Conference paper

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Synthesis of *Group B Streptococcus* type III polysaccharide fragments for evaluation of their interactions with monoclonal antibodies

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Abstract: *Group B Streptococcus* type III (GBSIII) is the most relevant serotype among GBS strains causing infections and the potential of its capsular polysaccharide conjugated to a protein carrier as vaccine is well documented. Polysaccharide from GBSIII (PSIII) can form helical structures in solution where negatively charged sialic acid residues would be disposed externally providing stabilization to the helix. A peculiar high affinity to specific monoclonal antibodies (mAbs) has been reported for PSIII, and fragments of diverse size bind to mAbs in a length dependent manner. These data have been rationalized in terms of conformational epitopes that would be formed by fragments with >4 saccharidic repeating units. Saturation Transfer Difference NMR experiments have demonstrated that the sialic acid residue is not involved in antibody recognition. However the molecular basis of the interaction between PSIII and mAbs has not been fully elucidated. An important prerequisite to achieve this would be the availability of the three possible sugar sequences representing the pentasaccharide PSIII repeating unit. Herein we established a [2+3] convergent approach leading to these three pentasaccharides (**1–3**) with the end terminal sugar bearing a linker for possible conjugation. The PSIII fragments were coupled to the genetically detoxified diphtheria toxin CRM_{197} to prove by ELISA that the three pentasaccharides are recognized by polyclonal anti-PSIII serum. The presence of the branching formed by a Glc residue β-(1→6) linked to GlcNAc was proven an important motif for antibody recognition.

Keywords: carbohydrates; conjugation; glycoconjugates; *Group B Streptococcus* type III; ICS-28.

Introduction

Streptococcus agalactiae or *Group B Streptococcus* (GBS) is a leading cause of bacterial sepsis and meningitis among neonates [1]. GBS type III is the most prevalent serotype among GBS strains causing infections and the potential of its capsular polysaccharide (PS) to act as an immunogen has been demonstrated [2, 3]. However, purified GBS PSs are only variably immunogenic in adults, therefore PS–protein conjugate vaccines have been developed to enhance their immunogenicity [3, 4].

Vaccination of pregnant mothers with GBS polysaccharides conjugated to carrier protein has been pursued as a strategy to protect newborns from infection [2]. A trivalent combination against types Ia, Ib

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and III has been proven to be safe and immunogenic in non-pregnant and pregnant women in phase-1 and phase-2 clinical trials, and maternal antibodies were efficiently transferred to neonates [5–7].

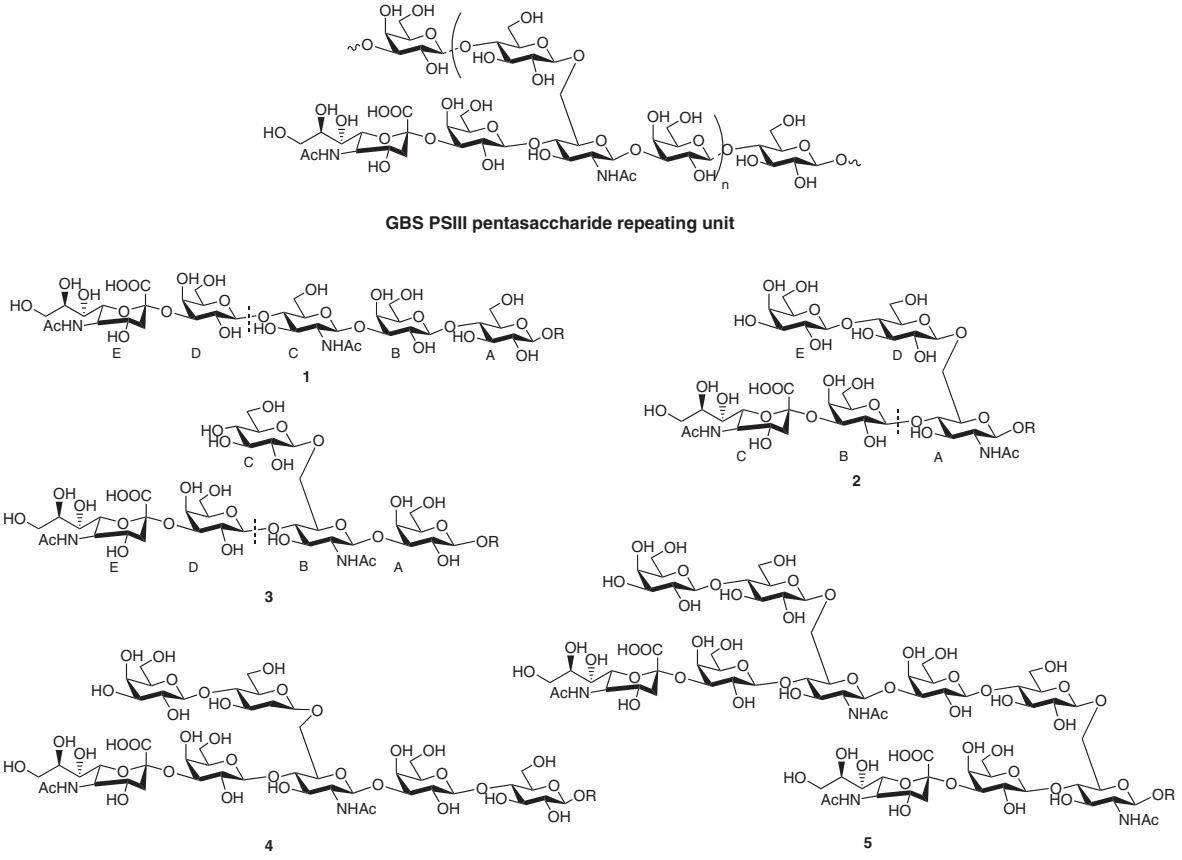
PSIII repeating unit is composed of the following pentasaccharide [8]:

$$\rightarrow 4\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow$$
$$\Big|$$
$$\alpha\text{-NeuNAc-}(2\rightarrow 3)\text{-}\beta\text{-D-Gal}p$$

By molecular dynamics simulations and NMR studies [9, 10], it has been shown that PSIII is flexible and exists predominantly as a random coil in solution, however it can locally form extended helical structures made by more than four repeating units (RUs) [11, 12]. This unique feature depends on the presence of the sialic acid residue as the structurally related *Streptococcus pneumoniae* type 14 polysaccharide, which differs from PSIII by the absence of the NeuNAc, results in a more disordered structure.

Furthermore, high affinity monoclonal antibodies (mAbs) were found, and PS fragments of different size were recognized by anti-PSIII mAbs in a length dependent manner. A dimer of 2RUs (**5**, Scheme 1) was shown to be sufficient to bind to a monoclonal IgG, although with a lower affinity as compared to fragments composed of a larger number of RUs [12]. These properties have been rationalized in terms of the presence of a conformational epitope situated on an extended segment of the GBS PSIII [15–17].

Interaction with a monoclonal IgM did not involve ring positions of the NeuNAc residue [18]. Therefore, NeuNAc would be relevant for induction of specific protective antibodies [19], however from a structural



Scheme 1: Chemical structure of PSIII and related repeating unit sequences **1–3**. Letters refer to the nomenclature used in the experimental section. In our approach R was a linker suited for conjugation ($CH_2CH_2NH_2$). A [2 + 3] convergent approach was envisaged for the synthesis of these fragments. Structures **4** and **5** have been reported in literature (ref. [13] and [14], respectively).

perspective this sugar residue would contribute to the stabilization of the backbone helical structure, rather than being directly part of the epitope.

The five residues composing the repeating unit can be combined according to three different sequences, the linear glycan (**1**) and the two branched structures (**2**) and (**3**) depicted in Scheme 1. Interactions with structures smaller than **5** have not been reported [12], and positions involved in antibody binding have not been identified in detail. Hence, the availability of short defined oligosaccharides related to GBS PSIII repeating unit is an important prerequisite to explore interactions between CPS and specific antibodies and to map the corresponding GBS PSIII epitopes.

Approaches to produce short PSIII fragments have been based on either depolymerization or chemical synthesis. Michon et al. used the partial N-deacetylation followed by nitrosation to attain large heterogeneous populations of different length fragments with an end terminal 2,5-anhydro-D-mannofuranose residue in place of the modified Glc*p*NAc [20]. Enzymatic degradation with endo-β-galactosidase from *Citrobacter freundii* has been also used to produce heterogeneous pools of oligosaccharides starting from native PSIII by cleavage of the β-D-Gal*p*-(1→4)-β-D-Glc*p* linkage of the backbone [3]. Size exclusion chromatography was then developed for the resolution of more homogeneous oligosaccharides in the range of 4 to 25 sugars [21].

Poszgay et al. reported the chemical synthesis of desialylated tri- and tetrasaccharide fragments [22]. This work set the basis for the incorporation of the α-NeuNAc residue in the deprotected tetraccharide acceptor by an enzymatic reaction, leading to the pentasaccharide repeating unit **1** [23]. Fully chemical assembly of a heptasaccharide **4** related to PSIII was described by Demchenko et al. [13] that used a highly convergent strategy based on incorporation of a protected α-NeuNAc-(2→3)-Gal*p* methylthiolgycoside previously reported [24] to a Glc*p*NAc acceptor. Further glycosylation the 6-OH position of this residue by a lactose donor gave a pentenyl pentasaccharide, which was in turn used as donor for glycosylation of a lactose acceptor affording the target fragment. The related desialylated hexasaccharide fragment was also described by the same authors [25].

The chemo-enzymatic approach was also used by Zou et al. to assemble the dimer of the branched unit **5** and N-propionyl substituted sialic acid analogs from a precursor core octasaccharide enzymatically sialylated by reaction with α-(2→3)-sialyltransferase and CMP-NeuNAc as donor [14]. A similar enzymatic approach has been also utilized to transform oligosaccharides from *S. pneumoniae* type 14 to PSIII counterparts [26].

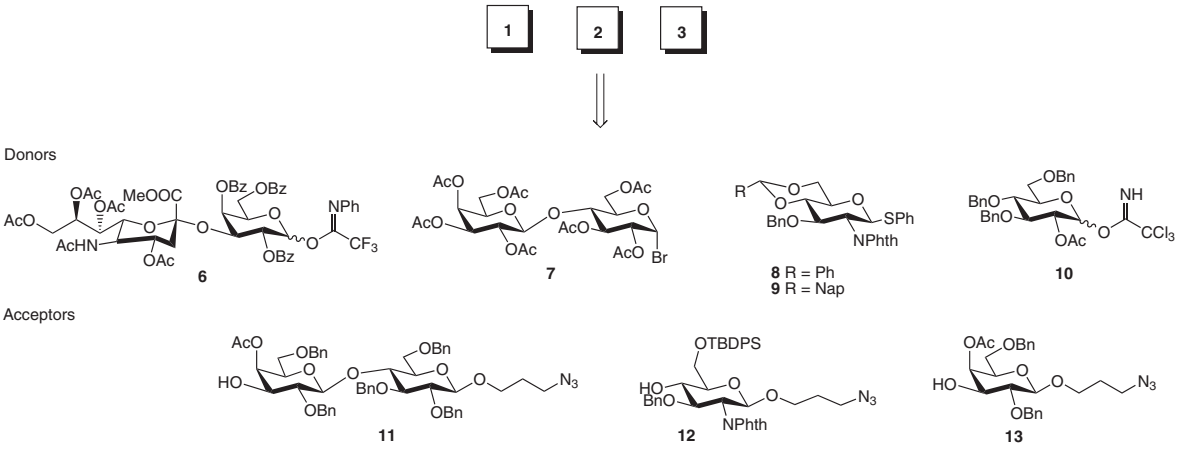
Syntheses of structures related to fragment **1**, a glycan that is commonly found in nature [27, 28], have also been reported. For instance Hsu et al. used a 5-*N*,4-*O*-oxazolidinone α-NeuNAc-(2→3)-Gal*p* phosphate donor to achieve the one-pot assembly of this oligosaccharide [29], whereas and N-Trichloroethoxycarbonyl protected α-NeuNAc-(2→3)-Gal*p* trifluoroacetimidate donor was utilized by Hanashima et al. for glycosylation of a trisaccharide acceptor [30].

Herein we established a [2 + 3] convergent approach leading to the repeating units **1–3** with the end terminal sugar bearing a linker for possible conjugation. The fragments were coupled to the genetically detoxified diphtheria toxin CRM_{197} to prove by ELISA recognition with polyclonal anti GBS PSIII serum.

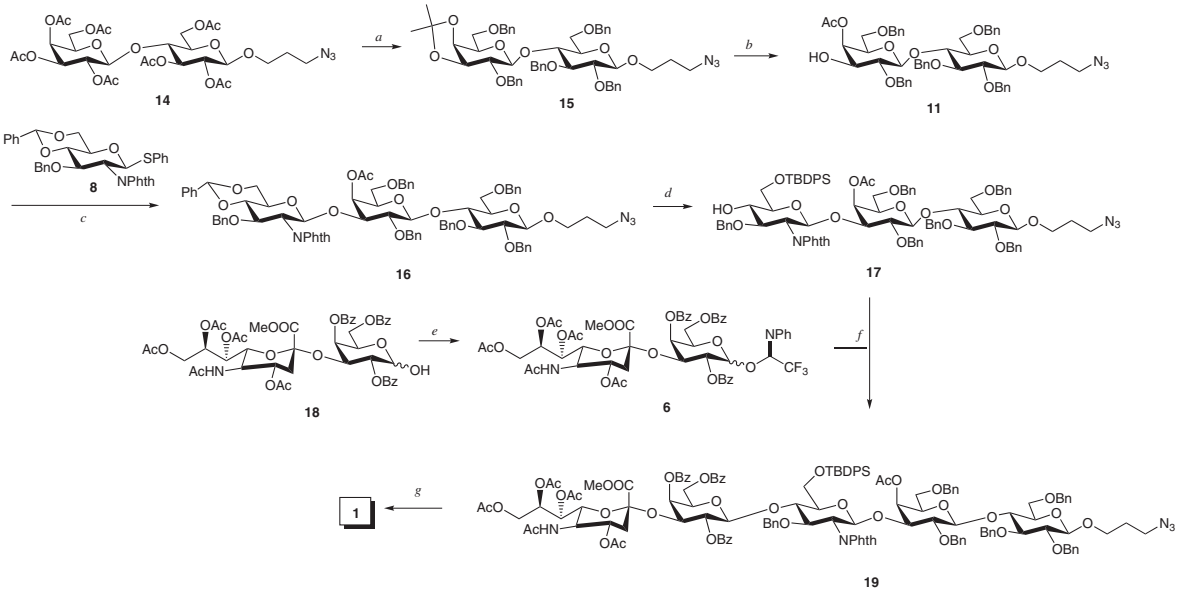
Assembly of PSIII repeating unit related glycans 1–3

Our retrosynthetic design of compounds **1–3** was based on the use of a protected α-NeuAc-(2→3)-Gal*p* trifluoroacetimidate donor for glycosylation of the 3-OH position in the Glc*p*NAc residue of a suitable trisaccharide acceptor. Typical approaches for glycan including the α-NeuNAc-(2→3)-Gal*p* motif are built realizing the challenging α-NeuNAc linkage in an early stage of the synthesis, consequently a variety of sialyl donors providing disaccharide donors have been developed over the years [31, 32]. We based our synthesis of the 1-OH α-NeuNAc-(2→3)-Gal described by Hasegawa's group in 1989 [33]. Despite more selective α-NeuNAc building blocks have been more recently reported [31, 32], we utilized this method because it renders our disaccharide donor available in a few steps with acceptable yields [34, 35].

The series of building blocks **6–13**, which can be prepared from reported protocols, were selected for the preparation of our target molecules **1–3**, as shown in Scheme 2.



Scheme 2: Desired building blocks for the preparation of compounds **1**–**3.**



Scheme 3: *Reagents and conditions*: (a) NaOMe, MeOH; $(CH_3)_2C(OCH_3)_2$, DMF, PTSA, 50 °C, then TEA, 9 : 1 MeOH-H_2O, 90 °C; BnBr, 60 % NaH, DMF, 57 % (over 3 steps); (b) 4 : 1 AcOH-H_2O, 70 °C; $(EtO)_3CCH_3$, *p*-TsOH, CH_3CN, then 4 : 1 AcOH-H_2O, 65 % (over 3 steps); (c) NIS, TfOH, DCM, – 20 °C, 72 %; (d) 4 : 1 AcOH-H_2O, 70 °C; TBDPSCl, DMAP, Py, 60 °C, 80 % (over 2 steps); (e) Cs_2CO_3, $CF_3CClNPh$, DCM, 82 %; (f) TMSOTf, DCM, 55 %; (g) LiI, Py, 120 °C; $H_2NCH_2CH_2NH_2$, EtOH, 90 °C; Ac_2O-Py; NaOMe, MeOH; H_2, Pd-C, 33 %.

The synthesis of fragment **1** commenced from the known spacer containing lactoside **14** [13] (Scheme 3). A sequence of deacetylation with NaOMe/MeOH, and regioselective 3,4-*O*-isopropylidene insertion in the Gal residue by treatment with dimethyl acetone and catalytic *p*-toluenesulfonic acid (PTSA) followed by benzylation with benzyl bromide and NaH of the purified isopropylinated intermediate gave compound **15** in overall 60 % yield.

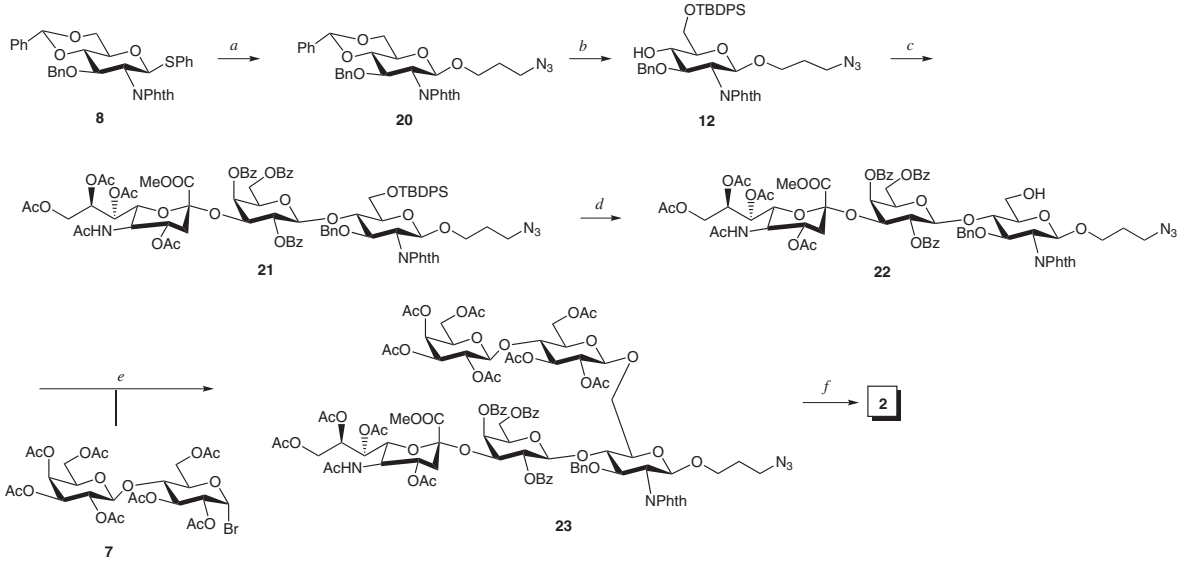
This disaccharide was regioselectively converted in the 4-*O*-acetylated acceptor 11 by removal of the isopropylidene ether with aqueous acetic acid at 50 °C and subsequent reaction with 1,1,1-triethoxyethane followed by acid hydrolysis of the formed orthoester (65 % yield, over 3 steps). Glycosylation of the 3-OH with the N-phthalimido glucosamine thiophenol donor **8** [36] using NIS-TfOH as promoters provided **16** in good yield (72 %). Benzylidene removal and selective 6-*O*-silylation with tertbutyldiphenylsilyl chloride and 4-(Dimethylamino)-pyridine (DMAP) in pyridine rendered the 4-OH of GlcNAc ready for coupling with the α-NeuNAc-(2→3)-Gal*p* trifluoroacetimidate donor **6**, prepared from the known **18** [34, 35] by classic

reaction with 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride and cesium carbonate. The trimethylsilyl triflate promoted glycosylation gave pentasaccharide **19** in acceptable 55 % yield. The higher stability of donor **6** resulted in improved yield compared to the trichloroacetimidate counterpart (29 % under the same conditions). Compound **19** was deprotected by a five-step procedure [13]. Saponification with Litium iodide in pyridine hydrolyzed the methyl ester of the sialic acid moiety of **19**. Next, reaction ethylenediamine in refluxing ethanol was used for concomitant removal of the O-acetyl esters and the phthalimido protecting groups. The intermediate trisaccharide was fully reacetylated with acetic anhydride in pyridine to install the acetamide group of the Glc*p*NAc residue. Final deacetylation with NaOMe/MeOH and catalytic hydrogenation over Pd/charcoal released the target linear pentasaccharide **1** where the azide of the spacer was reduced to amine. After purification by size exclusion column chromatography on Sephadex G-10, the final compound was obtained in overall 33 % yield from **19**, as estimated by spectrophotometric quantification of the sialic acid content.

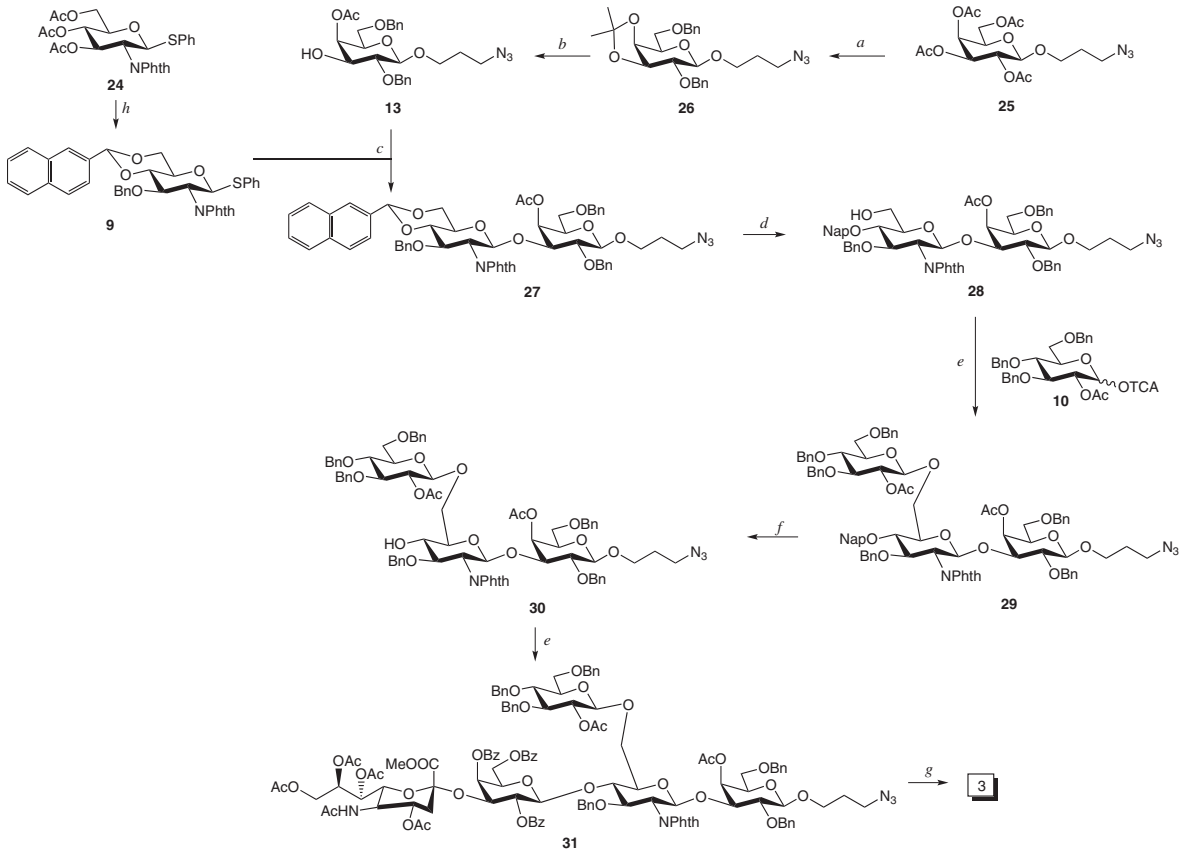
For the synthesis of the branched fragment **2** (Scheme 4), the phenylthio glucosamine **8** was used for glycosylation of 3-azidopropanol to obtain compound **20** in 84 % yield. This was converted into acceptor **22** by benzylidene removal and 6-*O*-protection with TBDPS as above described. Glycosylation using donor **6** in the presence of TMSOTf gave the trisaccharide **21** in 70 % yield. Following desilylation with HF·Pyridine uneventfully provided the acceptor **22** which was glycosylated by Koenigs and Knorr reaction with peracetate lactoside bromide **7** [37] and silver trifluoromethanesulfonate leading to **23** in 68 % yield.

The five-step deprotection sequence previously mentioned was used to obtain the target compound **2** in 42 % after purification as determined by NeuNAc quantification.

Finally, the Y-shaped fragment **3** was prepared starting from the monosaccharide acceptor **13** (Scheme 5), obtained in five steps from the peracetyl galactoside **25** [38] bearing the azido linker by deacetylation, 3,4-*O*-isopropylidination and 2,6-di-*O*-benzylation (→**26**), isopropylidene removal and regioselective 4-*O*-acetylation through 1,1,1-triethoxyethane followed by acid hydrolysis. NIS-TfOH promoted glycosylation of **13** with donor **9**, prepared from **24** [39] by deacetylation, PTSA catalyzed installation of the naphtylidene protection and 3-*O*-benzylation, proceeded in 72 % yield. Regioselective ring opening of the naphtylidene group with borontrimethylhydride·trimehtylamine complex rendered the 6-OH position available for glycosylation, while the 4-OH remained protected as naphtylmethylene ether in order to be liberated in a later stage of the synthesis. After TMSOTf promoted glycosylation with the glucosyl trichloracetimidate



Scheme 4: *Reagents and conditions*: (a) $HO(CH_2)_3N_3$, NIS, TfOH, 84 %; (b) 4.1 AcOH-H_2O, 70 °C; TBDPSCl, DMAP, Py, 60 °C, 70 % (over 2 steps); (c) TMSOTf, DCM, 70 %; (d) HFPy, 4 : 1 THF-Py, 0 °C to rt, 78 %; (e) AgOTf, DCM, 68 %; (f) LiI, Py, 120 °C; $H_2NCH_2CH_2NH_2$, EtOH, 90 °C; Ac_2O-Py; NaOMe, MeOH; H_2, Pd-C, 42 %.



Scheme 5: *Reagents and conditions*: (a) NaOMe, MeOH; 9 : 1 $(CH_3)_2C(OCH_3)_2$-DMF, PTSA, 50 °C, then TEA, 9 : 1 MeOH-H_2O, 90 °C; BnBr, 60 % NaH, DMF, 59 % (over 3 steps); (b) 4.1 AcOH-H_2O, 70 °C; $(EtO)_3CCH_3$, PTSA, CH_3CN, then 4 : 1 AcOH-H_2O, 80 % (over 3 steps); (c) NIS, TfOH, DCM, – 20 °C, 72 %; (d) $BH_3{\cdot}Me_3$, $BF_3{\cdot}Et_2O$, CH_3CN, 64 %; (e) TMSOTf, DCM, 72 %; (f) DDQ, 4 : 1 DCM-MeOH, 85 %; (e) 65 %; (g) LiI, Py, 120 °C; $H_2NCH_2CH_2NH_2$, EtOH, 90 °C; Ac_2O-Py; NaOMe, MeOH; H_2, Pd-C, 55 %; (h) NaOMe, MeOH; Naphthylidene dimethyl acetal, DMF, PTSA, 50 °C; BnBr, 60 % NaH, DMF, 63 % (over 3 steps).

10 [40] (72 % yield), the obtained trisaccharide **29** was subjected to Nap deprotection with 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). By doing so, the acceptor **30** was ready for glycosylation with donor **6** in the presence of TMSOTf, leading to the pentasaccharide **31** in 65 % yield. Again, application of the five-step deprotection procedure afforded purified compound **3** in 55 % yield from **31** as spectrophotometrically determined.

As shown in Table 1 and Fig. 1, the ^1H and ^{13}C chemical shifts for the signals of the synthesized fragments were in excellent agreement with literature NMR data for PSIII and different length fragments [9].

Antibody recognition of fragments 1–3 by ELISA

To confirm that the synthesized fragments could be used to study the interactions with PSIII specific mAbs, reactivity to the fragments with polyclonal anti-PSIII Abs was confirmed. To this end, compounds **1–3** were first conjugated to CRM_{197}, by treatment with an excess of di-N-hydroxysuccinimidyl adipate (Scheme 6) [41]. The isolated half esters **32–34** were incubated with the protein in sodium phosphate buffer at pH 7.2. The amount of coupled glycan was proportional to the active ester used in conjugation (Table 2). Thus the use of 15 equivalents led to incorporation of an average of 3 glycan moieties; when 75 equivalents were used the level of carbohydrate incorporation was increased to 24–27 mol/mol of protein.

Table 1: Chemical shift (ppm) of ^{1}H and ^{13}C NMR signals and 3J H_1-H_2 scalar coupling costants of compounds **1–3** in D_2O[a].

Residue		Compound 1		Compound 2		Compound 3		PSIII[c]	
		^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Gal	1	4.43 / 8.2 Hz	103.77	4.45 / 7.8 Hz	103.28	4.39 / 8.0 Hz	103.72	4.43	103.90
	2	3.57	70.68	3.55	71.58	3.57	70.49	3.59	70.82
	3	3.72	82.85	3.68	73.18	3.72	83.08	3.73	83.32
	4	4.16	69.05	3.93	69.48	4.16	69.16	4.17	69.06
	5	3.66	75.10	3.68	72.96	3.69	75.70	3.71	75.78
	6	3.65	63.16	3.71	61.58	3.73	62.35	3.74	61.87
	6′	3.88		3.76		3.76		3.79	
GlcNAc	1	4.69 / 8.2 Hz	103.72	4.52 / 7.8 Hz	101.98	4.71 / 8.0 Hz	103.63	4.70	103.97
	2	3.81	55.89	3.75	55.68	3.80	56.08	3.83	56.01
	3	3.73	72.82	3.72	74.23	3.73	73.40	3.73	72.85
	4	3.76	78.50	3.86	77.98	3.88	78.28	3.91	77.40
	5	3.72	75.69	3.72	76.02	3.73	74.38	3.73	73.90
	6	3.95	68.18	4.00	68.18	3.97	68.51	3.98	68.22
	6′	3.95		4.31		4.30		4.29	
Glc	1	4.50 / 8.5 Hz	102.74	4.55 / 7.8 Hz	103.00	4.52 / 8.0 Hz	103.66	4.54	103.44
	2	3.32	73.51	3.37	73.35	3.31	73.88	3.36	73.42
	3	3.64	75.38	3.67	75.15	3.52	76.78	3.67	75.12
	4	3.65	78.58	3.67	78.75	3.40	70.78	3.67	79.23
	5	3.66	75.38	3.68	75.54	3.53	76.58	3.67	75.53
	6	3.81	60.62	3.84	60.73	3.73	61.38	3.81	60.86
	6′	3.96		3.99		3.93		4.00	
Gal_s[b]	1	4.56 / 9.0 Hz	103.00	4.61 / 7.6 Hz	102.78	4.62 / 7.8 Hz	102.95	4.62	102.87
	2	3.57	70.22	3.56	69.89	3.57	70.28	3.57	70.23
	3	4.12	76.18	4.10	75.93	4.10	76.48	4.10	76.59
	4	3.92	68.78	3.96	68.27	3.97	68.40	3.97	68.43
	5	3.71	75.56	3.67	75.33	3.70	76.08	3.69	75.85
	6	3.74	61.85	3.71	61.80	3.73	61.78	3.74	61.76
	6′	3.71		3.75		3.76		3.77	
NeuNAc	3	2.76	40.35	2.76	40.36	2.76	40.38	2.76	40.47
	3′	1.80		1.83		1.82		1.82	
	4	3.68	69.05	3.67	69.30	3.68	69.38	3.69	69.21
	5	3.85	52.36	3.85	52.34	3.85	52.58	3.86	52.50
	6	3.62	73.70	3.63	73.60	3.64	74.05	3.66	73.80
	7	3.65	68.78	3.60	69.05	3.60	69.25	3.31	68.82
	8	3.87	72.59	3.87	72.45	3.88	72.70	3.89	72.65
	9	3.88	63.27	3.86	63.18	3.87	63.54	4.43	63.41
	9′	3.65		3.66		3.66		3.59	

[a]NMR experiments were carried out on a Bruker 500 MHz NMR instrument equipped with a TBI cooled probe at controlled temperature (± 0.1 K). Data acquisition and processing were performed using TOPSPIN 1.3 and 3.1 software, respectively.
[b]Gals refers to the residue linked to NeuNAc.
[c]These values were taken from ref. [9].

After purification by dialysis against sodium phosphate buffer, the glycoconjugates **35–37** were characterized by 4–12 % sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI TOF MS for the estimation of the carbohydrate/protein molar ratio (Fig. 2a,b and Table 2), and by microBCA for the protein content quantification. The degree of carbohydrate incorporation was corroborated by quantification of Gal present in the conjugated saccharide through high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) [42].

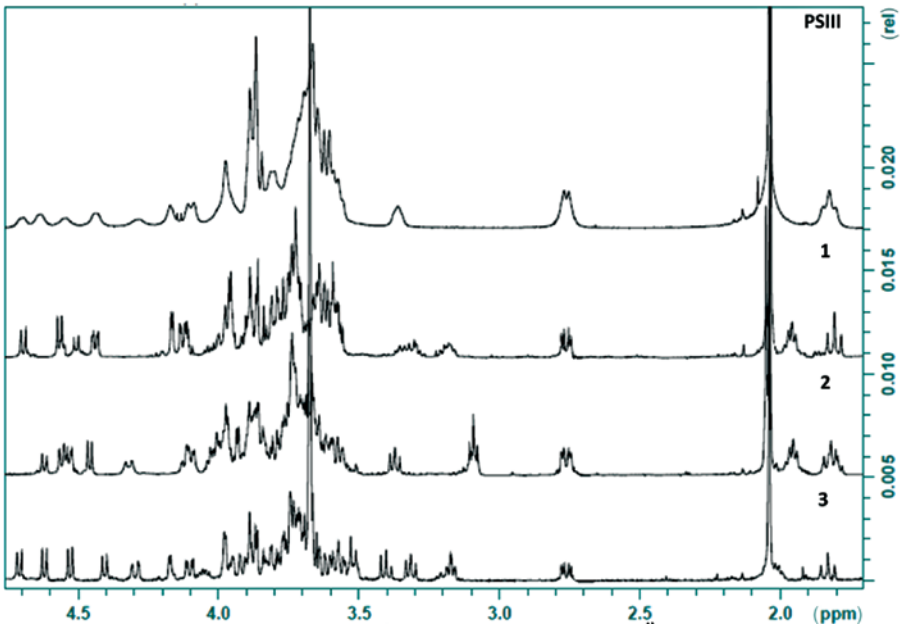
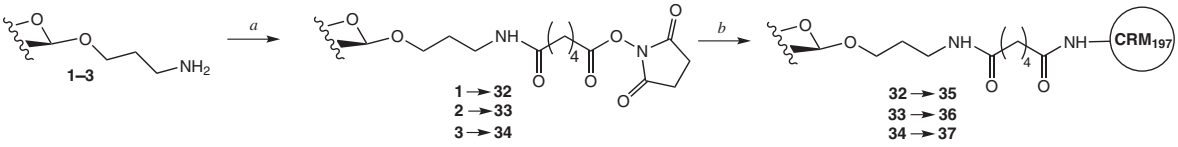


Fig. 1: ^{1}H NMR spectra of pentasaccharides **1–3** in comparison to PSIII in Tris buffer pH 7 (D_2O, 500 MHz, 298 k).



Scheme 6: *Reagents and conditions*: (a) Di-N-hydroxusuccinimidyl adipate, triethylamine, DMSO; (b) 100 mM sodium phosphate pH 7.2.

Table 2: Attributes of the synthesized glycoconjugates.

Glycoconjugate	mol NHS/mol protein	Protein conc. (µg/mL)	Sacch. conc. (µg/mL)	Average n° of saccharide chains per protein
CRM_{197}-DP1 linear **35**	15 : 1	1428	75	3.1
CRM_{197}-DP1 branched **36a**	15 : 1	1268	64	3.0
CRM_{197}-DP1 branched **36b**	75 : 1	1035	409	23.0
CRM_{197}-DP1 Y-shape **37**	75 : 1	1518	700	26.9

Next, the glycoconjugates **35–37** were used to measure by ELISA specific antibodies present in the anti-PSIII murine serum generated by immunization with the polysaccharide conjugated to a GBS pilus protein (Fig. 2c) [42]. The conjugated compounds **2** and **3**, presenting a Glc residue β-(1→6) linked to GlcNAc, exhibited the highest binding. The recognition of **2** appeared independent from its level of incorporation in the obtained glycoconjugates **36a,b**. On the opposite, the conjugated linear oligosaccharide **1** was recognized ~10-fold lower than **2** and **3**, and only slightly better than the negative control CRM_{197}. As expected, the highest level of anti-PSIII antibodies was detected for the positive control PSIII-CRM_{197} (6- to 10-fold higher than **36–37**). In sum, these data indicated that the presence of the branch is a structural relevant motif for the recognition of anti-PSIII antibodies.

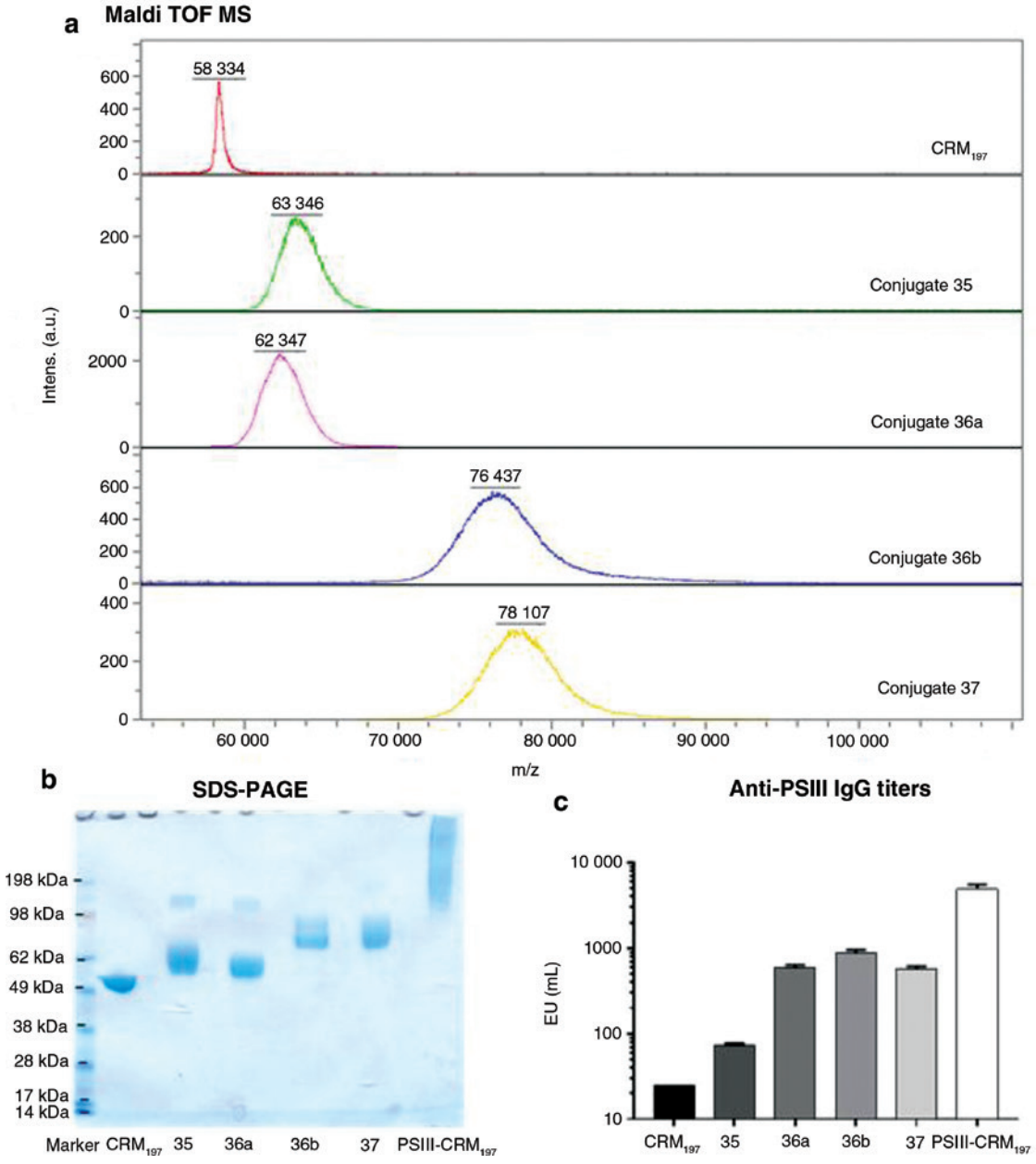


Fig. 2: (a) MALDI TOF MS spectra of synthesized glycoconjugates **35**–**37**; (b) SDS PAGE gel electrophoresis of glycoconjugates **35**–**37** and (c) anti-PSIII IgG titers measured by ELISA using glycoconjugates **35**–**37** for coating. Anti-PSIII murine serum was raised with the polysaccharide conjugated to a GBS pilus protein [42]; CRM_{197} and PSIII-CRM_{197} conjugate were the controls.

Conclusions

GBS PSIII has been reported to present a unique length dependency when fragments were used as inhibitors of the recognition of the polysaccharide with mAbs. In solution the polysaccharide tends to form a helical structure where the negatively charged sialic acid residue of the α-NeuNAc-(2→3)-β-D-Gal*p* branch would be positioned outside the trisaccharide backbone structure →4-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→. These features have been rationalized hypothesizing the existence of an extended

conformational epitope. A deep mapping of anti-PSIII mAbs would be required to better elucidate these PSIII features.

The three different pentasaccharides **1–3** are useful tools for mimicking the GBS PSIII repeating unit naturally produced from bacterial growth. In the present paper these glycans were synthesized via a [2+3] convergent approach. The structures, which were designed with chemical handle for conjugation, were coupled to the genetically detoxified diphtheria toxin CRM_{197}. Recognition of the sugars with polyclonal PSIII specific serum was demonstrated by ELISA. The presence of the branching formed by Glc β-(1→6) linked to GlcNAc was proven an important motif for antibody binding.

The synthetic glycans **1–3** will be used to define the molecular details of the interactions with anti-PSIII mAbs. Results will be reported at due course.

Experimental

General methods for chemical synthesis of oligosaccharides

All chemicals were of reagent grade, and were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F_{254} (Sigma Aldrich); after examination under UV light, compounds were visualized by heating with 10 % (v/v) ethanolic H_2SO_4. In the work up procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4, and concentrated under reduced pressure at 30–50 °C on a water bath. Column chromatography was performed on pre-packed silica cartridges RediSep (Teledyne-Isco, 0.040–0.063 nm) or Biotage SNAP Ultra (0.050 nm irregular silica). Unless otherwise specified, a gradient 0 → 100% of the elution mixture was applied in a CombiflashR_f (Teledyne-Isco) or Isolera (Biotage) instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 400 MHz and 298 K with a Bruker Avance^{III} spectrometer; δ_H values were reported in ppm, relative to the internal standard Me_4Si ($\delta_H = 0.00$, $CDCl_3$) or the water signal ($\delta_H = 4.79$ ppm, D_2O). ^{13}C NMR spectra were measured at 100 MHz and 298 K with a Bruker Avance^{III} spectrometer; δ_C values are reported in ppm relative to the signal of $CDCl_3$ ($\delta_C = 77.0$, $CDCl_3$). NMR signals were assigned by homonuclear and heteronuclear 2-dimensional correlation spectroscopy. When reporting assignments of NMR signals, sugar residues in oligosaccharides are indicated with capital letters, uncertain attributions are denoted "/". Nuclei associated with the linker are denoted with a prime.

Exact masses were measured by electron spray ionization cut-off spectroscopy, using a Q-Tof *micro*Macromass (Waters) instrument. Optical rotation was measured with a P-2000 Jasco polarimeter at 25 °C.

2,4,6-Tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-N-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-D-galactopyranosyl-*N*-phenyltrifluoroacetimidate (α,β) 6

To a solution of **18** (1.5 g, 1.4 mmol) in DCM (10 mL) and 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (681 µL, 4.2 mmol), Cs_2CO_3 (456 mg, 1.4 mmol) was added at 0 °C, and the reaction stirred at rt for 3 h. The solid was filtered off and the solvent evaporated. The crude was purified by flash chromatography (8 : 2 tol : acetone) to afford **6** as a brown foam in 82 % yield (1.15 g). $[\alpha]_D^{25} = +18.0^\circ$ (c 0.80 $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24–7.08 (m, 20H, H–Ar), 6.77 (d, $J = 6.9$ Hz, 1H, H-1^D), 5.71 (m, 1H, H-8^E), 5.68 (1 t, $J = 7.4$ Hz, 1H, H-2^D), 5.41 (d, $J = 2.1$ Hz, 1H, H-4^D), 5.18 (dd, $J = 2.1$, 9.4 Hz 1H, H-7^E), 5.04 (d, $J = 9.0$ Hz, 1H, H-3^D), 4.83 (m, 1H, H-4^E), 4.47–4.38 (m, 2H, 2×H-9^E), 4.37 (m, 1H, H-6$_a$^{D}), 4.33 (m, 1H, H-5^D), 3.92 (m, 1H, H-6$_b$^{D}), 3.85 (m, 4H, H-5^E, $COOCH_3$), 3.65 (dd, 1H, $J = 2.08$, 10.66, H-6^E), 2.50 (dd, 1H, $J = 4.52$, 12.58, H_{3eq}^{E}), 2.35, 2.17, 1.92,

1.78, 1.48 (s, 15H, CH_3CO) 1.69 (t, 1H, $J = 12.30$, $H_{3ax}{}^{E}$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.81, 170.71, 170.67, 170.27, 170.23, 168.19, 165.87, 165.73, 165.14, (CO), 133.45–125.32 (C–Ar), 119.41 (C-1^{D}), 96.90, 77.40, 77.08, 76.76, 72.19, 71.97, 71.17, 70.15, 69.34, 68.19, 67.59, 66.81, 62.75, 53.28, 48.77, 45.77, 37.37 (H-3^{E}), 23.13, 21.48, 20.76, 20.63, 20.29 (5 × CH_3CO). HR ESI-MS *m/z* $C_{55}H_{55}F_3N_2O_{21}$ $[M + Na]^+$ 1159.3147; found 1159.3065.

3-Azidopropyl 2,6-di-*O*-benzyl-3,4-di-*O*-isopropylidene-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside 15

Compound **14** (5.0 g, 11.7 mmol) was dissolved in 100 mL of 9 : 1 2,2-dimethoxypropane:DMF. Catalytic PTSA (0.2 equiv) was added and the reaction warmed at 50 °C for 3 h. A TLC (9 : 1 DCM : MeOH) showed the disappearance of the starting material and the formation of 2 major spots, along with other byproducts. The reaction was quenched with TEA until neutral pH, and the solvent removed under reduced pressure. The crude was dissolved in 150 mL of 9 : 1 MeOH : H_2O and warmed at 90 °C for 2 h, when the presence of one major spot was detected at TLC. The solvent was removed under reduced pressure, and the crude purified by flash chromatography (9 : 1 DCM : MeOH) to give the isopropylinated galactose in 72 % yield (3.9 g).

The forthcoming compound was dissolved in dry DMF (50 mL) under nitrogen atmosphere. The solution was cooled at 0 °C, and 60 % NaH (2.2 g, 55.25 mmol) was added portion-wise. After 20 min BnBr (10.3 mL, 85 mmol) and TBAI (7.8 g, 21.25 mmol) were added. The reaction was stirred overnight at rt, then quenched adding MeOH and solvent removed at reduced pressure. The crude was dissolved in CH_2Cl_2 washed 2 times with aq $NaHCO_3$ and one time with water. The organic phase was collected, dried with Na_2SO_4 and evaporated under reduced pressure. The crude was purified by flash chromatography (8 : 2 cyclohexane : EtOAc) to afford **15** in 79 % yield as a pale yellow oil (6.1 g). $[\alpha]_D^{25} = +25.4^{\circ}$ (c 0.12, $CHCl_3$).

^1H NMR (400 MHz, $CDCl_3$): δ 7.50–7.20 (m, 25H, H–Ar), 4.99 – 4.35 (m, 12H, CH_2Ph, includ. 4.45, d, H-1a, $J = 8.0$ Hz, 1H; 4.39, d, H-1b, $J = 8.7$ Hz, 1H), 4.15 (dd, 1H, $J = 5.5$, 1.1 Hz, H-4a), 4.07–3.96 (m, 3H, OCH_{2a}, H-3, H-4), 3.86 (dd, 1H, $J = 10.9$, 4.1 Hz, H-6_b), 3.80–3.70 (m, 3H, H-6_b, H-6_a, H-3), 3.67 (m, 1H, OCH_{2b}), 3.64–3.54 (m, 2H, H-6_a, H-5), 3.48–3.35 (m, 5H, H-2a, H-2b, CH_2N_3, H-5), 1.93 (m, CH_2CH_2N$_3$, 2H), 1.45 [s, 3H, C(CH_3)], 1.40 [s, 3H, C(CH_3)]. ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.95–126.96 [50 × C–Ar, *C*$(CH_3)_2$], 109.78, 103.58 (C1b), 101.85 (C1a), 82.98, 81.80 (C2b), 80.63 (C2a), 79.37, 77.25, 76.29, 75.43, 75.07–73.20 (5 × CH_2Ph), 72.01, 68.94 (C6a), 68.18 (C6b), 66.48 (OCH_2), 65.30, 48.33 (CH_2N_3), 29.27 (CH_2CH_2N_3), 27.98, 26.42 [2 × C(CH_3)]. HR ESI-MS *m/z* $C_{53}H_{61}N_3O_{11}$ $[M + Na]^+$ 938, 4204; found 938.4200.

3-Azidopropyl 4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside 11

Lactoside **15** (6.1 g, 6.7 mmol) was suspended in 4 : 1 AcOH : H_2O (200 mL). The reaction was warmed at 70 °C for 2 h. A TLC (7 : 3 cyclohexane : EtOAc) showed the disappearance of the starting material and the formation of a spot with a lower Rf. The solvent was removed at reduced pressure and the crude was co-evaporated with toluene (3 × 100 mL).

The crude was dissolved in CH_3CN (100 mL), then triethyl orthoacetate (3.7 mL, 20.1 mmol) and PTSA (270 mg, 1.34 mmol) were added. The reaction was stirred at rt for 4 h, then the solvent was removed under reduced pressure. The crude was dissolved in 4 : 1 AcOH : H_2O (100 mL) and after 2 h the solvent was removed at reduced pressure. The crude was purified by flash chromatography (6 : 4 cyclohexane : EtOAc) to afford **11** in 65% overall yield (3.9 g) as a pale yellow oil. $[\alpha]_D^{25} = -7.0^{\circ}$ (c 0.11, $CHCl_3$).

^1H NMR (400 MHz, $CDCl_3$) δ 7.47–7.13 (m, 25H, H–Ar), 5.37 (d, $J = 3.2$ Hz, 1H, H-4^{B}), 5.01–4.63 (m, 7H, 7 × C*H*HPh), 4.53–4.43 (m, 3H, includ. 2 × C*H*HPh, H-1a; 4.39, d, $J = 7.8$ Hz, 1H, H-1^{B}), 4.27 (d, $J = 12.0$ Hz, 1H, C*H*HPh), 4.01 (m, 2H, 1 × OCH_{2a}, H-4^{A}), 3.82 (dd, $J = 10.9$, 3.9 Hz, 1H, H-6^{A}), 3.75 (d, $J = 9.7$ Hz, 1H, H-6_a), 3.69–3.49 (m, 5H, H-3^{B}, OCH_{2b}, H-6_b, H-4^{B}, H-5^{B}), 3.48 – 3.31 (m, 7H, CH_2N_3, H-2^{A}, H-2^{B}, H-3^{A}, H-5^{A}, H-6^{A}), 2.06 (s, 3H,

C*H*$_3$CO), 1.92 (m, 2H, C*H*$_2$N$_3$). ^{13}C NMR (101 MHz, CDCl$_3$) δ 171.00 (*C*OCH$_3$), 138.99–126.97 (C–Ar), 103.57 (C-1^A), 102.30 (C-1^B), 82.71, 81.68, 80.08 (C-2^A, C-2^A), 76.28, 75.25, 75.04, 73.39, 73.23, 72.43, 71.98 (C-3^A, C-3A), 69.63 (C-4^B), 68.09 (C-6^B), 67.23 (O*C*H$_2$), 66.50 (C-6^A), 48.30 (*C*H$_2$N$_3$), 29.25 (*C*H$_2$CH$_2$N$_3$), 20.78 (*C*H$_3$CO). HR ESI-MS *m/z* C$_{52}$H$_{59}$N$_3$O$_{12}$ [M + Na]$^+$ 939.3996; found 940.4030.

3-Azidopropyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside 16

A solution of acceptor **11** (800 mg, 0.87 mmol) and donor **8** (655 mg, 1.13 mmol) with activated molecular sieves (4 Å, 1.0 g) in DCM (10 mL) was stirred for 20 min under nitrogen. NIS (508 mg, 2.26 mmol) and TfOH (20 µL, 0.23 mmol) were added at – 20 °C. After the reaction mixture was stirred for 24 h at room temperature, TEA was added until neutral pH, the solid filter off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (4 : 1 Tol : EtOAc) to afford **16** in 72 % yield (870 mg) as a colorless oil. $[\alpha]_D^{25} = +16.7^\circ$ (c 0.15, CHCl$_3$).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.47–7.13 (m, 39H, H–Ar), 5.55 (s, 1H, C*H*Ph), 5.31–5.27 (m, 2H, H-1^C, H-4^B), 4.83–4.52 (m, 5H, C*H*HPh), 4.45–4.33 (m, 5H, 4 × C*H*HPh, H-4^C), 4.21–3.39 (m, 6H, H-1^A, H-1^b, H-2^c, 3 × C*H*HPh), 3.85–3.71 (m, 5H, H-2$^{A–B}$, H-6$_a$$^{A–C}$), 3.62–3.18 (m, 15H, H3$^{A–C}$, H-4$^{A–C}$, 2 × H-5, H-6$_b$$^{A–C}$, OCH$_2$, CH$_2N_3$), 2.97–2.90 (m, 1H, H-5), 2.02 (s, 3H, C*H*$_3$CO), 1.82–1.69 (m, 2H, C*H*$_2$CH$_2$N$_3$). ^{13}C NMR (101 MHz, CDCl3) δ 169.90, 167.50 (CO), 139.04–123.14 (C–Ar), 103.46 (C-1^A), 101.87 (C-1^B), 101.27 (C*H*Ph), 99.20 (C-1c^C), 82.83 (C-2^A), 82.65, 78.88, 78.63, 75.66, 75.16, 75.04, 74.68, 74.43 (C-2^B), 74.31 (*C*H$_2$Ph), 74.26 (*C*H$_2$Ph), 74.04 (*C*H$_2$Ph), 73.55 (*C*H$_2$Ph), 73.11 (*C*H$_2$Ph), 72.82 (*C*H$_2$Ph), 72.49 (C-3^C), 69.85 (C-4^C), 68.76, 68.50, 68.21, 67.61, 66.25, 65.91 (C-6^A), 65.91 (C-6^B), 66.39 (O*C*H$_2$), 65.91 (C-6^C), 56.11 (C-2^C), 48.29 (*C*H$_2$N$_3$), 29.21 (*C*H$_2$CH$_2$N$_3$), 20.88 (*C*H$_3$CO). HR ESI-MS *m/z* C$_{80}$H$_{82}$N$_4$O$_{18}$ [M + Na]$^+$ 1409.5522; found 1409.5604.

3-Azidopropyl 3-*O*-benzyl-6-*O*-*t*-butyldiphenylsilyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside 17

Trisaccharide **16** (0.29 mmol, 400 mg) was suspended in AcOH:H$_2$O = 4 : 1 (25 mL). The reaction was warmed at 70 °C and stirred for 4 h. The solvent was removed at reduced pressure and the crude purified by flash chromatography (6 : 4 cyclohexane : EtOAc) to afford the debenzylidinated trisaccharide in 87 % yield (325 mg, 0.25 mmol) as a pale yellow oil.

The material was dissolved in pyridine (10 mL). TBDPSCl (0.50 mmol, 140 µL) and DMAP (0.05 mmol, 10 mg) were added and the reaction was stirred overnight at 60 °C, when TLC (7 : 3 cyclohexane : EtOAc) showed complete reaction. The solvent was removed at reduced pressure and the crude purified by flash chromatography (cyclohexane : EtOAc) to afford **17** in 92 % yield (675 mg) as a yellow oil. $[\alpha]_D^{25} = +5.7^\circ$ (c 1.23, CHCl$_3$).

^{1}H NMR (400 MHz, CDCl$_3$) δ 7.31–7.14 (m, 44H, H–Ar), 5.38–5.32 (m, 2H, H-1c, H-4b), 4.91 (d, *J* = 10.5 Hz, 1H, C*H*HPh), 4.86–4.36 (m, 7H, C*H*HPh), 4.33 (dd, *J* = 11.4, 2.8 Hz, 1H, H-4^C), 4.30–4.18 (m, 6H, H-1^A, H-1b, 4 × C*H*HPh), 4.17–4.08 (m, 2H, H-2^C, H-6$_a$), 4.02 (m, 2H, H-6^C, H-3), 3.95–3.83 (m, 3H, H-4^A, OCH$_{2a}$), 3.63 (m, 1H, H-5), 3.60–3.45 (m, 3H, H-5^B, H-6$_a$, OCH$_{2b}$), 3.45–3.23 (m, 9H, C*H*$_2$N$_3$, H-6$_a$, 2 × H-6$_b$, H-2^A, H-2^B, 2 × H-3), 3.04 (d, J = 9.5 Hz, 1H, H-5), 2.02 (s, 3H, C*H*$_3$CO), 1.92–1.82 (m, 2H, C*H*$_2$CH$_2$N$_3$), 1.12 (s, 9H, t-Bu). ^{13}C NMR (101 MHz, CDCl3) δ 169.80, 166.70 (CO), 135.59–120.48 (C–Ar), 103.41 (C-1^A), 101.82 (C-1^B), 98.41 (C-1^C), 82.58, 81.53, 79.11, 78.44, 77.72, 75.48, 75.09, 75.00, 74.96, 74.69, 74.33, 74.20, 73.90, 73.38, 73.07, 72.71, 69.93 (C-4^C), 68.34 (C-6b), 67.62 (C-6a), 66.35 (O*C*H$_2$), 65.31 (C-6^C), 55.75 (C-2^C), 48.25 (*C*H$_2$N$_3$), 31.07 (*C*(CH$_3$)$_3$), 29.18 (*C*H$_2$CH$_2$N$_3$), 26.83 (C(*C*H$_3$)$_3$), 20.66 (*C*H$_3$CO). HR ESI-MS *m/z* C$_{89}$H$_{96}$N$_4$O$_{18}$Si [M + Na]$^+$ 1159.6387; found 1559.6224.

3-Azidopropyl 2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-β-D-galactopyranosyl-(1→4)-3-*O*-benzyl-6-*O*-*t*-butyldiphenylsilyl-2-deoxy-2-phthalimido-β-D-glucopyranoside)-(1→3)-4-*O*-acetyl-2,6-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside 19

A solution of trisaccharide acceptor **17** (675 mg, 0.23 mmol) and disaccharide donor **6** (261 mg, 0.23 mmol) with activated 4 Å molecular sieves (800 mg) in DCM (8 mL) was stirred for 20 min under nitrogen. TMSOTf (0.046 mmol, 9 μL) was added at 0 °C. After the reaction mixture was stirred for 10 h at rt, when TLC (7 : 3 Tol : acetone) showed complete reaction. TEA was added until neutral pH, the solid filter off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (Tol:acetone) to afford **19** in 55% yield (314 mg) as an amorphous solid. $[\alpha]_D^{25} = +16.5^\circ$ (c 0.16, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28–7.11 (m, 59H, H-Ar), 5.73 (ddd, J = 2.2, 6.0, 9.2 Hz, 1H, H-8^E), 5.54 (dd, *J* = 8.0, 10.2 Hz, 1H, H-2^D), 5.37 (m, *J* = 3.5 Hz, 1H, H-7^E), 5.28–5.22 (m, 3H, H-1^D, H-4^B, H-4^D), 5.17 (d, *J* = 8.4 Hz, 1H, H-1^C), 4.96–4.61 (m, 9H, incl. m, 4.81, H-4^E and m, 4.62, H-6^E), 4.48–4.00 (m, 15H), 3.89–3.79 (m, 7H, incl. m, 5.02, H-5^E, and s, 3.83, COOCH_3), 3.65–3.62 (m, 1H), 3.59–3.50 (m, 1H, OCH_{2b}), 3.45–3.24 (m, 10H), 2.97–2.95 (m, 1H), 2.46 (dd, *J* = 4.5, 12.6 Hz, 1H, H-3$_e^E$), 2.18, 2.15, 2.11, 2.03, 1.96 (5 × s, 3H each, 5 CH_3CO), 1.89–1.80 (m, 5 H, CH_2CH$_2$N$_3$, incl. s, 1.83, CH_3CO), 1.70 (t, *J* = 12.0 Hz, H-3$_a^E$), 1.26 (s, 9H, t-Bu). ^{13}C NMR (101 MHz, CDCl3) δ 170.70–164.81 (C=O), 138.91–125.28 (C–Ar), 103.39 (C-1$^{A/B}$), 102.00 (C-1$^{A/B}$), 99.88 (C-1^D), 97.45 (C-1^C), 82.48, 81.61, 80.05, 78.43, 78.31, 77.32, 77.21, 77.01, 76.69, 75.52, 75.43, 75.08, 75.00, 74.85, 74.72, 74.30, 74.14, 73.31, 73.00, 72.61, 72.27, 72.21, 71.71, 70.80, 69.78, 69.30, 68.58, 68.14, 67.83, 67.69, 66.54, 66.35, 62.51, 62.11, 56.54 (C-2^C), 53.04 (C-5^E), 49.02 (COOCH_3), 48.27 (*C*H$_2$N$_3$), 37.39 (C-3^E), 29.26 [*C*(CH$_3$)$_3$], 29.21 (*C*H$_2$CH$_2$N$_3$), 26.80 (C(*C*H$_3$)$_3$), 23.16, 21.44, 21.21, 20.75, 20.71, 19.36 (6 × *C*H$_3$CO). HR ESI-MS *m/z* $C_{136}H_{145}N_5O_{38}Si$ $[M+Na]^+$ 2506.9235; found 2506.9224.

3-Azidopropyl 4,6-*O*-benzylidene-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 20

A solution of **8** (2.0 g, 3.45 mmol) and 3-azido-1-propanol (707 mg, 7.0 mmol) with activated molecular sieves (4 Å, 3.0 g) in DCM (25 mL) was stirred for 20 min under nitrogen. NIS (1.57 g, 7.0 mmol) and TfOH (61 μL, 0.7 mmol) were added at − 10 °C. After 12 h (TLC; 7 : 3 cyclohexane : EtOAc) the reaction was quenched with TEA, the solid filter off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (cyclohexane : EtOAc) to afford **20** in 84 % yield (1.65 g) as a yellow oil. NMR data were in agreement with those reported in literature [43].

3-Azidopropyl 3-*O*-benzyl-6-*O*-*t*-butyldiphenilsilyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 12

Monosaccharide **20** (1.65 g, 2.9 mmol) was suspended in AcOH : H_2O=4 : 1 (40 mL). The reaction was warmed at 70 °C and let stir for 4 h. The solvent was removed under reduced pressure and the crude purified by flash chromatography (6 : 4 cyclohexane : EtOAc) to afford 3-azidopropyl 3-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside in 89 % yield (1.24 g, 2.6 mmol) as a pale yellow oil.

The material was dissolved in pyridine (20 mL). TBDPSCl (1.34 mL, 5.2 mmol) and DMAP (65 mg, 0.52 mmol) were added and the solution was stirred overnight at 60 °C, at which time the reaction was complete (TLC, 8 : 2 cyclohexane : EtOAc). The mixture was diluted with DCM and washed with water. The organic phase were dried with Na_2SO_4 and evaporated at reduced pressure. The crude was purified by flash chromatography (cyclohexane : EtOAc) to afford **12** in 79 % (1.48 g) yield as a pale yellow oil. $[\alpha]_D^{25} = +12.9^\circ$ (c 0.29, $CHCl_3$). HR ESI-MS *m/z* $C_{40}H_{44}N_4O_7Si$ $[M+Na]^+$ 743.2877; found 743.2819.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12–6.84 (m, 19H, H–Ar), 5.17 (d, *J* = 8.4 Hz, 1H, H-1), 4.82, 4.59 (2 d, *J* = 12.2 Hz, 1H, CH_2Ph), 4.30 (dd, *J* = 10.7, 8.5 Hz, 1H, H-3), 4.17 (dd, *J* = 10.7, 8.5 Hz, 1H, H-2), 4.06–3.96 (m, 2H, 2 × H-6), 3.92

(t, J = 9.0 Hz, 1H, H-4), 3.76–3.82 (m, 1H, OCH_2a), 3.63 (dt, J = 9.8, 5.1 Hz, 1H, H-5), 3.54–3.40 (m, 1H, OCH_2b), 3.12 (m, 2H, $CH_2CH_2N_3$), 1.78–1.57 (m, 2H, CH_2N_3), 1.43 (s, 9H, t-Bu). ^{13}C NMR (101 MHz, CDCl3) δ 167.81 (CO), 138.22–127.41 (C–Ar), 98.14 (C-1), 78.79 (C-3), 74.60, 74.38, 74.33 (CH_2Ph, C-4, C-5), 65.82 (OCH_2), 65.09 (C-6), 55.35 (C-2), 48.00 (CH_2N_3), 31.04 ($C(CH_3)_3$), 28.81 ($CH_2CH_2N_3$), 26.82 ($C(CH_3)_3$).

3-Azidopropyl 2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-β-D-galactopyranosyl-(1→4)-3-*O*-benzyl-6-*O*-*t*-butyldiphenilsilyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 21

A solution of disaccharide donor **6** (500 mg, 0.44 mmol) and acceptor **12** (320 mg, 0.44 mmol) with activated molecular sieves (4 Å, 800 mg) in DCM (8 mL) was stirred for 20 min under nitrogen. TMSOTf (16 µL, 0.088 mmol) was added at −10 °C. After stirring for 10 h at rt, TLC showed complete reaction (7 : 3 Tol : acetone). TEA was added until neutral pH, the solid filter off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (Tol : acetone) to afford **21** in 70 % yield (520 mg) as a vitreous solid. $[\alpha]_D^{25} = +23.6^\circ$ (c 0.09, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32–6.52 (m, 34H), 5.57 (dd, J = 7.2, 9.0 Hz, 1H, H-2^B), 5.45 (d, J = 3.3 Hz, 1H, H-4^B), 5.35 (d, J = 7.8 Hz, 1H, H-1^A), 5.27 (dd, J = 9.2, 2.4 Hz, 1H, H-8^C), 5.02 (d, J = 10.0 Hz, H-1^B), 5.04–4.93 (m, 1H), 4.80–4.77 (m, 2H), 4.71 (d, J = 12.4 Hz, 1H, C*H*HPh), 4.43–4.17 (m, 8H), 4.10 (dd, J = 10.6, 8.6 Hz, 1H), 4.02 (dd, J = 12.6, 4.6 Hz, 1H), 3.92–3.77 (m, 3H), 3.73 (s, 3H, COOCH_3), 3.66 (dd, J = 10.8, 2.5 Hz, 1H), 3.58–3.53 (m, 1H, OCH_{2b}), 3.31 (d, J = 9.6 Hz, 1H, H-6$_b^A$), 3.23–3.17 (m, 1H, H-5^B), 3.01 (t, J = 6.8 Hz, 2H, CH_2N_3), 2.41 (dd, J = 12.7, 4.6 Hz, 1H, H-3$_e^C$), 2.12, 1.98, 1.91, 1.81 (5 × s, 3H each, 5 × CH_3CO), 1.70–1.67 (m, 2H, $CH_2CH_2N_3$), 1.62–1.60 (m, 4H, CH_3CO, H-3$_a^C$), 1.07 (s, 9H, t-Bu). HR ESI-MS *m/z* $C_{87}H_{93}N_5O_{27}Si$ $[M+Na]^+$ 1690.5275; found 1690.5801.

3-Azidopropyl 2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-β-D-galactopyranosyl)-(1→4)-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 22

Trisaccharide **21** (520 mg, 0.31 mmol) was dissolved in 4 : 1 THF:pyridine (10 mL). HF·py (930 µL) were added at 0 °C. The solution was stirred overnight (TLC, 7 : 3 Tol : acetone), then the reaction was diluted with DCM and washed with water. The organic phase were dried with Na_2SO_4 and evaporated at reduced pressure. The crude was purified by flash chromatography (Tol : acetone) to afford **22** (345 mg) in 78 % yield. $[\alpha]_D^{25} = +11.5^\circ$ (c 0.19, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51–6.53 (m, 24H, H–Ar), 5.83 (td, J = 9.3, 2.4 Hz, 1H, H-8^C), 5.55 (dd, J = 8.3, 10.5 Hz, 1H, H-2^B), 5.32 (d, J = 3.2 Hz, 1H, H-4^B), 5.20 (d, J = 10.2 Hz, 1H, H-1^A), 5.13 (m, 2H, H-7^C, NH), 5.02 (d, J = 8.5 Hz, 1H, H-1^B), 4.91 (d, J = 12.5 Hz, 1H, C*H*HPh), 4.87 (dd, J = 3.0, 10.5 Hz, 1H, H-3^B), 4.80 (dd, J = 4.5, 10.7 Hz, 1H, H-4^C), 4.61 (d, J = 12.5 Hz, 1H, C*H*HPh), 4.55 (dd, J = 11.9, 2.4 Hz, 1H, H-6^C), 4.49 (t, J = 9.0 Hz, 1H, H-6$_a^B$), 4.30–4.09 (m, 5H, H-2^B, H-3^A, H-5^B, H-6$_b^B$, H-6$_a^A$), 3.95 (dd, J = 3.2, 9.0 Hz, 1H, H-9$_a^C$), 3.89–3.75 (m, 8H, H-2^A, H-4^A, H-5^C, H-9$_b^C$, OCH_{2a}, incl. s, 3.82, COOCH_3), 3.63 (dd, J = 10.7, 2.7 Hz, 1H, H-5^A), 3.39–3.29 (m, 2H, OCH_{2b}, H-6$_b^A$), 3.16–2.99 (m, 2H, CH_2N_3), 2.47 (dt, J = 13.6, 6.8 Hz, 1H, H-3$_e^C$), 2.18, 2.12, 1.75 (4 × s, 3H each, 4 × CH_3), 1.70–1.57 (m, 3H, H-3$_a^E$, $CH_2CH_2N_3$). ^{13}C NMR (101 MHz, CDCl3) δ 172.22, 171.43, 170.98, 170.78, 170.60, 170.37, 170.25, 170.12, 169.17, 168.02, 167.58, 165.94, 165.83, 165.68, 165.49, 165.20 (C=O), 138.59– 123.18 (C–Ar), 100.97 (C-1^B), 98.19 (C-1^A), 96.82 (C-2^C), 78.04 (C-3^A), 76.47 (C-4^A), 75.20 (C-5^A), 74.44 (CH_2Ph), 71.73 (C-3^B), 71.59 (C-2^B), 71.46 (C-5^B), 70.62 (C-6^C), 69.39 (C-4^B), 68.30 (C-8^C), 67.36 (C-4^C), 66.78 (C-7^C), 65.87 (OCH_2), 63.77 (C-9^C), 61.71 (C-6$^{A/B}$), 60.16 (C-6$^{A/B}$), 55.67 (C-2^A), 53.17 (C-5^C), 48.53 (COOCH_3), 47.91 (CH_2N_3), 37.31 (C-3^C), 28.72 ($CH_2CH_2N_3$), 23.02, 21.41, 21.35, 20.81, 20.68, 20.45 (CH_3CO). HR ESI-MS *m/z* $C_{71}H_{75}N_5O_{27}$ $[M+Na]^+$ 1452.4547; found 1452.4557.

3-Azidopropyl [(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)]-[2,4,6-tri-*O*-benzoyl-3-*O*-(methyl 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-β-D-galactopyranosyl-(1→4)]-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 23

A solution of trisaccharide acceptor **22** (345 mg, 0.24 mmol) and donor **6** (420 mg, 0.60 mmol) with activated molecular sieves (4 Å, 800 mg) in DCM (8 mL) was stirred for 20 min under nitrogen. AgOTf (77 mg, 0.30 mmol) was added at 0 °C. After the reaction mixture was stirred for 10 h at rt, when TLC (7 : 3 Tol:acetone) showed complete reaction. TEA was added, the solid filter off and the solvent removed at reduced pressure. The crude was purified by flash chromatography (Tol : acetone) to afford **23** (300 mg, 0.14 mmol) in 68 % yield. $[\alpha]_D^{25} = +30.0^\circ$ (c 0.09, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26–6.73 (m, 24H, H–Ar), 5.73 (ddd, *J* = 2.2, 6.0, 9.2 Hz, 1H, H-8^E), 5.53 (dd, *J* = 8.3, 10.5 Hz, 1H, H-2^B), 5.36–5.31 (m, 2H), 5.25–4.79 (m, 11H), 4-59–4.40 (m, 5H), 4.30–3.62 (m, 20 H, incl. incl. s, 3.82, COOC*H*$_3$), 3.41–3.38 (m, 1H), 3.15–3.07 (m, 2H, CH_2N_3), 2.47 (dt, *J* = 12.8, 4.6 Hz, 1H, H-3$_e^C$), 2.28, 2.19, 2.18, 2.17, 2.16, 2.11, 2.06, 2.05, 2.03, 1.98, 1.91, 1.75 (12 × s, 3H each, 12 × CH_3), 1.70–1.59 (m, 3H, H-3$_a^E$, C*H*$_2$CH$_2$N$_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.91, 170.76, 170.52, 170.38, 170.22, 170.16, 170.10, 169.34, 168.15, 165.50, 164.99 (C=O), 133.76–123.28 (C–Ar), 101.34 (C-1$^{D/E}$), 101.03 (C-1$^{D/E}$), 100.72 (C-1^B), 97.69 (C-1^A), 96.91, 79.63, 77.18, 75.05, 74.69, 72.96, 72.37, 71.74, 71.59, 71.44, 71.35, 71.16, 71.05, 70.97, 70.86, 70.69, 70.57, 69.40, 69.00, 68.19, 66.69, 66.58, 66.17 (4 × C-6), 55.68 (C-2^A), 53.19 (C-5^C), 48.70 (COO*C*H$_3$), 47.96 (CH_2N_3), 37.25 (C-3^C), 28.67 (*C*H$_2$CH$_2$N$_3$), 23.11, 22.68, 21.44, 20.86, 20.80, 20.75, 20.73, 20.65, 20.54, 20.52 (12 × *C*H$_3$CO). HR ESI-MS *m/z* $C_{97}H_{109}N_5O_{44}$ $[M + Na]^+$ 2070.6343; found 2070.6296.

3-Azidopropyl 2,6-*O*-benzyl-3,4-*O*-isopropylidene-β-D-galactopyranoside 26

Compound **25** (3.0 g, 6.77 mmol) was dissolved in dry DMF (40 mL) under nitrogen atmosphere. The solution was cooled at 0 °C, and NaH 60 % mineral dispersion (704 mg, 17.6 mmol,) was added portion-wise. After 20 min BnBr (3.2 mL, 27.08 mmol) and TBAI (2.5 g, 6.7 mmol) were added. The reaction was stirred overnight at rt (TLC, 8 : 2 cyclohexane-EtOAc), then quenched by addition of MeOH and TEA. After removing the solvent under reduced pressure, the crude was dissolved in DCM and washed twice with aq. $NaHCO_3$ and twice with water. The organic layers were combined, dried with Na_2SO_4 filtered and evaporated under reduced pressure. The crude was purified by flash chromatography to afford **26** in 85 % yield (2.75 g). $[\alpha]_D^{25} = +31.8^\circ$ (c 1.00, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54–7.23 (m, 10H, C–Ar), 4.84 (s, 2H, CH_2Ph), 4.67, 4.60 (d, *J* = 12.0 Hz, 1H, C*H*$_2$Ph), 4.34 (d, *J* = 8.0 Hz, 1H, H-1), 4.24–4.12 (m, 2H, H-3, H-4), 4.05–4.03 (m, 1H, OCH$_{2a}$), 3.96 (t, *J* = 5.9 Hz, 1H, H-6$_a$), 3.86–3.79 (m, 2H, H-5, H-6$_b$), 3.68–3.64 (m, 1H, OCH$_{2b}$), 3.49–3.39 (m, 3H, CH_2N_3, H-2), 2.04–1.83 (m, 2H, C*H*$_2$CH$_2$N$_3$), 1.41, 1.37 (2 × s, 3H each, 2 × CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 128.50–127.62 (C–Ar, *C*(CH$_3$)$_2$), 102.81 (C-1), 79.59 (C-2), 79.06 (C-4), 73.81 (C-3), 73.58 (*C*H$_2$Ph), 73.55 (*C*H$_2$Ph), 72.24 (C-5), 69.51 (C-6), 66.37 (OCH$_2$), 48.33 (CH_2N_3), 29.22 (*C*H$_2$CH$_2$N$_3$), 27.79, 26.33 (2 × CH3). HR ESI-MS *m/z* $C_{26}H_{33}N_3O_6$ $[M + Na]^+$ 506.2267; found 506.2214.

3-Azidopropyl 4-*O*-acetyl-2,6-*O*-benzyl-β-D-galactopyranoside 13

Compound **26** (2.75 g, 5.7 mmol) was suspended in 4 : 1 AcOH : H_2O (50 mL). The reaction was warmed at 70 °C for 2h, when TLC (7 : 3 cyclohexane : EtOAc) showed the disappearance of the starting material and the formation of a spot with lower Rf. The solvent was removed at reduced pressure and the crude purified by flash chromatography (cyclohexane : EtOAc) to afford the 3-azidopropyl 2,6-O-benzyl-β-D-galactopyranoside in 92 % yield as an oil (2.30 g).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43–7.19 (m, 10H, H–Ar), 4.89, 4.67 (2 × d, *J* = 11.5 Hz, 1H, C*H*$_2$Ph), 4.56 (s, 1H, CH_2Ph), 4.33 (d, *J* = 7.6 Hz, 1H, H-1), 4.06–3.89 (m, 2H, H-4, OCH$_{2a}$), 3.74 (m, 2H, 2 × H-6), 3.60 (m, 3H, H-3, H-5,

OCH_{2b}), 3.49 (m, 1H, H-2), 3.38 (t, J = 6.8 Hz, 2H, CH_2N_3), 1.92–1.88 (m, 2H, $CH_2CH_2N_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.44–127.67 (C–Ar), 103.60 (C-1), 79.30 (C-2), 74.67 (CH_2Ph), 73.60 (CH_2Ph), 73.37 (C-5), 73.15 (C-3), 69.36 (C-6), 68.99 (C-4), 66.39 (OCH_2), 48.31 (CH_2N_3), 29.21($CH_2CH_2N_3$). HR ESI-MS *m/z* $C_{23}H_{29}N_3O_6$ $[M+Na]^+$ 446.1954; found 446.1954.

The diol was dissolved in CH_3CN (30 mL), then triethyl orthoacetate (2.8 mL, 15.6 mmol) and PTSA (208 mg, 1.04 mmol) were added. The reaction was stirred at rt for 4 h (TLC, 6 : 4 cyclohexane : EtOAc), then the solvent was removed under reduced pressure. The crude was dissolved in 4 : 1 AcOH : H_2O (50 mL) and after 2 h the mixture was concentrated. The crude was purified by flash chromatography (cyclohexane : EtOAc) to afford **23** in 87 % yield (2.20 g) as a pale yellow oil. $[\alpha]_D^{25} = +75.5^\circ$ (c 0.1 $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38–7.28 (m, 10H, H–Ar), 5.38 (dd, J = 3.6, 0.8 Hz, H-4), 4.94, 4.71 (2×d, J = 10.9 Hz, 2H, CH_2Ph), 4.58, 4.48 (2×d, J = 11.9 Hz, 2H, CH_2Ph), 4.41 (d, J = 7.8 Hz, 1H, H-1), 4.12–4.09 (m, 1H, H-6_a), 4.07–4.01 (m, 1H, OCH_{2a}), 3.79–3.76 (m, 2H, H-5, H-6_b), 3.69–3.67 (m, 1H, OCH_{2b}), 3.61–3.49 (m, 1H, H-2, H-3), 3.42 (t, J = 6.6 Hz, 2H, CH_2N_3), 2.09 (s, 3H, CH_3), 1.95–1.90 (m, 2H, $CH_2CH_2N_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.26 (CO), 138.27–127.78 (C–Ar), 103.88 (C-1), 79.37 (C-2), 74.93 (CH_2Ph), 73.64 (CH_2Ph), 72.46 (C-5), 71.94 (C-3), 68.48 (C-6), 68.06 (C-4), 64.99 (OCH_2), 48.30 (CH_2N_3), 29.19 ($CH_2CH_2N_3$), 21.07 (CH_3). HR ESI-MS *m/z* $C_{25}H_{31}N_3O_7$ $[M+Na]^+$ 508.2060; found 508.2072.

Phenylthio 4,6-*O*-naphtylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside 9

The protected phenylthio glucosamine **24** (5 g, 13.1 mmol) was deacetylated by treatment overnight with NaOMe in MeOH until pH was 9–10. The mixture was neutralized with Dowex H^+, then it was filtered. The filtrate was concentrated and dissolved in CH_3CN (20 mL) to which freshly prepared 2-naphthaldehyde dimethylacetal (12 mL, 65.5 mmol) and PTSA (498 mg, 2.62 mmol) were added. After stirring overnight, the crude mixture was purified on silica gel (cyclohexane-EtOAc) to give 3.5 g of product, which was directly used for benzylation.

To a solution of the 3-OH sugar (3.7 g, 9.4 mmol) in DMF (20 mL), 60 % NaH in mineral oil (587 mg, 14.1 mmol) was added at 0 °C under nitrogen atmosphere. After stirring for 20 min, BnBr (3.3 mL, 28.5 mmol) was added and mixture was agitated overnight. The crude mixture was partitioned in water (×3), and the combined organic layers were concentrated and purified on silica gel (cyclohexane-EtOAc) to provide the monosaccharide **9** (5.3 g, 69 % yield over three steps). $[\alpha]_D^{25} = +88.2^\circ$ (c 0.15, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78–6.77 (m, 21H, H–Ar), 5.70 (s, 1H, CHNap), 5.58 (d, J = 10.5 Hz, H-1), 4.70, 4.42 (2×d, J = 12.3 Hz, 2H, CH_2Ph), 4.41–4.32 (m, 2H, H-3, H-6_a), 4.24 (t, J = 10.0 Hz, H-2), 3.82 (t, J = 10.1 Hz, H-6_b), 3.79 (t, J = 8.9 Hz, H.4), 3.72–3.65 (m, 1H, H-5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.82 (CO), 137.70–123.40 (C–Ar), 101.53 (CHNap), 84.16 (C-1), 82.93 (C-4), 75.46 (C-3), 74.23 (CH_2Ph), 70.44 (C-5), 68.77 (C-6), 54.75 (C-2). HR ESI-MS *m/z* $C_{38}H_{31}NO_6S$ $[M+Na]^+$ 626.1613; found 626.1607.

3-Azidopropyl 3-*O*-benzyl-4,6-*O*-naphtylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranoside 27

A solution of donor **9** (800 mg, 1.27 mmol) and acceptor **13** (514 mg, 1.05 mmol) with activated molecular sieves (4 Å, 1.2 g) in DCM (12 mL) was stirred for 20 min under nitrogen. NIS (570 mg, 2.54 mmol) and TfOH (22 µL, 0.254 mmol) were added at – 20 °C. After stirring for 3 h (TLC, (7 : 3 Tol : EtOAc), the reaction mixture was quenched with TEA, the solid filter off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol : EtOAc) to afford **27** in 72 % yield (760 mg) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15–6.71 (m, 26H, H–Ar), 5.81 (s, 1H, CHNap), 5.46 (d, J = 8.3 Hz, 1H, H-1^B), 5.42 (d, J = 3.3 Hz, 1H, H-4^A), 4.83 (t, J = 11.4 Hz, 2H, 2×C*H*HPh), 4.69–4.33 (m, 5H, 4×C*H*HPh, H-3^B), 4.26 (d, J = 8.0 Hz, 1H, H-1^A), 4.22 (dd, J = 7.9, 10.2 Hz, 1H, H-2^B), 3.97–3.80 (m, 5H, 2×H-$6^{A,B}$, OCH_{2a}), 3.74 (dd, J = 9.6, 3.4 Hz, 1H, H-3^A),

3.72–3.60 (m, 1H, H-5^{A}), 3.58–3.42 (m, 4H, OCH_{2b}, H-5^{B}, H-2^{A}, H-4^{B}), 3.18 (dd, J = 10.1, 6.4 Hz, 2H, CH_2N_3), 2.14 (s, 3H, CH_3CO), 1.75–1.69 (m, 2H, $CH_2CH_2N_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.48, 167.38 (CO), 134.00–123.16 (C–Ar), 103.47 (C-1^{B}), 101.60 (CHNap), 99.07 (C-1^{A}), 82.91, 82.73, 78.71, 78.39, 74.50, 74.35, 74.30, 74.20, 74.01, 73.67, 72.76, 69.72 (C-4^{A}), 69.03, 68.71 (2 × C-6), 68.55 (OCH_2), 56.07 (C-2^{B}), 48.05 (CH_2N_3), 28.99 ($CH_2CH_2N_3$), 20.89 (CH_3CO). HR ESI-MS *m/z* $C_{57}H_{56}N_4O_{13}$ $[M+Na]^+$ 1027.3742; found 1027.3769. $[\alpha]_D^{25} = +32.3^\circ$ (c 0.10, $CHCl_3$).

3-Azidopropyl 3-*O*-benzyl-4-*O*-(2-naphtyl)methylene-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranoside 28

Disaccharide **27** (760 mg, 0.75 mmol) was dissolved in CH_3CN (15 mL). The solution was cooled to 0 °C and $BH_3{\cdot}NMe_3$ complex (275 mg, 3.75 mmol) and BF_3Et_2O (470 µL, 3.75 mmol) were added. The solution was stirred for 6 h maintaining the temperature at 0 °C (TLC, 7 : 3 Tol : EtAOc), then the reaction was quenched by addition of TEA and MeOH. The solvent was removed under reduced pressure and the crude was purified by flash chromatography (Tol : EtOAc) to afford **28** in 64 % yield (483 mg, 0.48 mmol) as a yellow oil. $[\alpha]_D^{25} = +22.7^\circ$ (c 0.11, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86–7.37 (m, 26H, H–Ar), 5.64 (d, J = 3.4 Hz, 1H, H-4^{A}), 5.41 (d, J = 8.5 Hz, 1H, H-1^{B}), 5.04, 4.92 (2 d, J = 11.1 Hz, 2H, CH_2Ar), 4.86–4.73 (m, 2H, 2 C*H*HPh), 4.48–4.25 (m, 5H, 4 C*H*HPh, H-3^{B}), 4.21 (d, J = 9.0 Hz, 1H, H-1^{A}), 4.18 (dd, J = 8.0, 10.1 Hz, 1H, H-2^{B}), 4.06–3.88 (m, 2H, 2 × H-6$_a^{A,B}$), 3.87–3.82 (m, 1H, OCH_{2a}), 3.72 (t, J = 9.0 Hz, 2H, H-6$_b^{A,B}$), 3.69–3.67 (m, 1H, OCH_{2b}), 3.50–3.34 (m, 5H, H-2^{A}, H-3^{A}, H-4^{B}, H-5^{A,B}), 3.17–2.98 (m, 2H, CH_2N_3), 2.08 (s, 3H, CH_3CO), 1.72–1.65 (m, 2H, $CH_2CH_2N_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.51, 167.42 (CO), 134.11–123.13 (C–Ar), 103.44 (C-1^{B}), 99.56 (C-1^{A}), 81.11, 79.15, 78.77, 78.42, 77.79, 77.23, 75.71, 75.20, 74.89, 73.76, 73.38, (4 × CH_2Ar), 72.38, 69.92 (C-4^{A}), 68.08, 68.81 (2 × C-6), 61.50 (OCH_2), 56.11 (C-2^{B}), 48.01 (CH_2N_3), 28.96 ($CH_2CH_2N_3$), 21.23 (CH_3CO). HR ESI-MS *m/z* $C_{57}H_{58}N_4O_{13}$ $[M+Na]^+$ 1029.3898; found 1029.3902.

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→6)-3-*O*-benzyl-4-*O*-(2-naphtyl) methylene-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranoside 29

A solution of **28** (483 mg, 0.48 mmol) and **10** (413 mg, 0.62 mmol) with activated molecular sieves (4 Å, 800 mg) in DCM (8 mL) was stirred for 20 min under nitrogen. TMSOTf (23 µL, 0.12 mmol) was added at – 10 °C. After the reaction mixture was stirred for 12 h at rt, TEA was added until neutral pH, the solid filter off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (8 : 2 Tol : EtOAc) to afford **29** in 72 % yield (504 mg). $[\alpha]_D^{25} = +19.9^\circ$ (c 0.12, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76–6.55 (m, 41H, H–Ar), 5.42 (d, J = 3.2 Hz, 1H, H-4^{A}), 5.34 (d, J = 8.3 Hz, 1H, H-1^{B}), 5.06 (t, J = 8.8 Hz, 1H, H-2^{C}), 4.97–4.72 (m, 5H, 5 × C*H*HPh), 4.65 (d, J = 12.5 Hz, 1H, C*H*HPh), 4.60–4.39 (m, 9H, 8 × C*H*HPh, H-1^{C}), 3.88–3.64 (m, 9H), 3.58–3.30 (m, 9H), 3.26–3.09 (m, 3H, incl. 3.10, CH_2N_3), 2.65, 2.10 (2 × s, 3H each, 2 × CH_3CO), 1.82–1.71 (m, 2H, $CH_2CH_2N_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.38, 170.37, 169.72, 169.49 (C=O), 133.59–123.10 (C–Ar), 103.54 (C-1^{A}), 101.34 (C-1^{C}), 98.56 (C-1^{B}), 82.88, 79.94, 79.07, 78.64, 78.07, 77.60, 77.23, 75.25, 74.92, 74.74, 74.10, 73.69, 73.47 (7 × CH_2Ar), 72.21, 69.78 (C-4^{A}), 68.68, 68.53, 68.04 (3 × C-6), 66.71 (OCH_2), 56.26 (C-2^{B}), 48.09 (CH_2N_3), 29.03 ($CH_2CH_2N_3$, 22.28, 22.10 (2 × CH_3CO). HR ESI-MS *m/z* $C_{86}H_{88}N_4O_{19}$ $[M+Na]^+$ 1503.5940; found 1503.5855.

3-Azidopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→6)-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranoside 30

To a solution of **29** (504 mg, 0.34 mmol) in 4 : 1 DCM : CH_3OH (12 mL), DDQ (235 mg, 1.02 mmol) was added. The reaction mixture was stirred at rt 5h (TLC, 7 : 3 cyclohexane : EtOAc), then it was diluted with DCM and

partitioned with aq $NaHCO_3$. The aqueous layer was extracted 3 times with 20 mL of DCM, then combined organic phases were dried with Na_2SO_4 and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane : EtOAc) to afford **30** as a yellow oil in 85 % yield (390 mg, 0.29 mmol). $[\alpha]_D^{25} = +2.5^\circ$ (c 0.50, $CHCl_3$).

^{1}H NMR (400 MHz, CDCl3) δ 7.76–6.55 (m, 34H, H–Ar), 5.36 (m, 2H, H-1^B, H-4^A), 5.07 (t, *J* = 8.2 Hz, 1H, H-2^C), 4.89–4.75 (m, 3H, 3 × C*H*HPh), 4.71–4.41 (m, 8 H, C*H*HPh, incl. d, 4.66, d, J = 7.9 Hz, H-1^C), 4.24–4.09 (m, 4H, H-6$_a^{A/C}$, 2 × C*H*HPh, incl. 4.12, d, *J* = 7.0 Hz, H-1^A), 4.03–3.61 (m, 10H), 3.59–3.67 (m, 7H), 3.15–3.09 (m, 2H, CH_2N_3), 2.02, 2.00 (2 × s, 3H each, 2 × CH_3CO), 1.73–1.66 (m, 2H, C$H_2CH_2N_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.40, 170.35, 169.72, 169.56 (C=O), 138.42–123.08 (C–Ar), 103.46 (C-1^A), 100.42 (C-1^C), 98.38 (C-1^B), 82.70, 78.49, 78.29, 77.91, 75.03, 74.78, 74.23, 74.17, 74.07, 73.88, 73.71, 73.57, 73.53, 72.80 (6 × CH_2Ph), 72.54, 72.26, 69.65 (C-4^A), 69.12, 68.20, 67.96 (3 × C-6), 66.76 (OCH_2), 55.72 (C-2^B), 48.07 (CH_2N_3), 29.23 (C$H_2CH_2N_3$), 20.96, 20.77 (2 × CH_3CO). HR ESI-MS *m/z* $C_{86}H_{84}N_4O_{19}$ $[M+H]^+$ 1341.5495; found 1341.5532.

3-Azidopropyl [(2-*O*-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl)-(1→6)]-{2,4,6-tri-*O*-benzoyl-*O*-[methyl 4,7,8,9-tetra-O-acetyl-5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate]-β-D-galactopyranosyl-(1→4)}-3-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranoside 31

A solution of trisaccharide **30** (390 mg, 0.29 mmol) and disaccharide donor **6** (329 mg, 0.29 mmol) with activated molecular sieves (4 Å, 700 mg) in DCM (8 mL) was stirred for 20 min under nitrogen. TMSOTf (11 µL, 0.058 mmol) was added at −10 °C. After the reaction mixture was stirred for 10h at rt, monitoring by TLC (7 : 3 Tol : acetone), TEA was added until neutral pH, the solid filter off and the solvent removed under reduced pressure. The crude was purified by flash chromatography (Tol : acetone) to afford **29** in 65 % yield (430 mg) as a foam. $[\alpha]_D^{25} = +18.2^\circ$ (c 0.15, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10–6.62 (m, 49H, H–Ar), 5.59–5.56 (m, 1 H, H-8^E), 5.41 (dd, J = 7.8, 9.2 Hz, 1H, H-2^D), 5.29–5.27 (s, 2H, H-7^E, NH), 5.18 (dd, *J* = 2.3, 9.4 Hz, 1H, H-3^D), 5.13 (d, *J* = 8.3 Hz, 1H, H-1^B), 5.02 (d, *J* = 7.8 Hz, 1H, H-1^D), 4.92–4.88 (m, 2H, H-4^A, H-4^D), 4.87–4.57 (m, 7H), 4.51–4.25 (m, 9H, incl. d, 4.46, *J* = 7.8 Hz, H-1^C, and d, 4.26, *J* = 9.0 Hz, H-1^A), 4.15–3.94 (m, 8H), 3.81–3.57 (m, 13H, incl. s, 3.74, $COOCH_3$), 3.53 (dd, *J* = 2.3, 10.8 Hz, 1H), 3.47–3.26 (m, 5H), 3.19 (t, *J* = 8.2 Hz, 1H), 3.07–3.02 (m, 2H, CH_2N_3), 2.45 (dd, *J* = 12.5, 4.4 Hz, 1H, H-3$_e^E$), 2.11, 1.96, 1.85, 1.83, 1.70 (5 × s, 3H each, 6 × CH_3CO), 1.64–1.53 (m, 3H, H-3$_a^E$, C$H_2CH_2N_3$), 1.35, 1.18 (2 × s, 3H each, 2 × CH_3CO). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.73, 170.55, 170.27, 170.21, 169.96, 169.13, 168.12, 167.77, 165.71, 165.55, 165.12 (C=O), 138.55 -122.99 (C–Ar), 103.41 (C-1^A), 101.54 (C-1^C), 101.00 (C-1^D), 98.45 (C-1^B), 96.91, 82.61, 79.17, 78.55, 78.18, 77.73, 76.82, 75.00, 74.82, 74.75, 74.62, 74.50, 74.02, 73.58, 73.48, 73.31, 72.48, 71.85, 71.80, 71.65, 70.67, 69.91, 69.37, 68.77, 68.68, 68.23, 67.93, 67.61, 66.61, 66.20, 62.10, 61.59 (4 × C-6), 56.11 (C-2^B), 53.09 (C-5^E), 48.87 ($COOCH_3$), 48.05 (CH_2N_3), 37.26 (C-3^C), 28.97 (C$H_2CH_2N_3$), 23.15, 21.43, 21.08, 20.80, 20.75, 20.71, 20.24 (7 × CH_3CO). HR ESI-MS *m/z* $C_{122}H_{129}N_3O_{39}$ $[M + Na]^+$ 2310.8162; found 2310.8175.

Procedure for final deprotection of oligosaccharides and 19, 23 and 31

A mixture of protected pentasaccharide **19**, **23** or **31** (0.1 mmol) and LiI (3 mmol) in pyridine (5 mL) was heated for 24h at 120 °C. The reaction mixture was concentrated under vacuum, and the residue was purified by silica gel column chromatography (gradient 2 % MeOH in DCM) to afford the demethylated product. This material was dissolved in ethanol (4 mL), and ethylenediamine (400 mL) was added. After being stirred for 16 h at 90 °C, the reaction mixture was then concentrated in vacuo, and the residue was coevaporated from toluene (2 × 10 mL) and EtOH (2 × 5 mL). The crude mixture was re-dissolved in pyridine (5 mL), and acetic anhydride (5 mL) was added. After being stirred for 16 h at room temperature, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in MeOH and MeONa was added until pH = 13. After 48 h the reaction was neutralized and the solvent removed under vacuum. The residue was dissolved in

MeOH and Pd/C (1 : 1 w/w in respect to the sugar) was added. The reaction mixture was stirred under pressure of H_2 (3 bar) for 72 h. Then, the catalyst was filtered off and the filtrate concentrated under reduced pressure. The reaction mixture was purified by G-10 size-exclusion column chromatography using water for elution. Fractions containing the sugar were quantified by sialic acid assay and freeze-dried to afford the deprotected oligosaccharide **1**–**3** as an amorphous powder (31–55 % yield).

3-Aminoopropyl 3-*O*-(5-*N*-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosyl)-β-D-galactopyranosyl]-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranoside 1

33 % yield. $[\alpha]_D^{25} = +46.0^{\circ}$ (c 0.03, H_2O). HR ESI-MS *m/z* $C_{40}H_{69}N_3O_9$ $[M+H]^+$ 1056.3971; found 1056.3966.

3-*Aminoopropyl* 3-*O*-(5-*N-acetamido*-3,5-*dideoxy*-D-*glycero*-α-D-*galacto-non*-2-*ulopyranosyl)*-β-D-*galactopyranosyl*)-(1→4)-*O*-[(β-D-*galactopyranosyl)*-(1→4)-*O*-(β-D-*glucopyranosyl*)-(1→6)]-*O*-2-*acetamido*-2-*deoxy*-β-D-*glucopyranoside 2*
42 % yield. $[\alpha]_D^{25} = -32.2^{\circ}$ (c 0.06, H_2O). HR ESI-MS *m/z* $C_{40}H_{69}N_3O_9$ $[M+Na]^+$ 1078.3810; found 1078.3810.

3-*Aminoopropyl* 3-*O*-(5-N-*acetamido*-3,5-*dideoxy*-D-*glycero*-α-D-*galacto-non*-2-*ulopyranosyl*)-β-D-*galactopyranosyl*]-(1→4)-*O*-[β-D-*glucopyranosyl*-(1→6)]-*O*-2-*acetamido*-2-*deoxy*-β-D-*glucopyranosyl*-(1→3)-*O*-β-D-*galactopyranoside 3*
55 % yield $[\alpha]_D^{25} = -17.2^{\circ}$ (c 0.14, H_2O). HR ESI-MS *m/z* $C_{40}H_{69}N_3O_9$ $[M+H]^+$ 1056.3969; found 1056.3966.

Conjugation to CRM_{197}

A solution of di-N-hydroxysuccinimidyl adipate (10 eq) and triethylamine (0.2 eq) in DMSO was added to the pentasaccharide **1–3**. The reaction was stirred for 3 h, then the product was precipitate at 0 °C by adding ethyl acetate (9 volumes). The solid was washed 10 times with ethyl acetate (2 volumes each) and lyophilized. The activated sugar was conjugated to CRM_{197} in sodium phosphate 100 mM at a protein concentration of 5 mg/mL, using the mol saccharide/mol protein ratio reported in Table 2.

After incubating overnight, the glycoconjugates **35–37** were purified by dialysis against 10 mM sodium phosphate buffer pH 7,2 (×10 washings) in 30 kDa Vivaspin Turbo (Sartorius) centrifugal concentrators and reconstituted in the same buffer.

MALDI-TOF mass spectra of CRM_{197} and glycoconjugate **35–37** were recorded by an UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics) in linear mode and with positive ion detection. The samples for analysis were prepared by mixing 2.5 µL of product and 2.5 µL of super DHB or sinapic acid matrix. 2.5 µL of each mixture were deposited on samples plate, dried at room temperature for 10 min and analyzed at the spectrometer.

SDS-PAGE was performed on 4–12 % pre-casted polyacrylamide gel (NuPAGE®Invitrogen) using MOPS 1x as running buffer (NuPAGE®Invitrogen). 5 µg of protein were loaded for each sample. After electrophoretic running with a voltage of 150 V for about 45 min, the gel was stained with blue coomassie.

ELISA analysis

Microtiter plates (96 wells, NUNC, Maxisorp) were coated with 100 µL of 1 µg/mL (saccharide concentration) of CRM_{197} conjugates or 20 µg/mL of CRM_{197} in PBS pH 7.4. Plates were incubated overnight at 2–8 °C, washed

three times with PBST (0.05 % Tween-20 in PBS pH 7.4) and saturated with 250 µL/well of PBST-B (2 % Bovine Serum Albumin-BSA in PBST) for 90 min at 37 °C. Two-fold serial dilutions of the serum (pool of sera from mice immunized with PSIII conjugated to a pilus protein) in PBST-B were added to each well and tested in duplicate. Plates were then incubated at 37 °C for 1 h, washed with PBST, and then incubated for 90 min at 37 °C with anti-mouse IgG-alkaline phosphatase (Sigma) diluted 1 : 2000. After washing, the plates were developed with a 4 mg/mL solution of *p*-Nitrophenyl Phosphate (pNPP) in 1 M diethanolamine (DEA) pH 9.8, at room temperature for 30 min. After blocking with 7 % EDTA, the absorbance was measured using a SPECTRAmax plate reader with wavelength set at 405 nm. IgG concentrations were expressed as the reciprocal serum dilution giving OD 1.0.

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Conflict of interest: All authors are GSK employees. FC, IM, FB and RA are owners of a patent on GBS conjugates.

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