (101 mg, 96%). $[\alpha]_D^{25}$ +14.4 (*c* 1.0, MeOH) (lit.,[49] $[\alpha]_D$ + 14.7); δ_H ($CDCl_3$) 7.73 (2H, d, $J = 7.3$ Hz, C*H* Fmoc Ph), 7.61 (2H, d, $J = 6.6$ Hz, C*H* Fmoc Ph), 7.38–7.24 (4H, m, C*H* Fmoc Ph), 6.12 (1H, d, $J = 9.2$ Hz, N*H*), 6.03 (1H, d, $J = 9.2$ Hz, N*H*), 5.26 (1H, t, $J_{2,3} = 9.6$ Hz, H-3), 5.09 (1H, t, $J_{3,4} = 9.6$ Hz, H-4), 4.64 (1H, d, $J_{1,2} = 8.3$ Hz, H-1), 4.50–3.60 (9H, m, C*H*$_2$ Fmoc, 2 C*H* Thr, H-6, C*H* Fmoc, H-6′, H-2, H-5), 2.04, 1.98, 1.97, 1.93 (12H, s, COC*H*$_3$), 1.18 (3H, d, $J = 6.3$ Hz, C*H*$_3$ Thr); δ_C ($CDCl_3$) 171.3, 170.9, 170.8, 169.3 (*C*OCH$_3$), 156.8 (*C*O Fmoc), 143.7, 141.1 (*C*quat.), 127.6, 127.0, 125.1, 119.9 (*C*H Ph), 99.4 (C-1), 71.7, 71.5, 70.9, 68.2, 67.3 (C-3, C-4, C-5, 2 *C*H Thr, *C*H$_2$ Fmoc), 62.0 (C-6), 52.3 (C-2), 47.0 (*C*H Fmoc), 22.9, 20.8, 20.7, 20.5 (CO*C*H$_3$), 17.3 (*C*H$_3$ Thr). ESI-HRMS: calcd. for $C_{33}H_{39}N_2O_{13}$ [M + NH_4]$^+$: 671.2452, found 671.2453.

H_2N-(Thr)$_2$-(GlcNAc-β-Thr)-(Thr)$_2$-Gly-OH (16)

Using the standard peptide synthesis procedure outlined, followed by preparative reverse-phase chromatography, gave compound (**16**) as an amorphous solid (4.5 mg, 14%). $[\alpha]_D^{25}$ +6.0 (*c* 0.1, MeOH); δ_H (D_2O) 4.42 (1H, d, $J_{1,2} = 8.1$ Hz, H-1), 4.40–4.25 (5H, m, αCHThr), 4.20–4.00 (5H, m, βCHThr), 3.85–3.55 (5H, m, CH_2Gly, H-6a, H-6b, H-5), 3.50 (1H, t, $J = 9.8$ Hz, H-3), 3.36 (1H, t, $J = 9.8$ Hz, H-4), 3.25 (1H, m, H-2), 1.87 (3H, s, NH*Ac*), 1.20–1.00 (15H, m, 5 × CH_3Thr). ESI-HSMS: calcd. for $C_{34}H_{70}N_7O_{17}$ [M + H]$^+$: 847.4705, found: 847.4705.

H_2N-(Thr)$_2$-(LacNAc-β-Thr)-(Thr)$_2$-Gly-OH (6)

To a solution of the glycopeptide H_2N-(Thr)$_2$-(βGlcNAc-Thr)-(Thr)$_2$-Gly-OH (**16**) (4.0 mg, 0.005 mmol) UDP-galactose (2.9 mg, 0.005 mmol), and alkaline phosphatase (0.4 mg) in HEPES buffer (0.4 mL, 50 mM, pH 7.0, containing 100 mM $MnCl_2$) was added the enzyme bovine β-1,4-galactosyltransferase (1.0 U), and the mixture was incubated for 24 h at 30 °C. After completion of the reaction the enzyme was denatured with ethanol and centrifuged before the supernatant was lyophilised. The dry residue was dissolved in water and purified by gel filtration (Toyopearl HW-40 S column, 15 × 850 mm, eluting with 0.1% aqueous TFA at 0.5 mL min^{-1}). Lyophilization of relevant fractions afforded glycopeptide (**6**) as an amorphous solid (3.5 mg, 80%). $[\alpha]_D^{25}$ −2.2 (*c* 0.1, MeOH). δ_H (D_2O) 4.45–4.40 (2H, m, H-1, H-1′), 4.38–4.21 (5H, m, βC*H*Thr), 4.15–4.00 (5H, m, αC*H*Thr), 3.85–3.45 (13H, m, C*H*$_2$Gly, H-6a, H-6b, H-6′a, H 6′b, H-5, H-5′, H-4, H-4′, H-3, H-3′, H-2), 3.37 (1H, m, H-2), 1.88 (3H, s, NH*Ac*), 1.20–1.00 (15H, m, 5 × C*H*$_3$Thr). ESI-HSMS: calcd. for $C_{36}H_{63}N_7O_{22}$ [M + H]$^+$: 946.4099, found: 946.4103.

Cell culture and expression of *T. cruzi trans*-sialidase

SOB media (Hanahan's broth)

Tryptone (20.0 g), yeast extract (5.0 g), sodium chloride (0.5 g) were dissolved in de-ionised water (1000 cm^3). The media was autoclaved and stored until required. Immediately prior to use, potassium chloride (10 cm^3, 250 mM stock solution, pH 7.0, sterile filtered) and magnesium chloride (5 cm^3, 2 mM stock solution, sterile filtered) were added.

Cell culture

Clones of TcTS (pTrcTs611/2)[50] in *E. coli* (XL1-Blue) were selected and raised on ampicillin (100 μg cm^{-3}) LB agar slopes. Single colonies were selected for the generation of glycerol stocks (20% glycerol; stored at −80 °C) using culture conditions given below. Overnight cultures were prepared directly from glycerol stocks in sterile SOB media (25 cm^3 in 50 ml culture tubes) with ampicillin (100 μl cm^{-3}, sterile filtered). The cultures were incubated overnight at 37 °C with shaking (200 rpm). SOB media (1000 cm^3 containing 100 μl cm^{-3} ampicillin, sterile filtered prior to addition, 2 L baffled plastic culture flasks) was warmed to 37 °C and inoculated to an optical density of 0.05–0.1 OD_{600} units. Cultures were left to incubate at 37 °C with shaking (250 rpm) until OD_{600} = 0.6–0.8 (4–6 h). Cells were induced with 1 mM IPTG (sterile filtered) and left for 12 h at 23 °C. The cells were

then harvested by centrifugation at 4 °C and the cell pellet was stored at −20 °C until required.

Preparation of a cell-free extract

The cell pellet was thawed on ice and re-suspended in 30 ml of phosphate buffer saline (PBS). After 30 minutes incubation on ice, the viscosity of the solution was decreased by addition of DNase (20 μg cm^{-3}). The cells were then sonicated on ice for 5 × 1 minutes (cycle 2, 50% power) with 1 minute intervals between bursts. The suspension was then centrifuged (10000 g for 30 min) to remove cell debris. The supernatant was then filtered through a 0.2 μm filter. The cleared protein lysate obtained was then employed in biotransformations.

General procedure for enzymatic reactions with *trans*-sialidase

Reactions were typically conducted with 1–2 mg of LacNAc-based acceptor substrate. To a solution of acceptor (1 eq.) and the calf serum fetuin (3 mol eq with respect to sialic acid), in phosphate buffer (0.2 M, pH 7.0) was added sufficient TcTS to effect complete conversion within 2 h at 30 °C. After completion of the reaction, as judged by TLC (DCM–MeOH–H_2O 10 : 10 : 3 v/v/v), protein was removed with a VIVASPIN 500 spin filter (MW cut-off 10000), and the filtrate was directly applied to a gel filtration column (Toyopearl HW-40 S column, 15 × 850 mm) using 5 mM NH_4HCO_3 buffer as eluent at 0.5 cm^3 min^{-1}. Data for sialylated compounds (**17–20**) can be found in Table 1.

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