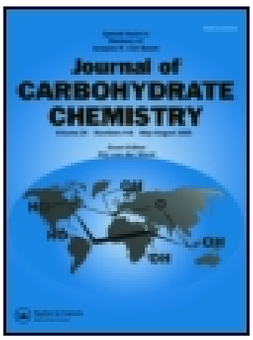

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Synthesis of glycoimmunogen Tn-Thr-PS A1 *via* hydrazone bond and stability optimization of PS A1 monosaccharide mimics under vaccine development conditions

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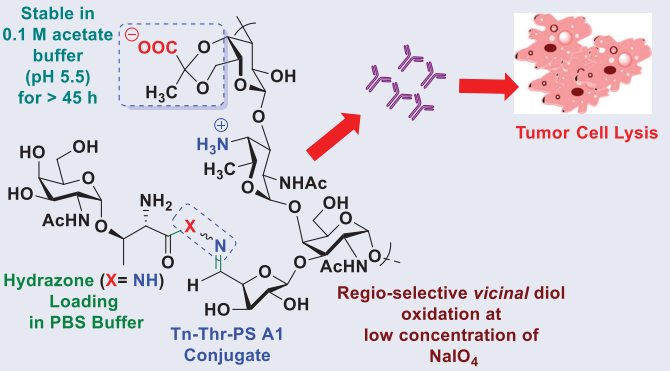
ABSTRACT

Previously, our group constructed several immunogens utilizing oxime linkage to conjugate a T-cell stimulatory zwitterionic polysaccharide PS A1 and tumor associated carbohydrate antigens (TACAs) in acetate buffer. Here, a semi-synthetic immunogen was synthesized using hydrazone conjugation between PS A1 and a glycopeptide hydrazide (α-D-GalNAc-L-Thr-NH-NH_2) with an excellent loading in PBS buffer. To get robust immune response, the retention of zwitterionic character of PS A1 under vaccine construction conditions is essential. In this regard, the stability of embedded pyruvate acetal moiety in tetrasaccharide repeating unit of PS A1 can validate the retention of the dual charges. Therefore, rather than utilizing this highly immunogenic PS A1 fully, stability studies were performed with synthetic 1-thiophenyl-4,6-*O*-pyruvate acetal-D-galacto*pyranose* in varying acetate buffer pHs and time intervals. Furthermore, 1-propyl-D-galacto*furanose* was synthesized to mimick the D-Gal*f* of PS A1 to examine regioselective hydrazone and oxime formation with α-D-GalNAc-L-Thr-NH-NH_2 and α-D-GalNAc-ONH_2 moieties respectively.

GRAPHICAL ABSTRACT



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Supplemental data for this article can be accessed on the publisher's website.

Introduction

Tumor associated carbohydrate antigens (TACAs),[1,2] as well as carbohydrate antigens of pathogens (bacterium, virus and fungus),[3] are significant bio-markers for the development of therapeutic and preventive vaccines because they are prominent on cell surfaces.[4] However, these B cell dependent, poorly immunogenic carbohydrate antigens are not able to induce cellular T (cluster of differentiation, CD 4+ and/or CD8+) cell dependent immunity on their own, critical for the presentation on major histocompatibility class (MHC) molecules of antigen presenting cells.[5] Over the past few years, a number of strategies have been devised to overcome the T-cell independent barrier for development of semi[6] or fully synthetic, multi-component carbohydrate-based cancer vaccines.[7] For example, conjugating TACAs (Tn, TF, STn, GM2, GD2, GD3, fucosyl-GM1, Globo-H and Lewis^y) with i) protein carriers such as keyhole limpet hemocyanin (KLH), tetanus toxoid (TT), bovine serum albumin (BSA), diphtheria toxin (CRM197),[4] ii) polysaccharides like zwitterionic polysaccharides (ZPSs), *Hemophilus influenzae* type b (*Hib*), *Streptococcus pneumoniae* (*Spn*),[5,8] iii) Toll like receptor 2 (TLR2) ligand, $Pam_3CysSerK_4$,[9] iv) immunological epitopes (T-epitope)[10] have been achieved. During optimization of vaccines, major challenges include antigen stability, high antigen loading levels, and construct/immunogen shelf-life stability. Herein, we have carried out stability tests by studying simple hydrolytic effects with synthesized monosaccharide units of the valuable immunogenic PS A1 polysaccharide T-cell stimulant.

In order to compensate for the T-cell independent nature of carbohydrate antigens, our group has been pursuing zwitterionic polysaccharide (ZPS), PS A1, as a carrier. This ZPS was isolated as a capsular polysaccharide from the commensal anaerobe *Bacteroides fragilis* (ATCC 25285/NCTC 9343) and consists of a tetrasaccharide core repeating unit (~120 repeat units, overall MW ~110 kD).[11–13] PS A1 was discovered to invoke MHC II mediated T-cell dependent immune responses.[14] The monosaccharide units of PS A1 are 4,6-*O*-pyruvate acetal D-galacto*pyranose*, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose, 2-acetamido-D-galacto*pyranose* and D-galacto*furanose*.[15]

Among a known number of TACAs, the Thomsen-nouveau (Tn) antigen (GalNAc-α-1-*O*-Ser/Thr) is the simplest and most widely studied.[16] Previously, our group prepared a Tn-PS A1 immunogen by conjugating a aminooxy α-D-GalNAc to an oxidized PS A1 *via* an oxime bond.[17] An immunogenicity profile of the vaccine was examined using C57BL/6 mice rendering a robust immune response against the Tn antigen.[17]

Although, oxime/hydrazone bond formation is reversible, the linkages are comparatively stable and faster than their corresponding imines due to the α-effect.[18,19] Because of the relative high stability, oxime conjugation strategies have been utilized in many biorthogonal conjugation reactions. Oximes, with greater inherent stability than hydrazones, are typically utilized for more robust linkage strategies, whereas acid labile hydrazones, which succumb to hydrolysis under more acidic conditions, are preferentially utilized for the release of biologically active molecules such as in antibody-drug conjugates.[20] In both cases, however, the commonly observed resistance to hydrolysis can be explained by the inductive effect of the more electronegative oxygen ($\chi_N = 3.5$) compared to the nitrogen atom ($\chi_N = 3.0$) (Fig. 1).[21]

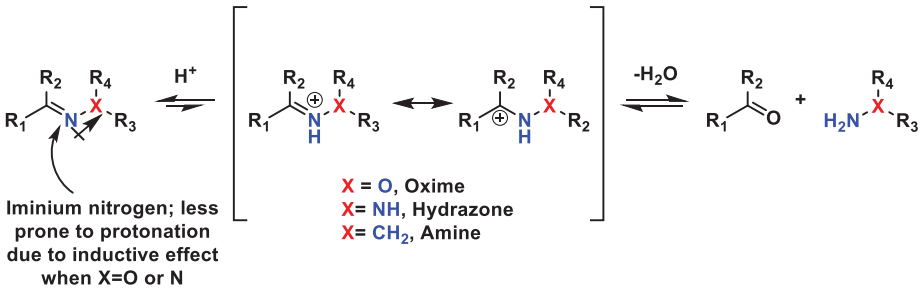


Figure 1. Hydrolysis of oxime/hydrazone/amine bonds.

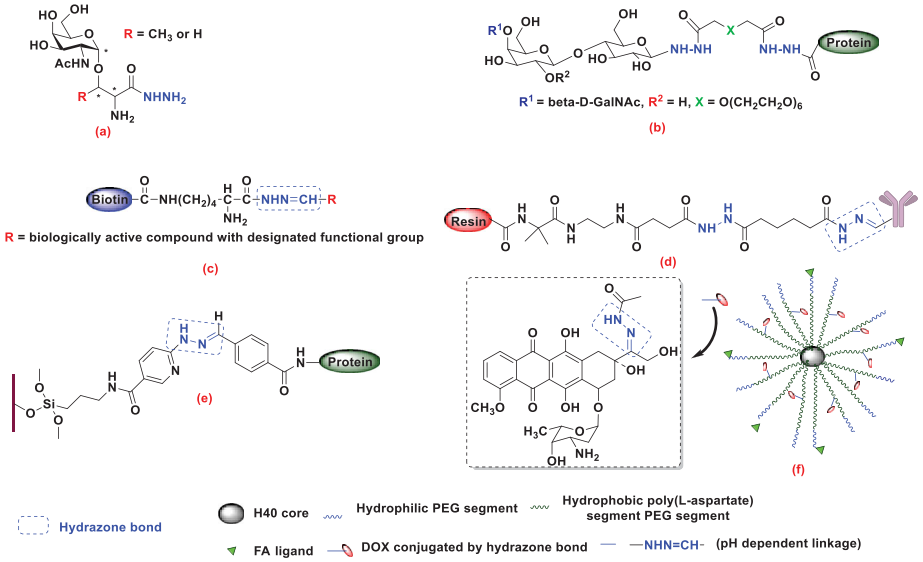
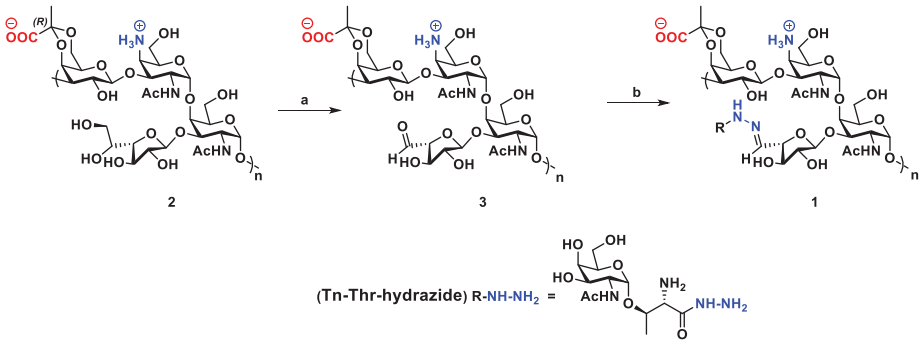


Figure 2. (a) Previously synthesized Tn-hydrazides,[22] (b) the structure of[23] glycan-bishydrazide conjugate,[24] (c) biotin reactive derivative,[25] (d) antibody immobilization with GlycoLink™ resin,[26] (e) conjugating protein with oxide-passivated silicon photonic microring resonators,[27] (f) the structure of H40-P (LA-DOX)-b-PEG-OH/FA copolymer.[20]



Scheme 1. Synthesis of Tn-Thr-PS A1 *via* hydrazone linkage. **Reagents and conditions:** (a) 0.1 M acetate buffer (pH 5.1), 2 mM $NaIO_4$ solution, dark room, rt, 90 min; (b) α-D-GalNAc-L-Thr-NH-NH_2, 1X PBS buffer (pH 7.2), rt, overnight.

Previously, our group synthesized a complete stereochemical set of α- and β-linked D-GalNAc-*O*-Ser/Thr antigens, with the aim of understanding structural aspects of the Tn antigen for immune recognition (Fig. 2a).[22] Currently, we are endeavoring to develop a vaccine through conjugation with one Tn antigen (α-D-GalNAc-L-Thr-NH-NH_2) and PS A1 (Sch. 1) employing a hydrazone link rather than an oxime. The aldehyde-reactive hydrazine group is commonly used for labeling and bioconjugation with a variety of biomolecules.[23] For example, with the aim of lectin binding and pathogen detection, glycoconjugates of bishydrazides were synthesized by conjugating α,ω-bishydrazides of carboxymethylated hexa (ethylene glycol) with unprotected saccharides at one end and a carrier protein (BSA) at the other (Fig. 2b).[24] For a variety of assay-based reasons, bacterial polysaccharides and glycoproteins have been labeled with biotin using hydrazide-biotin reactive derivatives (Fig. 2c).[25] Similarly, functionally modified antibodies or other proteins have been attached to affinity resins (e.g. GlycoLink™ coupling resin) (Fig. 2d)[26] or other solid support via the hydrazide moiety (Fig. 2e).[27] Furthermore, the utility of these acid-sensitive hydrazone linkages can be recognized by examining their application in various drug delivery vehicles such as nanoparticles, polymers, dendrimers, micelles, liposomes, and utilized in pH-responsive drug delivery (Fig. 2f).[20]

Results and discussions

PS A1 isolation

Bacteroides fragilis (ATCC 25285/NCTC 9343) was cultured in sterile and anaerobic conditions to isolate crude PS A1 and the crude was purified utilizing a previous protocol.[28] Recorded ^{1}H NMR at 22 °C and 60 °C and

COSY spectra of the purified PS A1 was comparable exactly with reported literature values.[28] It was then used for conjugation with α-D-GalNAc-L-Thr-NH-NH_2 to get our desire immunogen.

Synthesis of Tn-Thr-PS A1

The vaccine construct was assembled with a slight modification of our previously reported protocol.[29] We observed less antigen loading as compared to our previous oxime-based Tn-PS A1. To improve the loading, we conducted conjugation reactions in 1X PBS buffer (pH 7.2), which resulted in an increased loading of the Tn-Thr antigen to PS A1 (Sch. 1). The conjugation was validated by the presence of hydrazone doublets corresponding to the *E* and *Z* isomers at δ 7.47 and 7.12 ppm, respectively. Considering the molar fraction of the hydrazone doublets with respect to the molar fraction of PS A1 methyl group present on the pyruvate acetal of PS A1 and then analyzing the integration of ^{1}H NMR spectrum, we found a 9:1 *E/Z* ratio and an antigen loading of 8% (see Supplementary Information).

Factors effecting hydrolysis of pyruvate acetal on the D-galactopyranose

The tetrasaccharide repeating unit of PS A1 includes a pyruvate acetal ring, which contains an electron withdrawing carboxylate group adjacent to the acetal carbon center. A few factors, such as buffer,[30] solvent[30] and neighboring group participation,[31,32] can influence cleavage of this pyruvate acetal *via* a hydrolysis reaction.[33,34] However, it is known that the zwitterionic charge character is essential for immunogenicity of PS A1.[35] Since eliminating the carboxylate of the pyruvate acetal group *via* hydrolysis renders PS A1 non-zwitterionic, i.e. non-immunogenic,[35] so our focus turned to the stability of the pyruvate acetal group under biological conditions, including acetate buffer condition (pH 5.5). Hydrolysis of acetals involves

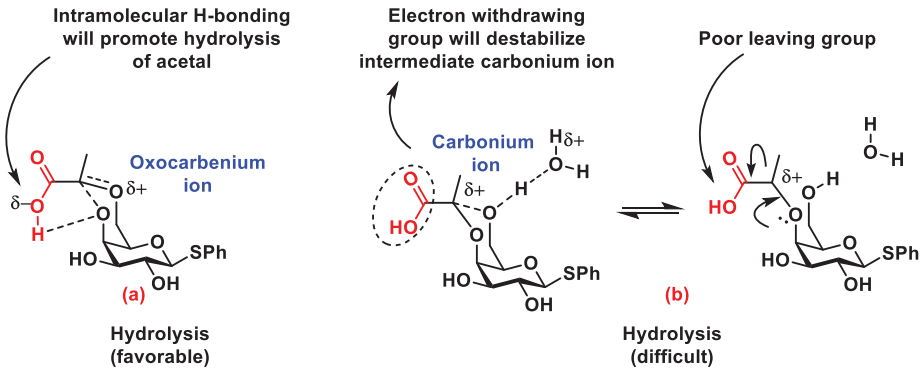


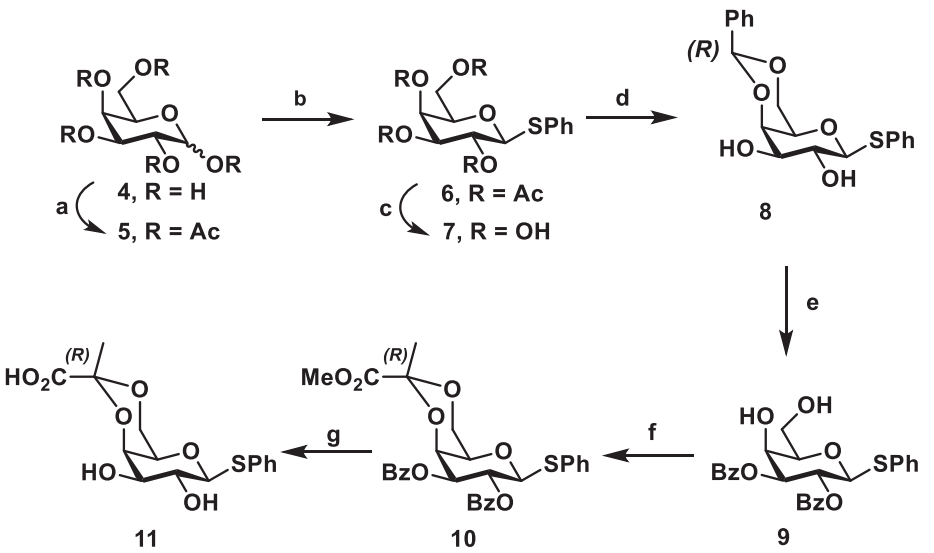
Figure 3. Hydrolysis of pyruvate acetal on the D-galacto*pyranose* sugar.

protonation followed by decomposition to alcohol and carbonium ion or oxocarbenium ion (Fig. 3). Generally, acetal bond cleavage is favored in the presence of a good leaving group or the stability of intermediate carbonium and oxocarbenium ion.[36,37] Furthermore, intramolecular participation of the neighboring acetamido group[31] or carboxyl group[32] is known to facilitate hydrolysis of glycosides and phenolic acetals. Therefore, as pyruvate acetal has a carboxyl functionality that might undergo neighboring group participation by intramolecular H-bonding with the oxygen, as noted in Figure 3a, hydrolysis of the acetal can be promoted even in the absence of hydronium ions.[38] Fortunately, carboxylic is a poor leaving group, and the presence of this electron withdrawing group adjacent to the intermediate carbonium or oxocarbenium center usually destabilize the ions,[39] which can potentially lead to the cleavage of this particular acetal difficult (Fig. 3b).

Since promising immunological responses have been obtained from entirely carbohydrate based Tn-PS A1 vaccine,[17] we always assumed that Tn-PS A1 was stable under various conditions. However, up until this point, our assumption was never experimentally proven. In comparison, many comparative hydrolytic stability studies were carried out by several groups on a series of hydrazone and oxime conjugates.[40,41] Kalia *et al.* reported that acceleration of oxime and hydrazone bond formation is acid-catalyzed, but slows at low pH due to protonation of either heteroatoms.[41] However, in order to address our concern regarding acetal stability, we designed and synthesized 1-thiophenyl-4,6-*O*-pyruvate acetal-D-galcto*pyranose* (**11**) to perform analogous stability studies.

Synthesis of phenyl 4,6-O-pyruvate acetal-1-thio-D-galactopyranosidee (11)

The synthesis of compound **11** commenced from monosaccharide D-galacto*pyranose* **4**, which was peracetylated by treatment with excessive acetic anhydride in pyridine in the presence of a catalytic amount of DMAP (Sch. 2). Peracetylated saccharide **5** was subsequently treated with thiophenol and boron trifluoride dietherate in anhydrous DCM at 0 °C overnight to afford compound **6**.[42] Following purification of compound **6**, Zemplén conditions were employed using freshly prepared NaOMe/MeOH from sodium metal and methanol to deprotect the acetate groups. After completion of reaction, NaOMe was neutralized using DOWEX® 50X8-100 and the resulting reaction mixture was evaporated to dryness. Subsequently, a 4,6-benzylidine group was installed by treatment of **7** with benzaldehyde dimethylacetal in the presence of camphorsulphonic acid to yield compound **8** in a 77% yield. Reaction of benzoyl chloride with compound **8** in the presence of catalytic DMAP in pyridine, followed by deprotection of



Scheme 2. Synthesis of 1-thiophenyl-4,6-*O*-pyruvate acetal-D-galacto*pyranose*. **Reagents and conditions:** (a) Ac_2O (10 eq), pyr, DMAP (cat.), rt, overnight, 75%; (b) $BF_3{\cdot}OEt_2$, thiophenol, anhyd. DCM, 0 °C, 80%; c) NaOMe, MeOH, rt, 1 h, 90%; (d) benzaldehyde dimethylacetal (1.2 eq), camphorsulphonic acid (pH ∼ 4–5), DMF, rt, 5 h, 77%; (e) (i) BzCl (7 eq), pyr, DMAP (cat.), overnight, rt, 82%; (ii) *p*TSA (cat.), MeOH, rt, 81%; (f) methyl pyruvate (2 eq), $BF_3{\cdot}OEt_2$ (2 eq), CH_3CN, rt, 2.5 h, 50%; (g) saturated NaOH solution, reflux, 2 h, 50%.

benzylidine acetal with *p*-toluene sulfonic acid in methanol, produced compound **9** in a very good overall yield for two steps. The 4,6-pyruvate acetal was installed employing the reaction with methyl pyruvate and $BF_3{\cdot}OEt_2$ in acetonitrile to generate (*R*)-pyruvate acetal **10** in a 50% yield.[13,43] Saponification of compound **10** at room temperature resulted in the target compound **11**, which, after purification, was used in stability studies.

NMR based stability study

We utilized compound **11** to examine the stability of the pyruvate acetal, as it is similar to the saccharide moiety present in the repeating unit of PS A1 (see Sch. 1). We understood that if cleavage of the acetal group took place, the chemical shift value of H-4, H-6, C-4 and C-6 of compound **11** would move upfield due to the loss of the electron withdrawing carboxylic group. ^{1}H and ^{13}C NMR spectra of compound **11** were recorded in deuterated acetate buffer (pD 5.51) at different time intervals for a total of 21 h. After the allotted time, no shift of the signals was noticed, which ensures no acetal cleavage (see Supplementary Information). The quaternary carbon peak of pyruvate acetal in the ^{13}C NMR spectra confirmed no hydrolysis of the ring either. Furthermore, HMBC spectrum revealed the correlation of H-6 (at δ 3.90–3.88) with quaternary carbon (at δ 100.84) present in the

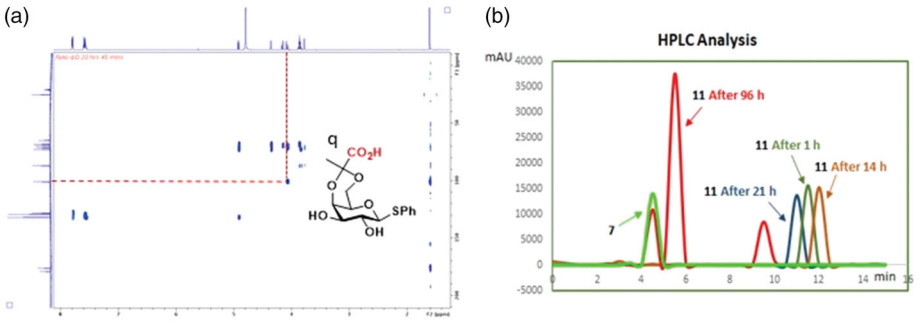


Figure 4. (a) HMQC spectrum indicated the presence of a quaternary carbon 21 h after heating compound **11** in acetate buffer (pD 5.5), (b) HPLC results at different time intervals of **11** in acetate buffer (pH = 5.5).

Table 1. NMR based stability studies of **11**.

Acetate buffer (pD)	Temperature (°C)	Time (h)	Significant signal shift
5.51	22	21	No
1.3	22	48	No
	37	24	No
	50	6	No
	70	1	No
	85	4	Yes

pyruvate acetal ring (Fig. 4a). To determine the stability under more acidic conditions, NMR spectra of compound **11** were recorded at pD 1.3 using deuterated chloroacetic acid buffer under a normal probe temperature (22 °C) in varying time intervals for 48 h (see Supplementary Information). No peak shifts were observed, indicating that bond cleavage did not take place. As pH decreased with an increase in temperature, the NMR probe temperature was raised to 37 °C and spectra were recorded multiple times within 24 h. The temperature was further increased to 50 °C for 6 h, followed by 70 °C for 1 h. The compound was stable under all the temperature conditions noted. Finally, the temperature was elevated to 85 °C and spectra were recorded at varying time intervals for 12 h. At time zero, no cleavage was observed; however, at around the 4 h time-frame, approximately 50% of cleavage was noted. This was confirmed by comparing the integration of new peaks to the actual peaks in recorded ^{1}H NMR spectrum of the compound (see Table 1).

We observed upfield shifts of relevant proton and carbon signals. After 4 h of heating, we analyzed 1 D (^{1}H, ^{13}C, DEPT135) and 2 D (H-H COSY, HMQC, HMBC) spectra of the compound to identify the peaks properly. The H-4 signal was observed to move from δ 4.20 to 3.97 and H-6 shifted from δ 4.00 to 3.73–3.70. The H-3 and H-5 peaks were also shifted upfield, respectfully. Similarly, a ^{13}C-4 signal shifted from δ 71.53 to 67.95 and the

C-6 peak shifted from δ 65.21 to 60.87. In addition, a new quaternary carbon signal appeared at δ 124.61, which represents a signal related to a hydrated acetal of the pyruvic acid. Considering all the chemical shifts, it is clear that pyruvate acetal is very stable at pD 5.51 and the stability is negatively correlated with temperature and positively correlated with pH.

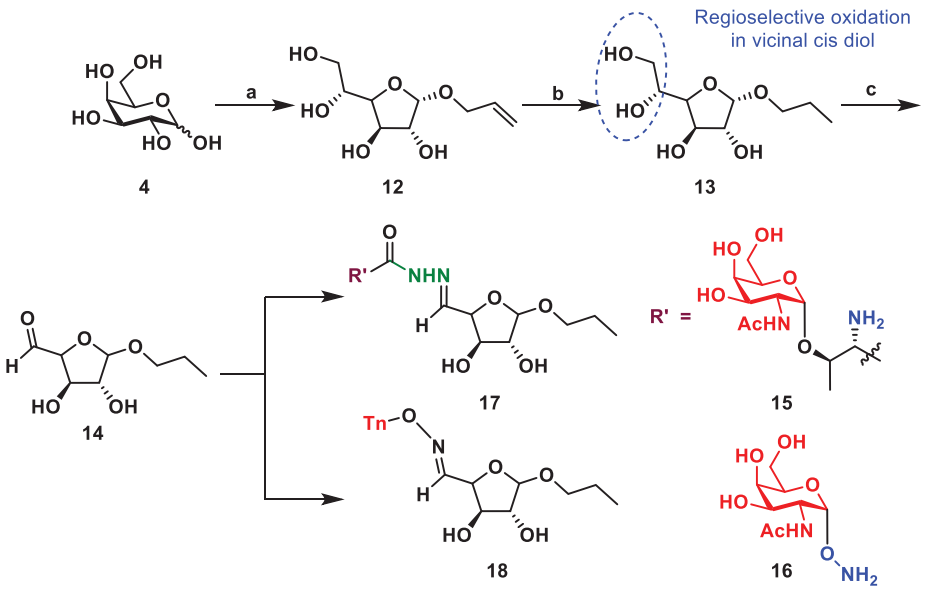
HPLC based stability study

High performance liquid chromatography (HPLC) was also used to trace the stability of compound **11** in 0.1 M acetate buffer (pH = 5.5) at different time intervals by closely followung a previously reported hydrolytic study.[44] A 600 µM solution of compound **11** was prepared in acetate buffer then approximately 10 µL of the sample was injected in HPLC after 1 h, 14 h, 21 h, and 96 h. We observed a corresponding single major peak with minor decrease in area and shift in retention time after 21 h compared to the initial injection. However, after 96 h, we observed multiple peaks. One of these peaks had a similar retention time (RT) with compound **7** (Fig. 4b), which might indicate hydrolysis.

Regioselective conjugation

To achieve regioselective conjugation of Tn-hydrazide antigen with PS A1, we conducted oxidation of PS A1 with 2 mM sodium metaperiodate solution. We anticipated that under this condition, only the vicinal *cis*-diol would be oxidized to leave the vicinal *trans* diol intact. In order to reveal the regioselective cleavage of *cis*-diol over *trans*- diol of galacto*furanose*, we synthesized propyl D-galacto*furanoside* **13** and subjected it to low to harsher oxidation conditions and analyzed the outcome. Thus, galacto*furanose* was converted into allyl galacto*furanoside* **12** by stirring with allyl bromide and sodium hydride in DMPU for 72 h (Sch. 3), which was followed by 10% Pd/C-catalyzed hydrogenolysis in methanol under 40 psi hydrogen pressure to produce **13**, which was purified by a silica gel column.

Thereafter, **13** was treated with a 4.5 mM $NaIO_4$ solution in 100 mM acetate buffer. The reaction was allowed to continue with stirring in the dark ($NaIO_4$ is light sensitive)[45] for 3 h in order to form the aldehyde. Only a 30% yield of aldehyde **14** was obtained as a result of the *cis*-diol cleavage. No *trans*-diol cleavage was observed (see Supplementary Information, compound **14** with a single aldehyde peak at 9.6 ppm), which is indicative of the selective oxidation under this reaction condition. Tn-hydrazide **15**[22] and aminoxy Tn **16**[17] were synthesized following previous literatures. Finally, the conjugation was examined by dissolving aldehyde **14** in acetate buffer (pH 5.0), followed by treatment with Tn-



Scheme 3. Synthesis of 1-propyl-D-galacto*furanose* and compound **17** and **18**. **Reagents and conditions:** (a) Allyl bromide (4 eq), NaH (3 eq), DMPU, rt, 72 h, 60%; (b) 10% Pd–C/H_2, MeOH, rt, 3 h, 70%; (c) $NaIO_4$ (1 eq, 4.5 mM), 100 mM NaOAc buffer (pH 5.0), rt, 6 h, dark, 30%; (d) **15**, 1X PBS buffer (pH 7.2), rt, overnight; (e) **16**, 0.1 M acetate buffer (pH = 5.5), rt, overnight.

hydrazide (**15**)[22] and aminoxy Tn (**16**) separately. Hydrazone and oxime formation was first detected by ESI-mass spectroscopy. Presence of peaks at m/z 531.2 and 432.9 corresponding to the (M + Na) peak of compound **17** and **18** were detected. Compound **17** and **18** were purified with a P2-Biogel column using water as eluent. ^{1}H NMR spectra of **17** and **18** revealed the presence hydrazone and oxime doublets at δ 7.62 and 7.38, respectively. Therefore, the synthesis of 1-allyl-galacto*furanose* and subsequent oxidation and conjugation validated the reaction outcome that was used for Tn-PS A1 conjugation. Furthermore, 1 D TOSCY of **18** was taken by radiating the peak at 7.34 ppm to confirm the oxime conjugation between aminooxy Tn and C-5 in compound **18**.

Conclusion

In summary, the Tn-Thr-PS A1 conjugate was synthesized by coupling a hydrazine derivative of α-D-GalNAc-*O*-L-Thr with oxidized PS A1, giving rise to the formation of a hydrazone conjugation. The synthesis of phenyl 4,6-*O*-pyruvate acetal-1-thio-D-galacto*pyranoside* was achieved to mimic PS A1 and used to evaluate the stability of pyruvate acetal. Stability studies were performed with NMR using varying pD, time, and temperature. The pyruvate acetal was revealed to be very stable at pD 5.1 and 1.3 at room temperature. The compound was stable for hours even after being heated to 70 °C at pD 1.3, but

cleavage was observed at pD 1.3 when the compound was heated at 85 °C. Another stability study was carried out with HPLC using varying time and showed that the pyruvate acetal was stable after our desired reaction time at pH 5.5. Finally, to reveal the cleavage of *cis*-diol over *trans*-diol in galacto*furanose*, propyl D-galacto*furanoside* was synthesized and subjected to oxidation conditions. Only the *cis*-diol was found to be cleaved with 4.5 mM $NaIO_4$ in 100 mM acetate buffer. These observations suggested that our strategy to oxidize PS A1 and conjugate it with hydrazine- or aminooxy-based antigens will not alter the structure of PS A1 and regioselective conjugation can be achieved to synthesize vaccines following this strategy.

Experimental

Materials and methods

All reagents and solvents were purchased from Aldrich, AK Scientific, Fisher Scientific, Chem Impex and EMD Millipore. All the solvents and reagents were used without further purification, unless otherwise stated. Thin layer chromatography (TLC) was performed on 0.25 mm Dynamic Adsorbents, L.L.C. pre-coated silica gel (particle size 0.03–0.07 mm, catalog no. 84111, lot # LA2006) to monitor the progress of reaction. TLC plates were visualized using UV light or by staining with *p*-anisaldehyde or ninhydrin solution followed by charring. Whatman Purasil 60 Å (230–400 mesh ASTM) silica gels were used for normal phase column chromatography. ^{1}H and ^{13}C NMR, DEPT135, HMQC, COSY spectra were acquired using Bruker 600 MHz spectrometers. The residual $CDCl_3$ singlet at δ 7.27 ppm (^{1}H NMR) and residual triplet at δ 77.23 ppm (^{13}C NMR) were used as the standard for ^{1}H NMR and ^{13}C NMR spectra respectively. The residual D_2O was referenced to 4.79 when spectra were taken in D_2O Signal patterns are indicated as s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet; dd: doublet of doublets; br: broad and coupling constants are reported in hertz (Hz). Low resolution mass spectra (LRMS) were recorded on an Esquire-LC electrospray ionization (ESI) mass spectrometer. High performance liquid chromatography (HPLC) chromatograms were analyzed with Agilent Technologies 1100 Series. Hypersil GOLD, 5 µm, 150 × 3 mm column from Thermo Fisher scientific, 254 nm wavelength and ACN/H_2O mobile phase were utilized elute the samples.

Isolation and purification of PS A1

B. fragilis ATCC 25285/NCTC 9343 was purchased from Presque Isle Cultures, which was lined on Blood Agar and Bacteroids Bile Esculin (BBE) plates.[15,46] An anaerobic glove bag was used to prepare the plate.

Initial bacterial growth was started by transferring agar plates to anaerobic jar filled with nitrogen (N_2) in presence of oxygen (O_2) indicator strips and incubated at 37 °C. The cultured bacteria were stored at −80 °C to use in future.

Culture of bacteria

1 L of nanopure water containing 20 g proteose-peptone, 5 g yeast extract, 5 g NaCl and 1 mg resazurin was autoclaved and 25% glucose, 25% potassium phosphate, 5% cysteine, 0.5% Hemin in 1 N NaOH and 0.5% Vitamin K1 in absolute ethanol solutions were prepared. Then 2 mL glucose, 2 mL potassium phosphate, 1 mL cysteine, 100 µL Hemin and 50 µL vitamin K1 were added to the autoclaved broth. Resazurin in presence of oxygen made the color of the entire solution red. To get the anaerobic condition the entire solution was degassed with nitrogen until the red color of the solution changed. After achieving the anaerobic condition, 5 mL of liquid media containing *B. fragilis* was transferred by cannulation to the degassed jar. The bacteria inoculated jar was incubated at 37 °C for 24 h.

Extraction of PS A1

After 48 h, the media was centrifuged at 8000 X g for 20 minutes. A 1X PBS buffer was prepared with 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. Then the pH was adjusted to 7.4. After washing with PBS solution, the cells were suspended with water and equal amount of 70% phenol was added to the suspension with continuous stirring at 68 °C. After 30 minutes of stirring the phenol layer was separated from aqueous layer by centrifuging at 5000 X g for 30 minutes at 4 °C. The aqueous layer after repeated extraction with diethyl ether was evaporated to dryness, dissolved in minimal amount of water and subjected to dialysis. After 3 days of dialysis followed by lyophilization, the crude was treated with DNase and RNase in 0.1 M acetate buffer. Then pH of the solution was adjusted to 7.0 by 2 M NaOH solution and then the solution was treated with pronase. Dialysis followed by lyophilization resulted crude polysaccharide, which was purified to obtain PS A1.

Purification of PS A1

The crude was subjected to purification by size exclusion column. At first sephacryl S-300 column was used and the crude was applied to the column by dissolving it in 0.5% sodium deoxycholate, 50 mM glycine and 10 mM EDTA (pH= 9.8).[47] Small fractions were pooled to measure UV absorbance at 220, 260 and 280 nm. Fractions having more than 0.1 absorbance at 260 and 280 nm were treated further with DNase, RNase and Pronase and

were subjected to extra purification. But the fractions having absorbance at 220 nm were pooled and dialyzed. Finally, it was purified by anion exchange column. Before loading on to the column, the crude PS A1 was treated with 3% acetic acid for 1 h at 70 °C. 50 mM Tris-HCl (pH 7.3) and increasing NaCl solution upto 2 M concentration was used as eluent. Pure PS A1 was obtained at the earlier fractions, before eluting with NaCl. PS A1 fractions were subjected to dialysis against distilled water for 3 days to remove buffer. After lyophilization, pure PS A1 was obtained as white fluffy solid. ^{1}H-NMR spectra of lyophilized PS A1 was recorded at 22 °C and 60 °C temperature and compared with the literature to determine and ascertain the presence and purity of the product.

Synthesis of designed conjugates and model compounds

Tn-Thr-PS A1 conjugate

PS A1 (0.001 g, 9.1×10^{-9} mol) was dissolved in 0.4 mL of 100 mM NaOAc buffer (pH 5.0) in an amber vial. To this solution was added 0.1 mL of 10 mM $NaIO_4$ to obtain overall 2 mM $NaIO_4$ concentration in the reaction mixture and the reaction was stirred at room temperature in dark for 60 minutes to afford PS A1 aldehyde. The reaction mixture was purified using a spin column with 3k molecular weight cut off. Nano-pure water was used to drain any residual $NaIO_4$ through the column by centrifuging it at 12000 rpm for 20 minutes and the process was repeated 4 times. Pure PS A1 aldehyde was dissolved in 2 mL of 1X PBS buffer (pH 7.2) and 0.5 mg Tn hydrazide (0.0014 mmol, 3 equiv. Considering PS A1 has an average MW of 110 K g/mol and 120 repeating units per mole) was added to the solution. The reaction was continued at room temperature for overnight in the dark and after that the desired product was purified using a 10k molecular weight cut off spin column. Nano pure water was used for spin column and the washing was repeated 5 times. The compound was lyophilized and ^{1}H NMR spectra was recorded. Formation of conjugate was confirmed by observing hydrazone doublets corresponding to the *E* and *Z* isomers at δ 7.47 and δ 7.12 ppm respectively.

The % loading was calculated based on integration value of hydrazone protons on ^{1}H-NMR spectrum and compared it with methyl proton present on pyruvate ring acetal of PS A1. The loading of antigen was obtained as about 8% by using the following formula:

$$[\text{MW of Tn}/(\text{MW of PSA1} + \text{MW of Tn})] \times (\text{Mole fraction of oxime H}) \times 100,$$

where mole fraction of hydrazone H = Hydrazone H integration/(methyl proton integration/3).

*1,2,3,4,6-penta-O-Acetyl-β-D-galactopyranose (**5**).* Galacto*pyranose* **4** (4 g, 22.22 mmol) was treated with Ac_2O (10 eq) and catalytic amount of DMAP in pyridine at room temperature and stirred overnight.[48] After completion of the reaction as noted by TLC, the solvent was evaporated and co-evaporated with toluene and partitioned with ethyl acetate (100 mL) and water (200 mL). The ethyl acetate layer was concentrated, and compound was purified over silica gel column using 40% ethyl acetate in hexane as eluent and peracetylated galacto*pyranose* **5** was obtained as a white solid (6.5 g, 16.67 mmol, 75%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.64 (d, 1H, $J = 5$ Hz, H-1), 5.42 (d, 1H, $J = 5$ Hz, H-4), 5.34–5.30 (m, 1H, H-2), 5.07 (dd, $J = 6$ Hz, H-3), 4.15–4.11 (m, 1H, H-6), 4.05–4.04 (m, 1H, H-5), 2.15 (s, 3H, OCH_3), 2.11 (s, 3H, OCH_3), 2.03 (s, 3H, OCH_3), 1.98 (s, 3H, OCH_3) ppm; ^{13}C NMR (150 MHz, $CDCl_3$): δ 170., 170.1, 169.9, 169.4,168.9, 92.1, 71.7, 70.8, 67.8, 66.8, 61.0, 20.8, 20.6, 20.6, 20.5 ppm; ESIMS $[(M + Na)^+]$ calcd for $C_{20}H_{24}NaO_9S$ is 463.1, found 462.8; ESIMS $[(M + Na)^+]$ calcd for $C_{16}H_{22}NaO_{11}$ is 390.34, found 390.1.

*Phenyl 2,3,4,6-tetra-O-acetyl-1-thio-β-D-galactopyranoside (**6**).* Compound **5** was again dissolved in anhydrous DCM (65 mL), thiophenol (1.5 eq) was added to it and stirred at 0 °C temperature.[48] Boron trifluoride dietherate (5 eq) was added to the cooled reaction mixture and the reaction was stirred for 24 h, as reaction competition was noted by TLC. Then the reaction mixture was quenched by saturated $NaHCO_3$ solution (until p^H shows neutral solution) and partitioned with DCM (150 mL). The DCM layer was collected, dried over anhydrous Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure. Silica gel column using 30% ethyl acetate in hexane as eluent resulted major beta product, 1-thiophenyl peracetylated galacto*pyranose* **6**, as a pale-yellow solid (5.87 g, 13.34 mmol, 80%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.51–7.49 (m, 1H, Ar-H), 7.32–7.26 (m, 2H, Ar-H), 5.41 (dd, $J_1 = 6$ Hz, $J_2 = 1.2$ Hz, 1H, H-4), 5.23 (t, $J = 12$ Hz, 1H, H-2), 5.05 (dd, $J_1 = 3.6$ Hz, $J_2 = 6$ Hz, 1H, H-3), 4.71 (d, $J = 12$ Hz, 1H, H-1), 4.19–4.16 (m, 1H, H-6), 4.12–4.09 (m, 1H, H-6′), 3.95–3.92 (m, 1H, H-5), 2.51 (s, 3H, OCH_3), 2.08 (s, 3H, OCH_3), 2.03 (s, 3H, OCH_3), 1.96 (s, 3H, OCH_3) ppm; ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.4, 170.2, 170.1,169.4, 132.5, 132.5, 128.9, 128.2. 86.–128.15, 86.58, 71.98, 67.22–67.20, 61.63, 20.86–20.59 ppm; ESIMS $[(M + Na)^+]$ calcd for $C_{20}H_{24}NaO_9S$ is 463.1, found 462.8.

*Phenyl 1-thio-β-D-galactopyranoside (**7**).* NaOMe was prepared in situ by dissolving 60 mg Na in anhydrous methanol at 0 °C. Compound **6** (4.5 g, 10.2 mmol) was dissolved in minimum volume of anhydrous methanol (30 mL) and NaOMe solution (until pH shows strong basic around 9–10) was slowly added to it. After completion of the reaction, the reaction mixture was quenched with amberlite 15 resin. Filtration followed by solvent

evaporation and co-evaporation with anhydrous toluene resulted fully deacetylated sugar **7** in 90% yield (2.5 g, 9.2 mmol). $[\alpha]_D^{22} = -52.1$ (c = 1.0, MeOH);[49] ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.42–7.41 (d, *J* = 6 Hz, 1H, Ar-H), 7.26–7.21 (m, 3H, Ar-H), 4.62 (d, *J* = 6Hz, 1H), 3.84 (d, *J* = 6 Hz, 1H), 3.61–3.57 (m, 4H), 3.55–3.46 (m, 1H) ppm; HRMS: TOF $[(M + Na)^+]$ calcd for $C_{12}H_{16}NaO_5S$ is 295.0616, found 295.0615.

*Phenyl 4,6-O-benzylidene-1-thio-β-D-galactopyranoside (**8**)*. Compound **7** was subsequently dissolved in DMF (20 mL) and further treated with benzaldehyde dimethylacetal (1.7 mL, 11.0 mmol) and camphor sulfonic acid (CSA) until solution got acidic pH. The reaction mixture was stirred for 5 h at 50 °C. After completion of the reaction, triethylamine (Et_3N) was added to quench CSA. The reaction mixture was evaporated to dryness and was purified over silica gel column using 45% EtOAc in hexane as the eluent to furnish pure compound **8** (2.5 g, 6.9 mmol, 77%) as a white solid. ^{1}H NMR (600 MHz, DMSO): δ 7.55–7.54 (m, 1H, Ar-H), 7.44–7.37 (m, 2H, Ar-H), 7.28–7.24 (m, 1H, Ar-H), 5.57–5.52 (m, 1H), 4.70–4.67 (m, 1H), 4.15 (d, *J* = 6 Hz, 1H), 4.07–4.02 (m, H), 3.65 (d, J = 12 Hz, 1H), 3.55–3.50 (m, 1H), 2.50 (d, J = 6 Hz, 1H) ppm; ^{13}C NMR (150 MHz, DMSO): δ 138.6, 133.9, 130.5, 128.7, 127.9, 126.5, 126.4, 99.8, 86.6, 76.1, 73.1, 69.3, 68.61, 67.88 ppm; HRMS: TOF $[(M + Na)^+]$ calcd for $C_{19}H_{20}NaO_5S$ is 383.0929, found 383.0925.

*Phenyl 2,3-di-O-benzoyl-1-thio-β-D-galactopyranoside (**9**)*. Compound **8** (0.9 g, 2.5 mmol) was dissolved in pyridine (12 mL). Benzoyl chloride (2.5 mL, 15 mmol) and catalytic amount of DMAP were added to the solution and stirred overnight at room temperature. After completion of the reaction, solvent was evaporated in reduced pressure and the crude solid was partitioned with DCM (30 mL) and saturated $NaHCO_3$ solution (50 mL). Organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give the crude product, which was purified over silica gel column using 25% EtOAc in hexane to afford Bz protected compound in 82% yield. To a solution of Bz protected compound (1.4 g, 2.5 mmol) in MeOH catalytic amount of TsOH was added and stirred at room temperature until full consumption of starting material was noticed by TLC. Then reaction mixture was quenched with Et_3N and evaporated to dryness. Crude mixture was subjected to silica gel column using 40% EtOAc in hexane as eluent to afford compound **9** (1.2 g, 2.5 mmol, 81%) as a white solid. $[\alpha]_D^{22} = +105$ (c = 1.0, $CHCl_3$);[50] ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.54–7.51 (m, 4H, Ar-H), 7.43–7.38 (m, 4H, Ar-H), 7.36–7.34 (m, 4H, Ar-H), 7.33–7.28 (m, 3H, Ar-H), 5.83–5.80 (m, 1H, H-2), 5.35 (dd, *J* = 3.42, 6.78 Hz, 1H, H-3), 5.00 (d, *J* = 10.02 Hz, 1H, H-1), 4.46–4.44 (m, 1H, H-4), 4.08–4.05 (m, 1H, H-6), 3.96 (dd, *J* = 4.2, 7.86 Hz, 1H, H-6′), 3.86–3.84 (m, 1H, H-5) ppm; ^{13}C NMR (150 MHz,

$CDCl_3$): δ 165.8, 165.3, 133.5, 133.3, 132.7, 132.3, 129.9, 129.8, 129.4, 129.0, 128.5, 128.4, 128.2, 86.6, 78.1, 75.4, 68.7, 67.8, 63.0 ppm; HRMS:TOF [$(M + Na)^+$] calcd for $C_{26}H_{24}NaO_7S$ is 503.1140, found 503.1136.

*Phenyl 4,6-O-[1-(R)-(methoxycarbonyl)-ethylidene]-2,3-di-O-benzoyl-1-thio-β-D-galactopyranoside (**10**)*. Compound **9** (0.25 g, 0.52 mmol) was dissolved in minimum volume of acetonitrile and the solution was cooled to 0 °C. Methyl pyruvate (0.094 mL, 1.04 mmol) and $BF_3{\cdot}OEt_2$ (0.13 mL, 1.04 mmol) was added to the cooled reaction mixture and stirred at room temperature for 2.5 h. After completion of the reaction, the reaction mixture was diluted with DCM (15 mL) and quenched with saturated $NaHCO_3$ (25 mL) solution. The compound was extracted with DCM from the aqueous layer and washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude mass was purified over silica gel column chromatography using 25% EtOAc in hexane as eluent to afford compound **10** as a white solid (0.15 g, 0.27 mmol, 50%). $[\alpha]_D^{22}$ +145; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.03–7.99 (m, 4H, Ar-H), 7.62–7.60 (m, 2H, Ar-H), 7.57–7.51 (m, 2H, Ar-H), 7.44–7.36 (m, 4H, Ar-H), 7.35–7.33 (m, 3H, Ar-H), 5.80–5.77 (m, 1H, H-2), 5.23 (dd, $J = 3.42$, 9.96 Hz, 1H, H-3), 4.93 (d, $J = 9.90$ Hz, 1H, H-1), 4.58 (d, $J = 3.3$ Hz, 1H, H-4), 4.23–4.21 (m, 1H, H-6), 4.06–4.04 (m, 1H, H-6), 3.67–3.66 (m, 1H, H-5) ppm; ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.1, 166.1, 165.0, 133.6, 133.3, 133.2, 131.5, 129.9, 129.8, 129.6, 129.3, 128.8, 128.4, 128.4, 128.3, 98.6, 85.7, 74.0, 69.2, 69.0, 67.0, 65.3, 52.5, 25.6 ppm; HRMS: TOF [$(M + Na)^+$] calcd for $C_{30}H_{28}NaO_9S$ is 587.1364, found 587.1352.

*Phenyl 4,6-O-[1-(R)-(methoxycarbonyl)-ethylidene]-1-thio-β-D-galactopyranoside (**11**)*. Compound **10** (0.15 g, 0.27 mmol) was stirred with saturated NaOH solution at room temperature for 2 h. After that, reaction mixture was quenched with DOWEX 50WX8-100 ion exchange (H^+) resin. Following neutralization, the reaction mixture was filtered, and the filtrate was evaporated to dryness. Silica gel column purification using 10% MeOH in DCM as eluent resulted compound **11** (0.06 g, 0.18 mmol, 50%) as a white solid. ^{1}H NMR (600 MHz, D_2O): δ 7.60–7.59 (m, 2H, Ar-H), 7.40–7.36 (m, 3H, Ar-H), 4.74 (d, $J = 9.02$Hz, 1H, H-1), 4.17 (d, $J = 3.00$ Hz, 1H, H-4), 3.97–3.95 (m, 1H, H-6), 3.90–3.88 (m, 1H, H-6′), 3.70–3.64 (m, 2H, H-2, H-3), 3.60 (br s, 1H, H-5), 1.42 (s, 3H, CH_3) ppm; ^{13}C NMR (150 MHz, D_2O): δ 176.1, 132.1, 132.0, 129.4, 128.3, 100.8, 87.2, 73.0, 71.3, 69.5, 68.4, 65.2, 25.4 ppm; HRMS: TOF [$(M + NH)^+$] calcd for $C_{15}H_{19}O_7S$ is 343.0852, found 343.0851.

*Allyl α-D-galactofuranoside (**12**)*. D-galacto*pyranose* (500 mg, 2.78 mmol) was partially dissolved in 7 mL DMPU in an inert atmosphere. Sodium hydride (NaH) (200 mg, 8.33 mmol) was added to the suspension and the reaction mixture was cooled to 0 °C. To the cooled reaction, allyl bromide

(1 mL, 11.12 mmol) was added slowly and the reaction mixture was stirred at room temperature for 72 h. After completion of the reaction, the reaction mixture was cooled to 0 °C and NaH was quenched with MeOH by adding dropwise (1 mL). Subsequently DMPU was extracted with hexane from the solution. A viscous sirup was obtained which was subjected to silica gel column chromatography using 10% MeOH in DCM as eluent to afford compound **12** (367 mg, 1.67 mmol, 60%) as a light-yellow solid. $[\alpha]_D^{22}$ +95.6 (*c* 1, H_2O);[51] ^{1}H NMR (600 MHz, MeOD): δ 5.98–5.95 (m, 1H, –CH=CH_2), 5.37–5.33 (m, 1H, –CH = CH_2), 5.20–5.17 (m, 1H, –CH = CH_2'), 4.91 (d, J = 4.56 Hz, 1H, H-1), 4.33–4.29 (m, 1H, –CH_2–CH = CH_2), 4.14–4.08 (m, 2H, H-3, –CH_2'–CH = CH_2), 3.98 (dd, J = 4.56, 7.92 Hz, 1H, H-2), 3.75–3.73 (m, 1H, H-6), 3.66–3.62 (m, 2H, H-4, H-6′), 3.58–3.55 (m, 1H, H-5) ppm; ^{13}C NMR (150 MHz, MeOD): δ 134.3, 116.1, 100.5, 82.2, 77.5, 74.9, 73.1, 68.4, 62.7 ppm; HRMS: TOF [$(M + Na)^+$] calcd for $C_9H_{16}NaO_6$ is 243.0845 found 243.0813.

*Propyl α-D-galactofuranoside (**13**).* To a solution of compound **12** (100 mg, 0.45 mmol) in anhydrous methanol (10 mL) was added 10% Pd–C (50 mg) and the mixture was stirred for 3 h at room temperature in 40 psi hydrogen pressure. The reaction mixture was filtered through a Celite®-545 bed, then washed with plenty of MeOH and the combined filtrate was evaporated under reduced pressure to obtain compound **13** (70 mg, 0.32 mmol, 70%) as a white solid. $[\alpha]_D^{22} = -12$ (*c* 1, MeOH) ^{1}H NMR (600 MHz, D_2O): δ 4.97 (d, J = 4.19 Hz, 1H, H-1), 4.12–4.08 (m, 2H, H-2, H-6), 3.76–3.67 (m, 4H, H-3, H-5, H-6′, CH_3–CH_2–CH_2), 3.58–3.55 (m, 1H, H-4), 3.48–3.44 (m, 1H, CH_3–CH_2–CH_2'), 1.61–1.55 (m, 2H, CH_3–CH_2–CH_2), 0.88 (t, J = 7.29 Hz, CH_3–CH_2–CH_2) ppm. ^{13}C NMR (150 MHz, D_2O): δ 100.79, 81.09, 76.29, 74.55, 72.99, 70.25, 62.08, 22.06, 9.75 ppm; EIMS [$(M + Na)^+$] calcd for $C_9H_{18}NaO_6$ is 245.1 found 244.9.

*Propyl α-D-xylo-pentodialdo-1,4-furanoside (**14**).* To a solution of compound **13** (20 mg, 0.09 mmol) in 20 mL 100 mM NaOAc buffer (pH 5.0), $NaIO_4$ (19.3 mg, 0.09 mmol) was added slowly to obtain overall 4.5 mM $NaIO_4$ concentration in the reaction mixture and the reaction was stirred at room temperature in dark for 3 h. Thin layer chromatography revealed partial consumption of starting material. The product was extracted with EtOAc from the buffer solution, evaporated to dryness and finally purified over silica gel column using 10% MeOH in DCM as eluent to afford compound **14** (5.1 mg, 0.03 mmol, 30%) as colorless gum. ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.71 (s, 1H, CHO), 5.17 (d, J = 4.34 Hz, 1H, H-1), 4.36–4.35 (m, 1H, H-3), 4.22–4.21 (m, 1H, H-4), 4.12–4.11 (m, 1H, H-2), 3.89–3.86 (m, 1H, CH_3–CH_2–CH_2), 3.58–3.54 (m, 1H, CH_3–CH_2–CH_2'), 1.67–1.62 (m, 2H, CH_3–CH_2–CH_2), 0.94 (t, J = 7.43 Hz, 1H, CH_3–CH_2–CH_2) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ 201.53, 102.82, 87.14, 78.46, 78.15, 71.22,

23.09, 10.84 ppm; ESIMS $[(M + Na)^+]$ calcd for $C_8H_{14}NaO_5$ is 213.1, found 213.3.

*(Propyl α-D-xylofuranoside)-5-aldehyde {O-[2-(acetylamino)-2-deoxy-α-D-galactopyranosyl]-L-threonyl}hydrazone (**17**).* Compound **14** (3 mg, 0.015 mmol) was dissolved in 100 mM acetate buffer (pH 5.0) and Tn hydrazide **15** (5.3 mg, 0.015 mmol) was added to it. The reaction mixture was stirred overnight and formation of the hydrazone was monitored by mass spectroscopy. After obtaining the desired hydrazide, the reaction mixture was lyophilized and subjected to P2-Biogel column using water as eluent for the purification of product **17**. ^{1}H NMR (600 MHz, CD_3OD): δ 7.55, (d, $J = 6.0$ Hz, 1H, N = CH), 4.85 (d, $J = 6.0$ Hz, 1H), 4.25–4.24 (d, $J = 6.0$ Hz, 1H), 4.03–4.00 (m, 1H), 3.95–3.93 (m, 1H), 3.87 (d, $J = 6$ Hz, 1H), 3.76 (dd, $J = 6.0$ Hz, 1H), 3.67–3.63 (m, 2H), 3.54–3.44 (m, 2H), 1.95 (s, 3H), 1.28–1.27 (s, 3H); ESIMS $[(M + Na)^+]$ calcd for $C_{20}H_{36}N_4NaO_{11}$ is 531.2, found 531.5.

*(Propyl α-D-xylofuranoside)-5-aldehyde O-[2-(acetylamino)-2-deoxy-α-D-galactopyranosyl]oxime (**18**).* Compound **14** (3.6 mg, 0.016 mmol) was dissolved in 100 mM acetate buffer (pH 5.2) and aminooxy Tn (4.1 mg, 0.016 mmol) was added to it. The reaction mixture was stirred overnight, and formation of the oxime was monitored by mass spectroscopy. After obtaining the desired oxime *compound* **18** the reaction mixture was subjected to P2-Biogel column using water as eluent for the purification of product and finally lyophilized. ^{1}H NMR (600 MHz, CD_3OD): δ 7.11–7.10 (d, $J = 6.80$ Hz, 1H, N = CH), 4.94–4.93 (d, $J = 6$ Hz, 1H), 4.11–4.10 (d, $J = 6$ Hz, 1H), 4.04–4.01 (m, 1H), 3.60–3.57 (m, 1H), 3.41–3.40 (m, 1H), 2.65 (s, 3H), 2.50–2.49 (m, 1H), 2.40–2.30 (m, 4H), 1.50–1.48 (q, 2H), 0.80–0.78 (m, 2H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ 181.46, 149.15, 174.84, 172.58, 146.52, 101.15, 80.41, 70.49, 28.92, 26.15, 23.18, 9.78 ppm. ESI-MS $[(M + Na + H)^+]$ calcd for $C_{20}H_{37}N_4NaO_{11}$ is 432.4, found 432.9.

Stability study

Preparation of buffer solutions

To prepare 100 mM acetate buffer of pD 5.50, 20 mg NaOH was dissolved in D_2O and pH was adjusted by adding deuterated acetic acid (CD_3COOD) dropwise and pH was measured using a pH meter. When the pH meter reading showed pH 5.10, the volume of the entire the following solution was made 5 mL. Then pD of the solution was calculated as 5.51 using formula:

$$\text{pD} = \text{pH meter reading} + 0.41$$

653.5 mg trichloroacetic acid was dissolved in 5 mL D_2O and the pH was adjusted to 0.89 by slowly adding 1 M NaOH solution (prepared in D_2O). After obtaining the desired pH, the volume of the entire solution was made 10 mL (overall trichloroacetic acid concentration was 0.40 M). Then pD of the solution was calculated as 1.30 using the above formula.

NMR analysis at different temperatures

NMR spectra were recorded at 22 °C only when stability of compound **11** was evaluated at acetate buffer of pD 5.51. However, variable temperatures were used to examine the stability of compound **11** at pD 1.30. At first, spectra were recorded at 22 °C in different time interval for 48 h. Then temperature was increased to 37 °C and the solution was heated in an incubator for 24 h at the same temperature. Subsequently temperature was increased to 50 °C and allowed to continue the same temperature for 6 h in a water bath. As no change was noticed in the NMR peak, temperature was again increased to 70 °C followed by 85 °C in water bath. NMR spectra were recorded after 4 h of heating at 85 °C temperature. A small fraction of pyruvate acetal cleavage was observed; therefore, the solution was again heated at 85 °C for 12 h and NMR spectra were recorded.

HPLC analysis at different temperatures

0.1 M of acetate buffer at pH 5.2 was prepared following Henderson-Hasselbalch equation. Then, around 0.86 mg of compound **11** was dissolved in 5 mL of this acetate buffer to get 500 µM concentration. From the same vial 10 µL of solution was injected to the HPLC system after 1 h, 14 h, 21 h respectively and recorded the chromatogram. Since, most of Tn-Ps A1 conjugation were carried out for overnight, we also decided to monitor the chromatogram till 21 h. However, after 96 h, 50 µL was injected to get better observations of the multiple peaks. Most of the samples were eluted around 40/60 water and acetonitrile mobile phase system.

Disclosure statement

No potential conflict of interest was reported by the authors.

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