Synthesis and applications of cell wall glycopolymer fragments from Staphilococci and Enterococci

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Chapter 6

Summary and future prospects

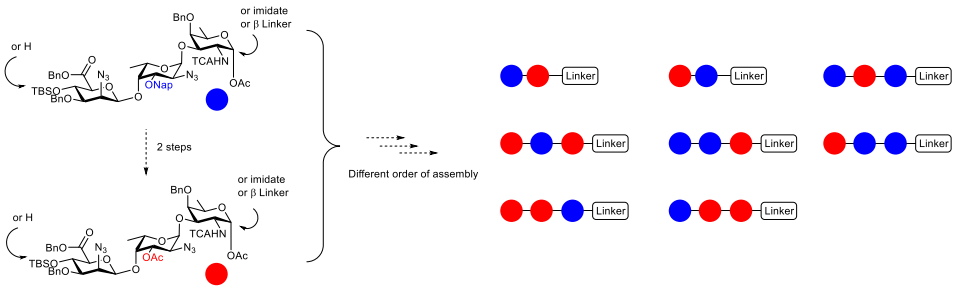
This thesis deals with the synthesis and antibody-recognition evaluation of well-defined fragments of carbohydrate-based cell-wall components from staphylococci and enterococci. The availability of libraries, comprising similar yet minutely diverse molecules, allows to evaluate the impact of all structural elements involved in protein-ligand interactions.[1] In the context of active and passive immunization as alternative strategies against multi-drug resistant staphylococci and enterococci infections,[2] structure-immunogenicity relationship studies can provide insights for rationalized optimization of glycoconjugate based vaccines or antibody engineering. Therefore, alongside advanced and efficient synthetic strategies, methodologies for antigenic evaluation need to be properly chosen or developed.

In **chapter 1** an overview is given on the synthetic efforts for delivering well-defined fragments of different carbohydrate-based oligomers belonging to *S. aureus* species.[3] The review is divided in three major parts as glycopolymers can be found at three different bacterial surface levels: the biofilm (PNAG and dPNAG), capsular polysaccharides (CP5, CP8 and Strain M) and teichoic acids (WTA and LTA).[4] The well-defined synthetic structures have been used for a variety of applications, such as the evaluation of the antigenic properties of PNAG *vs* dPNAG or the detection of antibodies in human sera against different glycotypes of RboP-based WTA.[5]

The most abundant *S. aureus* clinical isolates are serotype 5 and 8, of which the CPs have been already employed in the past for vaccine development.[6] Although the preclinical trial results appeared to be promising, the development of glycoconjugate based vaccines has been discontinued.[7] In the last decade, efforts have been focused on the synthesis of well-defined fragments, delivering different strategies to generate the trisaccharide repeating unit of both polysaccharides.[8] To date, only one synthesis has been reported on a protected CP8-based hexasaccharide, but deprotection of this fragment failed.[9] In **Chapter 2** the development of an efficient synthetic route is reported to deliver fragments of the capsular polysaccharide of *S. aureus* type 5, with and without the *O*-acetyl ester in the *N*-acetyl-L-fucose moiety. The strategy relied on a key protected trisaccharide building block that allows to selectively and easily introduce the acetyl substituent as well as elongation to the corresponding hexamer following a [3+3] coupling approach. To achieve the desired trisaccharide building block, both methyl and benzyl mannuronate donors were explored for the [1+2] glycosylation. No differences were observed during the coupling steps, both for the assembly of the trisaccharide unit as well as the elongation to the hexasaccharide, but the benzyl group outperformed its methyl counterpart at the deprotection stage. The fragments thus obtained, two trimers and two hexamers, will be employed for accessing the structural requirements for recognition by antibodies raised against the native CP5 and in particular to evaluate the role of acetyl substituent in antigenicity. The library can be further expanded by generating longer fragments, different acetyl substitution patterns or related zwitterionic compounds, in which part of the amino groups do not carry the acetyl functionality. The *O*-acetyl ester can be introduced in the trisaccharide building block before elongation, allowing the [3n+3] coupling using trisaccharides bearing either the

nathyl protecting group or the *O*-acetyl ester to give access to a library with different *O*-acetylation patterns (Figure 1).

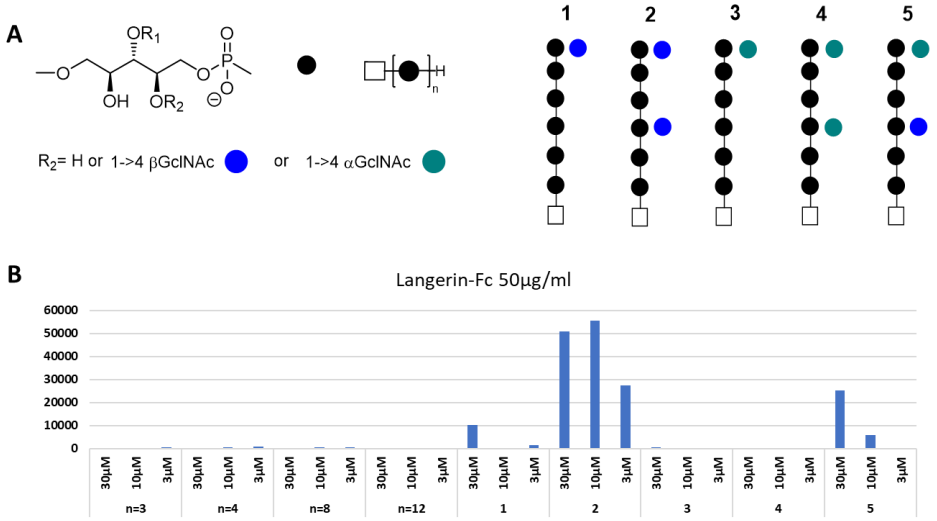
Figure 1: *Synthetic strategy to deliver hexamers and nonamers with different acetyl ester substitution patterns*



Chapter 3 describes the development of a TA-microarray to probe the binding of monoclonal and polyclonal antibodies from different sources.[10] Since both GroP- and RboP-based well defined fragments from the in-house library are equipped with an aminohexanol linker, epoxide functionalized microarray glass slides were used to immobilize the compounds. In order to assess the feasibility of the technology, at first a commercially available monoclonal antibody generated against native *S. epidermidis* LTA was used.[11] It was revealed that the antibody was specifically recognizing the GroP backbone as signals for short unsubstituted GroP-fragments were observed. Of note, the measured fluorescence was not always linearly proportional to the different dilutions of the mAb or the concentration of the printed TAs, and the developed assay was thus only used further for qualitative binding screening. In the case of RboP-based fragments, the assessment was performed using generated monoclonal antibodies from B-cells of patients infected by *S. aureus*.[12] Next, the TA-microarray was applied to reveal the preferential binding of more complex biological samples, such as rabbit sera raised against native LTA from *E. faecalis* or different synthetic TA-conjugates.[13] Generally, IgG antibodies were directed towards GroP-based fragments bearing a glycosyl substituent and the antibodies raised against synthetic TA-conjugates were shown to be specific for the glucosyl appendages. This technology was also used to detect pre-existing antibodies towards TAs in pre-bleed sera, highlighting the possibility to introduce this type of assay in immunization protocol workflows. Finally, screening of serum from healthy donors was performed, revealing strong binding against the different RboP-glycotypes. The arrays can be used to probe binding to many other biomolecules such as biosynthesis enzymes and lectins. A preliminary study was conducted to probe the binding of langerin (CD207), a C-type lectin receptor (CLRs) that is found on Langerhans cells (LC).[14] In particular, langerin is able to recognize the β-glucosamine and glucose residues in a calcium-dependent manner and thus hypothesized to be involved in *S. aureus* sensing via binding to the different β-GlcNAc substituted RboP WTA.[15] In Figure 2A an overview is given on the fully synthetic RboP-based fragments immobilized on epoxide-functionalized glass slide as described in Chapter 3. Two different protein derivatives

were probed, where the recombinant construct of the extracellular carbohydrate domain (ECD) of human langerin was either labelled with the FITC fluorophore[16] or the Fc of a IgG1 human antibody.[17] In the first case no fluorescent signal was observed at different protein concentrations (25, 50 or 100 µg/ml). Using the Fc-labelled ECD, interaction with RboP hexamers bearing the 1,4-β-GlcNAc substituent (**1**, **2**, **5**, Figure 2B) was detected. Since the Fc-derivative differs from the FITC-labelled ECD by presenting the protein domain as a dimer instead of a monomer, the success of the latter results might be attributed to the establishment of multivalent interactions. This would also explain the higher signal intensity for the double substituted RboP hexamer **2** compared to the monosubstituted **1** and **5**. These results are in line with the findings of Hendriks *et al.*, who showed that human langerin CD207 is able to recognize regioisomeric β-GlcNAc substituents on a RboP backbone.[18] This latter study was performed using the FITC-labelled ECD in combination with an enzymatically modified RboP hexamer and dodecamer for which a higher degree of glycosyl substitution was obtained as compared to the study described above.

***Figure 2**: A) Overview of RboP based synthetic fragments; B) Array results showing binding between Langerin and 1→4βGlcNAc substituted RboP fragments*



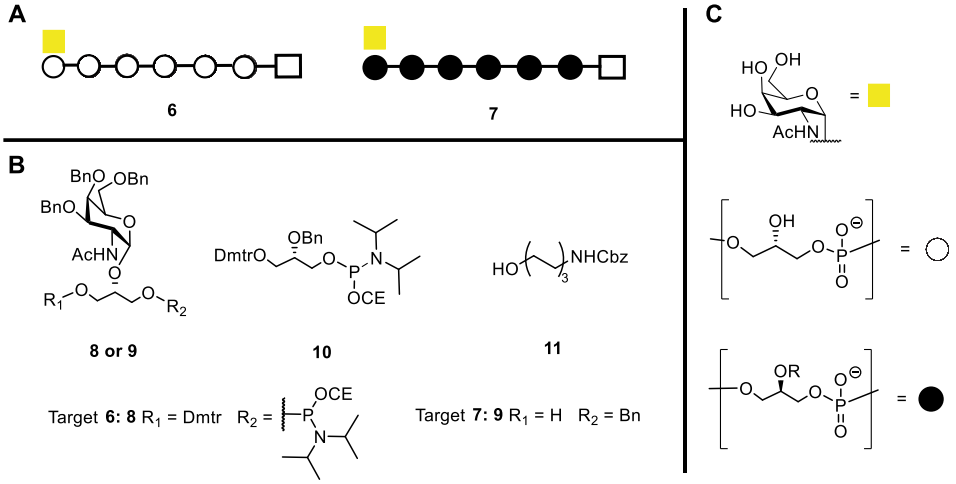
In **Chapter 4**, the synthesis of a new set of glucosylated GroP-LTA-fragments is reported.[19] The compounds differ from the previous generated library as the linker was attached to the side of the naturally occurring lipid anchor. Thus, the fragments feature a *sn*-Gro-1-P backbone with an α-glucosyl substituent at different positions along the chain. In order to improve the synthesis of the key glucosyl-glycerol phosphoramidite building block, an additive-mediated glycosylation was employed on a perbenzylated glucosyl imidate donor.[20] It was observed that the glycosylation stereochemistry outcome strongly depends on the stereochemistry and protecting groups of the glycerol acceptor. The new set of GroP TAs was evaluated alongside an unsubstituted *sn*-Gro-3-P

hexamer and a glucosylated *sn*-Gro-3-P one (**WH7**) using the microarray technology as described in Chapter 3. Two rabbit sera were probed, one raised against native LTA from *E. faecalis* and one against the **WH7**-BSA conjugate. As seen in Chapter 3, the IgG antibodies specifically recognized the fragments bearing glycosyl substituents. However, for the serum against the native LTA high IgG binding was observed for the *sn*-1-GroP fragments while no signals were detected for the two *sn*-3-GroP hexamers. The reverse situation was observed for the serum raised against **WH7**-BSA, indicating that the stereochemistry of the GroP backbone influences TA-antibody recognition.

As shown in Chapter 3 and 4, microarray technology can be used to qualitatively assess the binding of anti-TA antibodies from different sources. In order to further evaluate the interaction at the molecular level, this tool can be combined with other qualitative and quantitative techniques. **Chapter 5** deals with the epitope mapping of a monoclonal antibody (WH7.01 mAb) that was raised against **WH7**-BSA, a fully synthetic GroP-based TA-conjugate.[21] After generation through hybridoma technology,[22] the WH7.01 mAb was probed by ELISA, showing binding of the **WH7** antigen and LTA from *S. aureus*, and by an OPA assay showing higher opsonic activity against *S. aureus* then to *E. faecalis*. Next antigen-antibody recognition was examined using different well-defined GroP fragments. A first screening using the microarray revealed the GroP backbone as the main recognized structural element. It became prominent by ELISA that the length, the glucosyl substituent and the glycerol stereochemistry also had an impact on the antibody interaction. Subsequently, the binding was quantitatively evaluated via SPR analysis using the **WH7** antigen, a non-substituted hexamer and a non-substituted pentamer. Based on this study it was concluded that the higher affinity of WH7.01 mAb towards the glucosylated hexamer was a result of increasing K_{on} and decreasing K_{off} values. Finally, STD-NMR spectroscopy was used to identify the structural antigenic elements involved in the binding of the generated mAb. STD effects were clearly observed for proton signals of the GroP backbone, while no signals were detected from the glucosyl moiety. These results suggest that the glucosyl moiety plays an indirect, yet favourable, role in the interaction, probably by influencing the conformational freedom of the GroP residues to provide a better binder. With more TA-based fragments in hand, a library of different mAbs can be generated. In particular, the workflow here presented can be used in the future to link the structural elements required for antibody interaction with the activity of the antibodies against a target bacterium. The library of GroP-based TA fragments can be further expanded with D-alanine or other glycosyl substituents. For instance, the WTA structure from *S. aureus* ST395 lineage is characterized by the presence of an α-GalNAc substituent on the C-2 position of an *sn*-3-GroP backbone as based on biosynthesis pathway studies (see Chapter 1).[23] Recently it was shown that macrophage galactose-type lectin (MGL), a C-type lectin receptor found abundantly on dermal dendritic cells and dermal macrophages, is able to recognized WTA from *S. aureus* ST395.[24] The results were carried out using wild type and isogenic mutant strains, demonstrating that the presence of the α-GalNAc is crucial for binding by MGL. The availability of well-defined fragments can help to identify the structural requirements for the interaction with different biomolecules such as MGL as well as antibodies from different sources. As described in Chapter 4, not only the carbohydrate appendage but

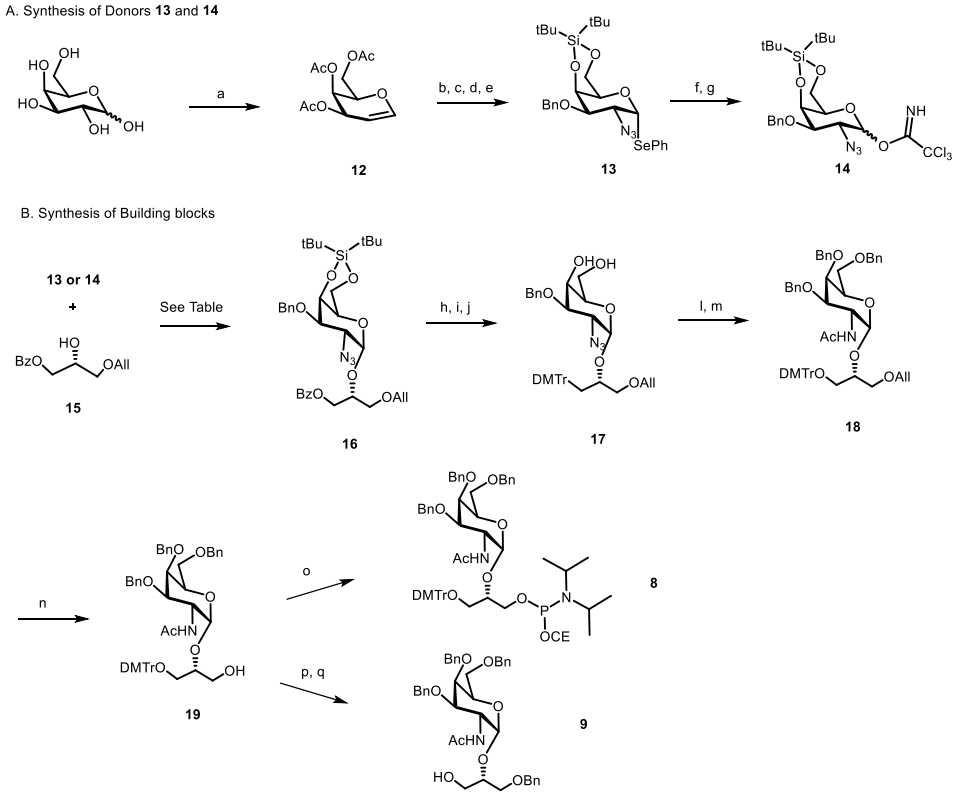
also the stereochemistry of the GroP backbone is pivotal for antibody binding. In Figure 3 a strategy is shown to generate well-defined structures in both the *sn*-1- and the *sn*-3-GroP series. The synthetic approach is based on the use of the same phosphoramidate strategy (Chapter 4) with opposite starting points: either from the linker side or from the terminal GroP residue **9**, to generate the *sn*-1 or *sn*-3 GroP residues, respectively.

Figure 3: *A) Target compounds **6** and **7**; B) Building blocks for the synthesis of the target compounds; C) Structure - symbols correlation of compounds in A*



Scheme 1 shows the synthetic pathway towards building blocks **8** and **9**, for which the key glycosylation step was accomplished using either galactosyl donor **13** or **14** bearing a 4-6-*O*-silylidine protecting group, which ensures the α-selectivity in the glycosylation reaction.[25] The synthesis of donor **13** commenced with acetylation of D-galactose, followed by bromination and zinc-mediated reductive elimination affording galactal intermediate **12** in 65% over three steps.[26] A regio- and stereo-selective azidophenylselenation (APS) was performed using $TMSN_3$, $(PhSe)_2$ and BAIB as described in Chapter 2. After removal of the acetyl groups under Zémplen conditions, a silylidene-ketal was installed at the 4,6-O-position in good yields, using di-tert-butylsilyl bis(trifluoromethanesulfonate) ($DTBS(OTf)_2$) and pyridine.[27] Finally, benzylation of the remaining 3-OH gave fully protected donor **13** in high yield. Subsequently, imidate donor **14** was afforded in two steps starting from compound **13**. Synthesis of compound **15** was achieved using the same strategy and conditions as described in Chapter 4.

Scheme 1: *Synthesis of GalNAc-GroP building blocks*



Reagents and conditions*: a) (i) Ac_2O, Pyridine, DMAP (cat.); (ii) HBr 33% in AcOH, DCM, 0° C to r.t.; (iii) Zn, NH_4Cl, EtOAc, 70° C, 72% (over three steps); b) $(PhSe)_2$, Me_3SiN_3, DCM, -8°C (±2°C), 65%; c) Na(s), MeOH, quant.; d) DTBS(OTf)$_2$, Pyridine, DMF, 82%; e) BnBr, NaH, DMF, 0° C to r.t., 88%; f) NIS, THF, H_2O, quant.; g) Cl_3CCN, K_2CO_3, DCM, 0°C to r.t., 92%; h) NaOMe, MeOH, 0°C to r.t., 98%; i) DMTrCl, DMAP, DCM, 85%; j) TBAF, THF, 92%; l) BnBr, NaH, DMF, 0° C to r.t., 97%; m) (i) PPh_3, THF, 40° C (3h), H_2O, 60° C (o.n.); (ii) AcCl, Pyridine, 88% (over two steps); n) (i) Ir(COD)$(PPh_2Me)_2PF_6$, H_2, THF; (ii) $NaHCO_3$(aq), I_2, THF, 82%; o) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, TEA, DCM, 88%; p) BnBr, NaH, DMF, 0° C to r.t., 97%; q) TCA, CH_2Cl_2, quant.*

With donor **13** and acceptor **15** in hand, two conditions were explored for the glycosylation reaction (Table 1).[28] Coupling under pre-activation conditions using Ph_2SO and Tf_2O in DCM at -78°C affords compound **16** in 71% and the yield was consistent after scale up from 0.5 mmol to 5 mmol (entry 1 and 2). Using NIS as activating agent and TMSOTf as promoter at 0°C, similar results were obtained when the reaction was performed at 0.5 mmol but a drop in yield was observed during scale up (entry 3 and 4). Coupling between **14** and **15** was performed using TMSOTf as activating agent in DCM, affording target compound **16** in excellent yield. This procedure was chosen for further scale up and this proved to be reliable even at 20 mmol scale.

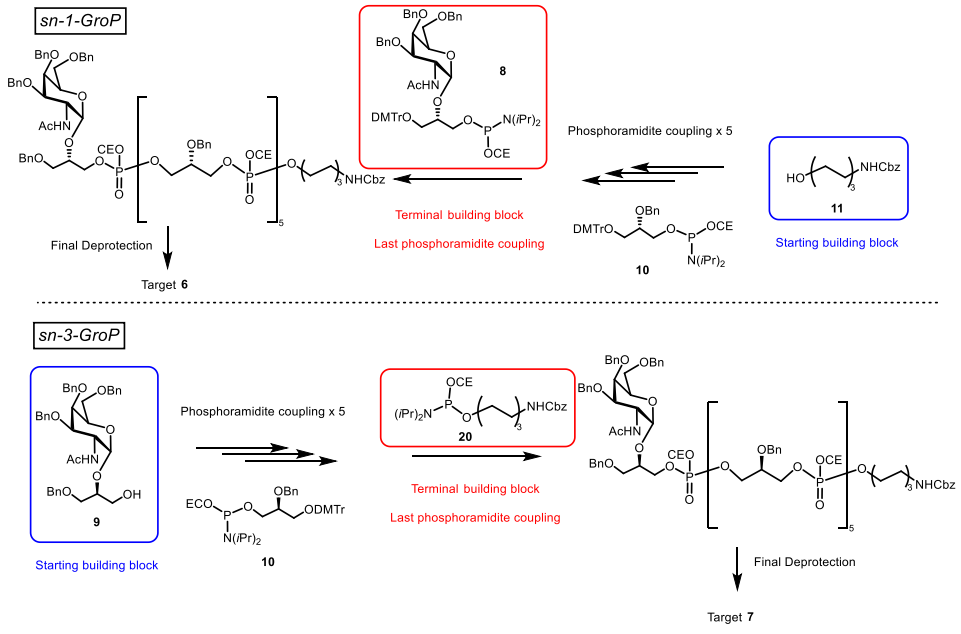
Table 1: *Reagents and conditions for coupling between donor* ***13*** *or* ***14*** *and acceptor* ***15***

Entry	Donor	Scale (mmol)	Conditions*	Temp	Time	Yield
1	**13**	0,5	A	-78°C to -30°C	2 h	71%
2	**13**	5	A	-78°C to -30°C	2 h	64%
3	**13**	0,5	B	-50°C	1.5 h	69%
4	**13**	5	B	-50°C	2 h	57%
5	**14**	0,5	C	-78°C to -10°C.	1 h	92%
6	**14**	5	C	-78°C to -10°C.	1 h	90%
7	**14**	20	C	-78°C to -10°C.	1.5 h	88%

* A: Ph_2SO, Tf_2O, TTBP, DCM; B: NIS, TMSOTf, DCM; C: TMSOTf, DCM

With compound **16** in hand, protecting group manipulation commenced to afford final building blocks **8** and **9**. At first the benzoyl ester was removed under Zémplen conditions after which the acid labile DMT group was installed. Using TBAF the intermediate **17** was obtained to subsequently introduce two benzyl groups at the galactosyl 4- and 6-*O* positions. The azide moiety was reduced under Staudinger conditions and after acetylation intermediate **18** was obtained in 88% yield over two steps. Finally, a two-step procedure was followed as described previously to afford alcohol intermediate **19**, from which both building blocks **8** and **9** could be obtained. Scheme 2 shows how the final targets were accomplished, using the same phosphoramidite coupling approach and deprotection sequences as described in Chapter 4.

Scheme 2*: Synthetic strategy to deliver GalNAc substituted GroP fragments.*



Overall, this thesis has provided a toolbox of synthetic methods and evaluation techniques to assemble and evaluate structures mimicking the CP5 from *S. aureus* as well as glycerol phosphate based teichoic acids. The generated libraries, that can be readily extended in the future, contain compounds that differ in very small structural features to allow the detailed characterization of the molecular elements recognized by immune system proteins and defining the antigenic epitopes that can be recognized. Moreover, from the results presented in chapter 5, a workflow is presented that can be used in the future to build a library of monoclonal antibodies generated with synthetic structures. This work can be used in the future to link the recognized epitope to the activities of these monoclonals.

EXPERIMENTAL

Microarray binding assay with Fc-labelled ECD

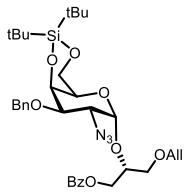
The amino-spacer equipped GTA-fragments were dissolved in spotting buffer (Nexterion Spot, Schott Nexterion) with 10% DMSO in 384-wells V-bottom plates (Genetix, New Milton, UK). The GTA-fragments were printed in three final concentrations (30μM, 10μM and 3μM) in triplicate on epoxysilane-coated glass slides (Slide E, Schott, Nexterion) by contact printing using the Omnigrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI) equipped with SMP3 pins with uptake channels that deposit 0.7 nl at each contact. The slides were rested in a high humidity chamber for 18 hours and were stored in the dark until used. The slides were washed with PBS (3x) and subsequently all unreacted sites on the arrays were blocked by shaking the slides for 1 hour with ethanolamine (0.25 ml, 0.05M in PBS containing 20 mg/ml of BSA). The slides were flushed with PBS and PBS containing 5% of Tween® 20 subsequently and finally each array was rinsed with PBS containing 1% of Tween® 20. After removal of the PBS containing 1% of Tween® 20, the arrays were shaken with 0.25 ml of Fc-labelled ECD diluted with TSM buffer containing 1% of Tween® 20 and 10 mg/ml of BSA for 60 minutes. Three different concentrations were used 25, 50 or 100 μg/ml. The slides were flushed with TSM and TSM containing 5% of Tween® 20 subsequently and finally rinsed with TSM containing 1% of Tween® 20 subsequently. After removal of the TSM containing 1% of Tween® 20, the arrays were shaken with 0.25 ml of goat anti-human IgG secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, A-11013) at 0.5 μg/ml concentration, diluted with TSM containing 1% of Tween® 20 and 10 mg/ml of BSA for 30 minutes in the dark. The slides were flushed with PBS, PBS containing 5% of Tween® 20 and MilliQ subsequently. The slides were dried by centrifugation and fluorescent measurements were performed using Agilent G2565BA microarray scanner system (Agilent technologies) with 10 μm resolution, using two lasers (532 nm and 635 nm). Data and image analyses were performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, USA) as described previously.[8] Fluorescence intensities were quantified and corrected for background/non-specific protein adhesion by subtracting the fluorescence at blank spots, where only spotting buffer was printed without RTA fragment. The average of the triplicate spots was calculated and visualized in bar graphs using Microsoft Excel.

General

All chemicals (Acros, Fluka, Merck, Sigma-Aldrich, etc.) were used as received and reactions were carried out dry, under an argon atmosphere, at ambient temperature, unless stated otherwise. Column chromatography was performed on Screening Devices silica gel 60 (0.040-0.063 mm). TLC analysis was conducted on HPTLC aluminium sheets (Merck, silica gel 60, F245). Compounds were visualized by UV absorption (245 nm), by spraying with 20% H_2SO_4 in ethanol or with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 25 g/l and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ 10 g/l, in 10% aqueous H_2SO_4 or with a solution of $KMnO_4$ (2%) and K_2CO_3 (1%) in water followed by charring at +/- 140 °C. Optical rotation measurements ($[\alpha]_D^{20}$) were performed on a Propol automated polarimeter (Sodium D-line, λ = 589 nm) with a concentration of 10 mg/ml (c = 1), unless stated otherwise and the reported value was calculated as the mean of 10 measurements. Infrared spectra were recorded on a Shimadzu FT-IR 8300. ^1H, ^{13}C and ^{31}P NMR spectra were recorded with a Bruker AV 400 (400, 101 and 162 MHz respectively), a Bruker AV 500 (500, 125 and 202 MHz respectively) or a Bruker DMX 850 (850, 214 and 344 MHz respectively). NMR spectra were recorded in $CDCl_3$ with chemical shift (δ) relative to tetramethylsilane for both ^1H and ^{13}C. When D_2O or CD_3CN were used, ^1H-NMR were recorded with chemical shift (δ) relative to the proton of residual solvent (4.75 ppm and 1.94 ppm respectevely). ^{13}C-NMR spectra were recorded with chemical shift (δ) relative to TMS (external standard) in case of D_2O and 1.32 ppm as residual solvent in CD_3CN.The ^{31}P- NMR spectra were recorded with chemical shift (δ) relative to H_3PO_4. (external standard). High resolution mass spectra were recorded by direct injection (2 µl of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1 % formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylphthalate (*m/z* = 391.28428) as a lock mass. High resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

Synthesis of building blocks **8** and **9**

2-*O*-(2-azido-4,6-*O*-silylidene-3-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-allyl-1-*O*-benzoyl-*sn*-glycerol (16)



Method A: Donor **13** (5 mmol), TTBP (10 mmol, 2 eq) and Ph_2SO (12.5 mmol, 2.5 eq) were coevaporated with toluene three times and subsequently dissolved in dry DCM (50 ml, 0.1 M). Flame-dried 3Å molecular sieves were added and the mixture was stirred at room temperature for 30 min. After cooling to -80°C, Tf_2O (12.5 mmol, 2.5 eq) was slowly added and the mixture was allowed to warm to -60°C. After re-cooling to -80°C, a solution of acceptor **15** (10 mmol, 2 eq) in dry DCM (10 ml)

was slowly added into the reaction mixture. The reaction was allowed to warm up to -30°C and after 2 h TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of donor **13**. The reaction was quenched using 7 ml NEt_3 and diluted with DCM. The solution was filtered over a bed of Celite®, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude was then subjected to column chromatography (Pentane:EtOAc; 95:5 → 9:1→8:2), yielding compound **16** in 64% yield (3.2 mmol).

Method B: Donor **13** (5 mmol) and acceptor **15** (10 mmol, 2 eq) were coevaporated three times using toluene and dissolved in dry DCM (50 ml, 0.1 M). After addition of flame-dried 3Å molecular sieves, the solution was cooled to -80°C, NIS (6 mmol, 1.2 mmol) and TMSOTf (0.5 mmol, 0.1eq) were added and the mixture was stirred at -50°C until TLC (Pentane:EtOAc, 9:1) showed full conversion of donor **13**. The reaction was quenched by addition of 7 ml NEt_3 and diluted with DCM, filtered over a pad of Celite®, washed with sat. aq. $Na_2S_2O_3$, $NaHCO_3$, brine and dried over $MgSO_4$. The desiccant was filtered off and the crude was concentrated in vacuo. The crude was then subjected to column chromatography (Pentane:EtOAc; 95:5 → 9:1→8:2), yielding compound **16** in 57% yield (1.6 mmol).

Method C: Imidate donor **14** (5 mmol) and acceptor **15** (6 mmol, 1.2 eq) were coevaporated with toluene three times and dissolved in dry DCM (50 ml, 0.1M). After addition of flame-dried 3Å molecular sieves, the solution was cooled to -80°C and TBSOTf (1.5 mmol, 1.3 eq) was added slowly. The temperature was allowed to warm up to -10°C and after 1 h TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of donor **14**. The reaction was quenched by addition of 7 ml NEt_3 and diluted with DCM, filtered over a pad of Celite®, washed with sat. aq. $NaHCO_3$, brine and dried over $MgSO_4$. The desiccant was filtered off and the crude was concentrated in vacuo. The crude was then subjected to column chromatography (Pentane:EtOAc; 95:5 → 9:1→8:2), yielding compound **16** in 90% yield (4.5 mmol).

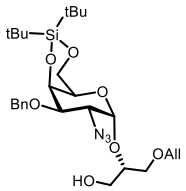
TLC analysis: R_f = 0.38 (Pentane:EtOAc; 95:5)

^{1}H NMR (400 MHz, $CDCl_3$), δ: 8.00-7.87 (2H, H_{arom}, m), 7.49-7.45 (1H, H_{arom}, m), 7.38-7.30 (4H, H_{arom}, m), 7.27-7.23 (2H, H_{arom}, m), 7.22-7.18 (1H, H_{arom}, m), 5.79 – 5.69 (1H, H_{allyl}, m), 5.17 – 5.04 (3H, 2 x H_{allyl}, H_1, m), 4.66 (1H, CHH_{Bn}, *J*=11.5 Hz, d), 4.56 (1H, CHH_{Bn}, *J*=11.5 Hz, d), 4.53-4.50 (1H, H_4, m), 4.42 (1H, CH$H_{glycerol}$, *J*=11.9 Hz, *J*=4.5 Hz, dd), 4.32 (1H, CH$H_{glycerol}$, *J*=11.9 Hz, *J*=4.5 Hz, dd), 4.16-4.02 (3H, 2xH_6, $H_{glycerol}$, m), 3.89-3.80 (4H, 2x CH$H_{glycerol}$, H_5, H_3, m), 3.69 (1H, H_2, *J*=3.6 Hz, *J*=10.6 Hz, dd), 3.54-3.49 (2H, CH_{2_Allyl}, m), 0.98 (9H, 3xCH_{3_tBu}, s), 0.95 (9H, 3xCH_{3_tBu}, s)

^{13}C NMR (101 MHz, $CDCl_3$) δ: 166.3 (C_q), 137.9 (C_q), 134.3x2 (CH_{allyl}, CH_{arom}), 129.9 (C_q), 129.7, 128.55, 128.52, 128.49, 127.95, 127.89 (CH_{arom}), 117.3 (CH_{2allyl}), 98.4 (C_1), 75.3 (C_3), 75.2 ($CH_{glycerol}$), 72.3(CH_{2_allyl}), 70.4 (CH_{2Bn}), 70.2 ($CH_{2\_glycerol}$), 69.9 (C_4), 67.7 (C_5), 67.3 (C_6), 63.91 ($C_{2\_glycerol}$), 58.3 (C_2), 27.7, 27.4 (CH_{3_tBu}), 23.5, 20.8 (C_{q_tBu}).

HRMS: calcd for $C_{34}H_{47}N_3O_8Si$ 654.3205, found 654.3200.

2-O-(2-azido-4,6-O-silylidene-3-O-benzyl-2-deoxy-α-d-galactopyranosyl)-3-O-allyl-sn-glycerol (21)



A chip of Na metal was added to a stirring solution of **16** (3.18 mmol, 1.0 eq) in dry MeOH (32 ml, 0.1 M). The reaction was left to stir at room temperature until TLC analysis (Pentane:EtOAc, 9:1) showed complete conversion of the starting material. The reaction mixture was neutralized by addition of Amberlite IR-120 (H^+ form), filtered and concentrated *in vacuo*. The title compound **21** was obtained after column chromatography (Pentane:EtOAc; 9:1→8:2→7:3) as colorless oil in 95% yield (3.0 mmol).

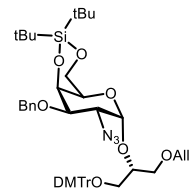
TLC analysis: R_f = 0.34 (Pentane:EtOAc; 8:2)

^{1}H NMR (400 MHz, CD_3Cl), δ: 7.46 – 7.27 (5H, CH_{arom}, m), 5.89-5.76 (1H, H_{allyl}, m), 5.26 – 5.14 (2H, 2xH_{allyl}, m), 5.07 (1H, H_1, *J* = 3.6 Hz, d), 4.76 (1H, CH*H*$_{Bn}$, *J*=11.5 Hz, d), 4.67 (1H, CH*H*$_{Bn}$, d), 4.58 – 4.55(1H, H_4, m), 4.22 (1H, H_6, *J*=12.6, Hz, *J*=2.2 Hz, dd), 4.12 (1H, H_6, *J*=1.7 Hz, dd), 4.01-3.83 (6H, H_2, CH_{2_Allyl}, H_5, H_3, $CH_{glycerol}$, m), 3.78-3.70 (1H, CH*H*$_{glycerol}$, m), 3.67-3.58 (1H, CH*H*$_{glycerol}$, m), 3.53-3.43 (2H, $CH_{2\_glycerol}$), 2.78-2.67 (1H, OH, bs), 1.06 (9H, 3xCH_{3_tBu}, s), 1.02 (9H, 3xCH_{3_tBu}, s).

^{13}C NMR (101 MHz, CD_3Cl) δ: 138.0 (C_q), 134.5 (CH_{allyl}), 128.7, 128.0 x2 (CH_{arom}),117.4 (CH_{2Allyl}), 98.7 (C_1), 79.1 (C_3), 76.9 ($CH_{glycerol}$), 72.3 (CH_{2_Allyl}), 70.6 (CH_{2_Bn}), 70.2 ($CH_{2\_glycerol}$), 69.7 (C_4), 67.8 (C_5), 67.4 (C_6), 62.8 ($CH_{2\_glycerol}$), 59.5 (C_2), 27.8, 27.5 (CH_{3_tBu}), 23.6, 20.9 (C_{q_tBu}).

HRMS: calcd for $C_{27}H_{43}N_3O_7Si$ + Na^+ 572.2763, found 572.2761.

2-*O*-(2-azido-4,6-*O*-silylidene-3-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-allyl-1-4,4'-dimethoxytrityl-*sn*-glycerol (22)



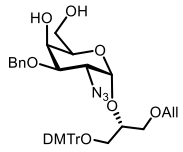
Alcohol **21** (2.74 mmol, 1.0 eq) was dissolved in dry DCM (9 ml, 0.3 M) and the reaction cooled to 0°C. Under inert atmosphere, 4,4'-dimethoxytrityl chloride (3.15 mmol, 1.15 eq) and Et_3N (4.11 mmol, 1.5veq) were added and the reaction mixture was left stirring, allowing to warm up to room temperature. After 3 h, TLC analysis (Pentane:EtOAc; 7:3:0, 1% TEA) showed complete consumption of starting material. The reaction was quenched by addition of MeOH (0.1 mL), diluted with DCM and washed with a 1:1 mixture of $NaHCO_3$ and brine. The aqueous layer was extracted with DCM twice and the combined organic layer were dried with $Na_2S_2O_4$, filtered and concentrated *in vacuo*. Compound **22** was isolated in 86% yield (2.35 mmol) after column chromatography (Pentane:EtOAc; 98:2→95:5→80:20, 1%TEA).

TLC analysis: R_f= 0.31 (Pentane:EtOAc; 95:5)

^{1}H NMR (400 MHz, CD_3CN), δ: 7.50 – 6.76 (18H, H_{arom}, m), 5.84-5.80 (1H, H_{Allyl}, m), 5.23 – 5.14 (2H, CH_{2Allyl}, m), 5.11 (1H, H_1, *J*=3.4 Hz, d), 4.77-4.71 (2H, CH*H*$_{Bn}$, H_2, m), 4.58 (1H, CH*H*$_{Bn}$, *J* = 11.4 Hz, d), 4.25 (1H, H_6, *J* = 12.5, 2.1 Hz, dd), 4.02 (1H, H_6, *J* = 12.5, 1.7 Hz, dd), 3.94 (1H, H_4, m), 3.91 – 3.82 (3H, $H_{glycerol}$, CH_{2_Allyl}, m), 3.76 (6H, 2x CH_{3DMTr}, s), 3.75-3.69

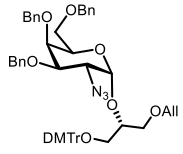
(2H, H_5, H_3,m), 3.51-3.47 (2H, $CH_{2\_glycerol}$, m), 3.23 – 3.13 (2H, $CH_{2\_glycerol}$, m), 1.06 (9H, 3xCH_{3_tBu}, s), 1.03 (9H, 3xCH_{3_tBu}, s).
^{13}C NMR (101 MHz, CD_3CN) δ: 136.0 (C_{allyl}), 131.0, 129.4 (C_q), 129.0 x2, 128.8, 128.7, 127.8 (C_{arom}), 117.0 (CH_{2_allyl}), 114.0 (C_{arom}), 98.3 (C_1), 76.4 (C_3), 76.3 ($CH_{glycerol}$), 72.5 (CH_{2Bn}), 71.2 (CH_{2_Allyl}), 70.7 (C_4), 70.3 (C_5), 68.3 (C_6), 68.0 ($CH_{2\_glycerol}$), 64.0 ($CH_{2\_glycerol}$), 59.6 (C_2), 55.9 (CH_{3DMTr}), 28.1, 27.8 (CH_{3_tBu}), 23.8, 21.3 (C_{q_tBu}).
HRMS: calcd for $C_{48}H_{61}N_3O_9Si + Na^+$ 874.4069, found 874.4066.

2-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-allyl-1-4,4'-dimethoxytrityl-*sn*-glycerol (17)



Compound **22** (0.91 mmol, 1.0 eq) was dissolved in THF (9 ml, 0.1 M) and a 1M solution of TBAF in THF (2.28 mmol, 2.5 eq) was added. The reaction was left to stir at room temperature and after 3h, TLC analysis (Pentane:EtOAc; 9:1, 1%TEA) showed complete conversion of starting material. The reaction mixture was diluted with EtOAc and washed with $NaHCO_3$ and H_2O. The aqueous layers were re-extracted and the combined organic one dried over Na_2SO_4, filtered and concentrated *in vacuo.* The title compound **17** (0.82 mmol) was isolated after column chromatography (DCM:acetone; 99:1→8:2, TEA 1%) in 91% yield.
TLC analysis: R_f= 0.39 (DCM:Acetone; 85:15)
^{1}H NMR (400 MHz, Acetonitrile-d_3) δ 7.51 – 6.82 (18H, H_{arom}, m), 5.85-5.81 (1H, H_{Allyl}, m), 5.23 – 5.08 (3H, CH_{2Allyl}, H_1, m), 4.77 (1H, CHH_{Bn}, *J* = 11.5 Hz, d), 4.55 (1H, CHH_{Bn}, *J* = 11.5 Hz, d), 4.23 (1H, H_4, m), 3.96 – 3.87 (4H, H_5, H_2, CH_{2_Allyl}, m), 3.83 (1H, H_3, *J* = 10.7, 3.0 Hz, dd), 3.76 (6H, 2x CH_{3DMTr}, s), 3.67 – 3.62 (3H, $H_{glycerol}$, 2xH_6, m), 3.57 – 3.47 (2H, $CH_{2\_glycerol}$, m), 3.29 (1H, OH, bs), 3.24 – 3.12 (2H, $CH_{2\_glycerol}$, m), 2.18 (1H, OH, bs).
^{13}C NMR (101 MHz, CD_3CN) δ: 136.1 (C_{allyl}), 131.0, 129.3, 129.0, 129.0, 128.8, 128.7x2 (C_{arom}), 116.9 (CH_{2_allyl}), 114.0 (C_{arom}), 98.5 (C_1), 76.7 (C_3), 76.5 ($CH_{glycerol}$), 72.6 (CH_{2Bn}), 71.6 (C_5), 71.2 (CH_{2Bn}), 71.0 ($CH_{2\_glycerol}$), 66.4 (C_4), 64.0 ($CH_{2\_glycerol}$), 62.5 (C_6), 60.2 (C_2), 55.9 (CH_{3DMTr}).
HRMS: calcd for $C_{40}H_{45}N_3O_9 + H^+$ 712.3229, found 712.3225.

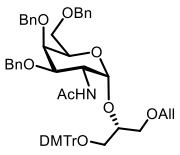
2-*O*-(2-azido-3,4,6-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-allyl-1-4,4'-dimethoxytrityl-*sn*-glycerol (23)



Diol **17** (0.82 mmol, 1 eq) was dissolved in DMF (7.5 ml, 0.1 M) and BnBr (1.8 mmol, 2.2 eq) was added. The reaction mixture was cooled to 0°C and 60% NaH in mineral oil (1.8 mmol, 2.2 eq) was added in small portions over the course of 15 minutes. The reaction mixture was left to react for two hours allowing to slowly warm up to room temperature, after which TLC analysis (Pentane:EtOAc, 55:45) showed complete conversion of starting material. The reaction was quenched at 0°C by addition of water and diluted with Et_2O. After washing with H_2O and brine, the aqueous layers were extracted twice more with Et_2O and all combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The resulting crude was subjected to column chromatography (95:5→7:3 pentane:EtOAc, TEA 1%) to yield fully protected glycoside **23** in 83% (0.68 mmol) yield.

TLC analysis: R_f= 0.33 (Pentane:EtOAc; 9:1, 1% TEA)
^{1}H NMR (400 MHz, Acetonitrile-d_3) δ 7.51 – 6.75 (28H, H_{arom}, m), 5.83-5.79 (1H, H_{Allyl}, m), 5.21 – 5.06 (3H, CH_{2Allyl}, H_1, m), 4.82 (1H, CHH_{Bn}, *J* = 11.2 Hz, d), 4.63 (1H, CHH_{Bn}, *J* = 11.3 Hz, d), 4.55-4.47 (2H, 2xCH_{2_Bn}, m), 4.23-4.17 (1H, H_5, m), 4.16 – 4.13 (1H, H_4, m), 4.00-3.96 (1H, H_3, m), 3.93 – 3.80 (3H, $H_{glycerol}$, $CH_{2\_glycerol}$, m), 3.81-3.77 (1H, H_2, m), 3.75 (6H, 2x CH_{3DMTr}, s), 3.72 – 3.47 (4H, $CH_{2\_glycerol}$, 2xH_6), 3.23 – 3.13 (2H, $CH_{2\_glycerol}$, m).
^{13}C NMR (101 MHz, CD_3CN) δ 136.1 (C_{allyl}), 131.0, 129.4, 129.3, 129.2, 129.0, 129.0, 128.8, 128.6x2, 127.7 (C_{arom}), 116.9 (CH_{2_allyl}), 114.0 (C_{arom}), 98.5 (C_1), 77.8 (C_3), 76.6 ($CH_{glycerol}$), 75.7 (CH_{2Bn}), 74.8 (CH_4), 73.9 (CH_{2Bn}), 72.6 (CH_{2_allyl}), 72.4 (CH_{2Bn}), 71.0 ($CH_{2\_glycerol}$), 70.5 (C_5), 70.0 (C_6), 64.1 ($CH_{2\_glycerol}$), 60.9 (C_2), 55.9 (CH_{3DMTr}).
HRMS: calcd for $C_{54}H_{57}N_3O_9$ + H^+ 892.4168, found 892.4167.

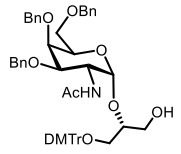
2-*O*-(2-*N*-acetylamine-3,4,6-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-allyl-1-4,4'-dimethoxytrityl-*sn*-glycerol(18)



Galactosazide **23** (5.1g, 5.8 mmol) was dissolved in 60mL of THF (0.1 M). PMe_3 (1 M solution in toluene, 17 ml, 17 mmol, 3 eq) was added and the reaction was left to stir at 45°C for 3h, after which H_2O (2 ml, 111 mmol, 19 eq) was added. After TLC (Pentane:EtOAc; 8:2, 1% TEA) indicated complete consumption of starting material, the reaction mixture was concentrated *in vacuo*. The resulting crude was dissolved in 50 mL pyridine (0.18 M) and the solution was cooled to 0°C. Ac_2O (1,2 ml, 11.6 mmol, 2 eq) was slowly added and the reaction mixture was left to stir overnight. After completion, the reaction mixture was concentrated *in vacuo* and purified by means of column chromatography (9:1→1:1 pentane:EtOAc, 1% TEA), yielding acetylated compound **18** in 83% (1.38g, 1.52mmol) yield as a slight yellow foam.
TLC analysis: R_f= 0.27 (Pentane:EtOAc; 9:1, 1% TEA)
^{1}H NMR (400 MHz, CD_3CN), δ: 7.46 – 6.82 (28H, H_{arom}, m), 6.23-6.18 (1H, NH, m), 5.86-5.80 (1H, H_{Allyl}, m), 5.18-5.11 (3H, CH_{2Allyl}, H_1, m), 4.86-4.44 (6H, 3xCH_{2_Bn}, m) d, 4.42-4.35 (1H, H_2, m), 4.14-4.09 (1H, H_5, m), 4.05-4.02 (1H, H_4, m), 3.93 – 3.77 (3H, CH_{2_allyl}, $H_{glycerol}$, m), 3.75 (6H, 2x CH_{3DMTr}, s), 3.68 (1H, H_3, *J* = 11.1, 2.7 Hz, dd), 3.60 (1H, CH$H_{2_glycerol}$, *J* = 9.5, 6.6 Hz, dd), 3.56-3.48 (3H, CH$H_{2_glycerol}$, 2xC_6, m), 3.21-3.11 (2H, $CH_{2\_glycerol}$, m), 1.66 (3H, CH_3, s).
^{13}C NMR (101 MHz, CD_3CN), δ: 170.4 (C_q), 136.2 (C_{allyl}), 130.9, 129.3, 129.3, 129.2, 128.9, 128.8x2, 128.7, 128.5x2, 127.7 (C_{arom}), 116.8 (CH_{2_allyl}), 114.0 (C_{arom}), 98.5 (C_1), 78.1 (C_3), 76.2 ($CH_{glycerol}$), 75.5 (CH_{2Bn}), 74.8 (C_4), 73.8 (CH_{2Bn}), 72.6 (CH_{2_allyl}), 72.5 (CH_{2Bn}), 71.1 ($CH_{2\_glycerol}$), 70.6 (C_5), 70.1 (C_6), 63.5 ($CH_{2\_glycerol}$), 55.9 (CH_{3DMTr}), 49.9 (C_2), 23.4 (CH_3).
HRMS: calcd for $C_{56}H_{61}NO_{10}$ + Na^+ 930.4188, found 930.4182.

2-*O*-(2-*N*-acetylamine-3,4,6-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-1-4,4'-dimethoxytrityl-*sn*-glycerol (19)

Fully protected galactose intermediate **18** (1.38 g, 1.52 mmol) was coevaporated with toluene three times and dissolved in 15mL freshly distilled THF (0.1 M). The resulting solution was purged with Argon for ten minutes after which $Ir(COD)(PPh_2Me)_2PF_6$ (0.02 mmol, 0.01 eq) was added. H_2 was bubbled through the solution for 5 seconds during



which a distinct colour changes from deep red to light yellow occurred. The solution was purged with Argon for 1 minute and left to stir under inert atmosphere. After 1.5 hour TLC analysis, (Pentane:Toluene:EtOAc; 3:3:4 R_f=0.66) showed complete conversion of starting material. The reaction mixture was diluted with 5mL of both THF and sat. aq. $NaHCO_3$ and vigorously stirring for 5 minutes. I_2 (80 mg, 2.28 mmol) was added and was left to stir for 30 minutes after which TLC analysis showed complete conversion of isomerized intermediate. The mixture was diluted using EtOAc and washed with sat. aq. $Na_2S_2O_3$, brine and dried over Na_2SO_4. The desiccant was filtered off, the organic layer was concentrated *in vacuo* and the resulting crude was purified using column chromatography (Pentane:EtOAc; 8:2→1:9, 1% TEA), yielding **19** in 76% (998 mg, 1.15 mmol) yield.

TLC analysis: R_f=0.66 -(Pentane:Toluene:EtOAc; 3:3:4 1% TEA) isomerized intermediate

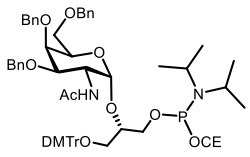
TLC analysis: R_f=0.28 -(Pentane:EtOAc; 7:3, 1% TEA) compound **19**

^{1}H NMR (400 MHz, Acetonitrile-d_3) δ: 7.46 – 7.16 (28H, H_{arom}, m), 6.19-6.16 (1H, NH, m), 4.86 (1H, H_1, *J* = 3.8 Hz, d), 4.80 (1H, CHH_{Bn}, *J* = 11.2 Hz, d), 4.73 (1H, CHH_{Bn}, *J* = 11.7 Hz, d), 4.57 – 4.39 (4H, 2xCH_{2_Bn}, H_2, m), 4.13-4.09 (1H, H_5, m), 4.05-4.02 (1H, H_4, s), 3.76 (6H, 2x CH_{3DMTr}, s), 3.72 (1H, H3, *J* = 11.2, 2.7 Hz, dd), 3.69 – 3.59 (2H, $H_{glycerol}$, $CH_{2\_glycerol}$), 3.59 – 3.50 (3H, $CH_{2\_glycerol}$, 2xH_6, m), 3.18-3.12 (2H, $CH_{2\_glycerol}$, m), 2.88-2.83 (1H, OH, bs), 1.67 (3H, CH_3, s).

^{13}C NMR (101 MHz, CD_3CN) δ: 170.4, 139.9, 139.8, 139.4, 137.1, 137.0 (C_q), 131.0, 130.9, 129.3, 129.2, 128.9x2, 128.8x2, 128.6, 128.6, 128.5, 127.8, 114.0 (C_{arom}), 98.63 (C_1), 87.03 (C_{q_DMTr}), 79.25 ($CH_{glycerol}$), 78.21 (C_3), 75.41 (CH_{2Bn}), 74.78 (C_4), 73.82 (CH_{2Bn}), 72.57 (CH_{2Bn}), 70.83 (C_5), 70.39 (C_6), 63.70 ($CH_{2\_glycerol}$), 63.43 ($CH_{2\_glycerol}$), 55.88 (CH_{3DMTr}), 49.88 (C_2), 23.33 (CH_3).

HRMS: calcd for $C_{53}H_{57}NO_{10}$ + Na^+ 890.3875, found 890.3877.

1-([*N,N*-Diisopropylamino]-2-cyanoethylphosphite)-2-*O*-[2-*O*-acetyl-3,4,6-tri-*O*-benzyl galactosamine]-3-*O*-(4,4-dimethoxytrityl)-*sn*-glycerol (8)



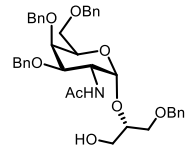
Galactose **19** (891 mg, 1.03 mmol) was in dry DCM (7 mL, 0.14 M) and Et_3N (1.545 mmol, 1.5 eq) was added At 0 °C 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.2 mmol, 1.2 eq) was added and the reaction was left for 2 hours after which TLC analysis (Pentane:EtOAc:Et_3N, 7:3:0,1) showed complete consumption of the starting material. After diluting the reaction mixture with DCM, a wash with a mixture of $NaHCO_3$ and brine (1:1) was performed and the organic layer was dried over $Na_2S_2O_4$, filtered and concentrated *in vacuo*. The desired product was purified by column chromatography (Pentane:EtOAc:Et_3N, 90:19:1→75:25:0), affording compound **8** in 79% (4.96g, 7.9mmol) yield as a slight yellow oil.

TLC analysis: Rf = 0.74 (Pentane:EtOAc; 7:3).

^{1}H NMR (400 MHz, CD_3CN) δ 7.49 – 7.20 (28H, H_{arom}, m), 6.88 (2H, H_{arom}, *J* = 2.2 Hz, d), 6.19 (1H, NH, *J* = 9.6 Hz, d), 4.93 (1H, H_1, *J* = 15.7, 3.7 Hz, dd), 4.83 (1H, CHH_{Bn}, *J* = 11.2, 2.6 Hz, dd), 4.75-4.40 (6H, 5x CHH_{Bn},, H_2, m), 4.19-4.12 (1H, H_5, m), 4.08-3.82 (2H, H_4, $H_{Glycerol}$, m), 3.77 (6H, 2xCH_{3_Dmtr}, s), 3.76 – 3.71 (2H, H_3, $CH_{2\_glycerol}$, m), 3.71 – 3.31 (6H,

2xH_6, CH_{2_oce}, $CH_{2\_glycerol}$, m), 3.29-3.11 (2H, 2xH_{iPr}, m), 2.56 – 2.49 (2H, CH_{2_oce}, m), 1.68 (3H, CH_3, s), 1.15-1.07 (12H, 4xCH_{3_iPr}, m).
^{13}C NMR (101 MHz, CD_3CN) δ 169.13, 169.08, 158.28 (C_q), 138.66, 135.64, 129.67, 129.65, 128.03, 128.00, 127.99, 127.91, 127.61, 127.60, 127.55, 127.41, 127.36, 127.34, 127.23, 127.21, 126.46 (C_{arom}), 112.74 (C_q), 97.29 (C_1), 76.87 (C_3), 75.77 ($CH_{glycerol}$), 75.56 (CH_{2_OCE}), 73.47 (CH_{2_Bn}), 73.38 (H_4), 72.63 (CH_{2_Bn}), 71.26 (CH_{2_Bn}), 69.35 (C_5), 68.78 (C_6), 63.16 ($CH_{2\_Glycerol}$), 62.29 ($CH_{2\_glycerol}$), 58.15 (CH_{2_OCE}), 54.58 (CH_{3_OMe}), 48.53 (C_2), 23.80, 23.77, 23.73, 23.69, 23.60, 23.52 (CH_{3_iPr}), 22.07 (CH_3),19.70 (CH_{iPr}).
^{31}P NMR (161.7 MHz, CD3CN): δ: 148.5, 148.7.

2-*O*-(2-*N*-acetylamine-3,4,6-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-benzyl-*sn*-glycerol (19)



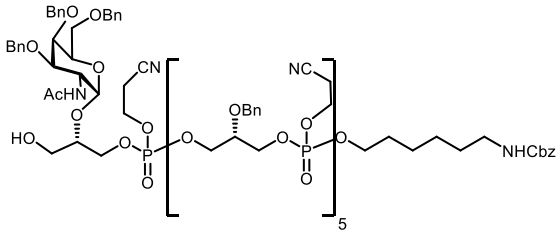
Galactose **19** (865 mg, 1.0 mmol) was dissolved in DMF (10 ml, 0.1 M) and the mixture was cooled to 0°C. Subsequently, BnBr (1,3 mmol, 1,3 eq) and NaH (1,3 mmol, 1 eq, 60% w/w) were added. The reaction mixture was stirred overnight reaching room temperature, after which TLC analysis (PE:EtOAc; 6:4, 1% TEA) showed complete consumption of starting material. After cooling at 0°C, the reaction was quenched with slowly addition of water until bubbling stopped. The mixture was diluted with Et_2O and washed 3 times with water. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The crude was dissolved in DCM (2 ml, 0.5 M) and a solution of TCA (0.18 M in DCM, 27,8 ml, 5 eq) was slowly added. After 2h of stirring, TLC analysis (Pentane:EtOAc; 7:3) showed complete consumption of starting material. The reaction mixture was diluted with DCM and washed with a solution of $NaHCO_3$ and brine (1:1). The final compound **9** was obtained after column chromatography (Pentane:EtOAc; 7:3→1:1→3:7) in 93% yield.
TLC Analysis: Rf = 0.35 (DCM:Acetone; 7:3).
^{1}H NMR (400 MHz, CD_3CN) δ: 7.40-7.26 (20H, H_{arom}, m), 6.50 (1H, NH, *J*=9.4 Hz, d), 4.94 (1H, H_1, J=3.7 Hz, d), 4.83(1H, CHH_{Bn}, J=11.2 Hz, d), 4.75 (1H, CHH_{Bn}, J=11.7 Hz, d), 4.59-4.37 (7H, 3x CHH_{Bn}, CH_2, m), 4.18-4.13 (1H, H_5, m), 4.06-4.04 (1H, H_4, m), 3.79-3.69 (2H, H_3, $CH_{glycerol}$, m), 3.66-3.49 (6H, 2x$CH_{2_glycerol}$, 2xH_6, m), 2.9 (1H, OH, bs), 1.90 (3H, CH_3, s)
^{13}C NMR (101 MHz, CD_3CN) δ: 170.8, 140.0, 139.8, 139.7, 139.5 (C_q), 129.3, 129.3 x3, 129.2, 128.9, 128.8, 128.7, 128.6, 128.5, 128.5 x2 (CH_{arom}), 99.3 (C_1), 79.4 ($CH_{glycerol}$), 78.2 (C_3), 75.5 (CH_{2_Bn}), 74.8 (C_4), 73.8, 73.7, 72.5, (CH_{2_Bn}), 71.0 ($CH_{2\_glycerol}$), 70.6 (C_5), 70.2 (C_6), 62.4 ($CH_{2\_glycerol}$), 23.3 (CH_3)
HRMS: calcd for $C_{39}H_{45}NO_8$ + H^+ 656.3218, found 656.3217

Phosphoramidite couplings

General procedure

The starting material alcohol is co-evaporated three times with dry ACN. Once dissolved in dry ACN (0.1M), a solution of DCI in ACN (0.25 M, 1.5-2.5 eq) is added together with 3Å MS and the reaction mixture is stirred for 15 min at room temperature. A solution of phosphoramidite **8** or **10** (0.176 M in ACN) is added (1.2-2.0 eq) under inert atmosphere. After TLC analysis shows complete consumption of starting material, a solution of CSO (0.5 M in ACN) is added (2.0-3.0 eq) and the reaction is allowed to stir at r.t. for 15 min, after which the reaction is diluted with EtOAc and washed once with a mixture of $NaHCO_3$ and brine (1:1). The organic layer is dried over $Na_2S_2O_4$, filtered and concentrated *in vacuo*. The crude is then dissolved in DCM (0.1 M) and a solution of TCA (0.18 M in DCM) is added (5 eq). Once TLC analysis show complete conversion to a lower running spot, the reaction mixture is diluted in DCM and washed with a solution of $NaHCO_3$ and brine (1:1), dried over $Na_2S_2O_4$, filtered and concentrated *in vacuo*. The desired product is isolated by column chromatography.

(Protected) (GalNAc-sn1-GroP)(*sn*1-GroP)$_5$-Spacer 24



Alcohol **S10** (80 µmol; See Experimental Chapter 4) was coupled with phosphoramidite **8** (120 µmol, 1.5 eq) following the general procedure. Compound **24** was obtained after column chromatography (DCM:Acetone, 1:1) in 75% yield (60 µmol).

TLC analysis: Rf = 0.28 (DCM:Acetone; 1:1).

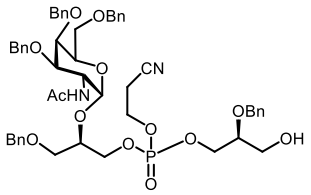
^{1}H NMR (400 MHz, CD_3CN) δ: 7.41 – 7.11 (45H, H_{arom}, m), 7.01-6.76 (1H, NH, m), 5.72-5.63 (1H, NH, bs), 5.03 (s, 2H, H-26), 4.90-4.35 (20H, H_1, 18x$CH_{Bn}H$, H_2, m), 4.32 – 3.97 (36H, H_5, H_4, 6xCH_{2_OCE}, $CH_{2\_OSpacer}$, 10x$CH_{2_glycerol}$, m), 3.97 – 3.80 (5H, 5x$H_{glycerol}$, m), 3.80 – 3.52 (8H, H_3, 2xH_6, $H_{glycerol}$, 2x$CH_{2_glycerol}$), 3.10-3.00 (3H, CH_{2_NHCbz}, OH, m), 2.76 – 2.60 (12H, 6xCH_{2_OCE}, m), 1.88 (3H, CH_3, s), 1.66 – 1.55 (2H, CH_{2_Spacer}, m), 1.48 – 1.38 (2H, CH_{2_Spacer}, m), 1.38 – 1.28 (4H, 2xCH_{2_Spacer}, m).

^{13}C NMR (101 MHz, CD_3CN) δ 171.1, 170.9, 139.9x2, 139.8, 139.5, 139.0, 129.9 (C_q), 129.4x2, 129.3, 129.2, 128.9x2, 128.8x2, 128.6x2, 128.6, 128.5x2, 126.2 (C_{arom}), 99.0 (C_1), 78.2 ($CH_{glycerol}$), 76.9 (C_3), 76.8, 76.7, 76.6 ($CH_{glycerol}$), 75.5 (CH_{2_Bn}), 74.7 (C_4), 73.9x2, 72.7x2, 72.61 (CH_{2_Bn}), 71.1 (C_5), 69.7 (C_6), 68.4-66.9 (CH_{2_OCE}, $CH_{2\_glycerol}$), 66.6 (CH_{2_Cbz}), 63.7-63.2 ($CH_{2\_glycerol}$), 61.0 ($CH_{2\_glycerol}$), 49.7 (C_2), 41.4 ($CH_{2\_Nspacer}$), 30.7, 30.4, 26.8, 25.7 (CH_{2_spacer}), 23.3 (CH_{3Ac}), 20.2-20.1 (CH_{2_OCE}).

^{31}P NMR (162 MHz, CD_3CN) δ 0.16, 0.09, 0.02, 0.00, -0.10, -0.16, -0.19, -0.20, -0.22, -0.23, -0.26, -0.29, -0.30, -0.31, -0.34, -0.35.

HRMS: calcd for $C_{136}H_{167}N_9O_{43}P_6 + H^+$ 2800.9656, found 2800.9652.

(Protected) (GalNAc-sn3-GroP)(*sn*3-GroP) 25



Alcohol **9** (350 µmol) was coupled with phosphoramidite **10** (450 µmol, 1.5 eq) following the general procedure. Compound **25** was obtained after column chromatography (DCM:Acetone, 1:1) in 83% yield (290 µmol).

TLC analysis: Rf = 0.26 (DCM:Acetone; 6:4).

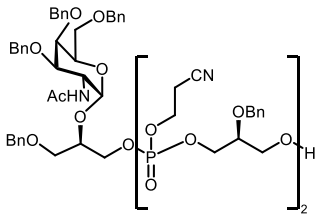
^{1}H NMR (400 MHz, CD_3CN) δ: 7.32 – 7.13 (25H, H_{arom}, m), 7.01 – 7.00 (1H, NH, m), 4.81-4.76 (1H, H_1, m), 4.75-4.66 (1H, CHH_{Bn}, m), 4.65-4.56 (1H, CHH_{Bn}, m), 4.55 – 4.49 (2H, CHH_{Bn}, m), 4.48 – 4.26 (7H, 3x CHH_{Bn}, H_2), 4.21 – 3.84 (8H, H_5, H_4, CH_{2_OCE}, 2x$CH_{2_glycerol}$, m), 3.80 – 3.71 (1H, $H_{glycerol}$, m), 3.63 – 3.38 (8H, H_3, $H_{glycerol}$, 2xH_6, 2x$CH_{2_glycerol}$), 3.11 – 3.00 (1H, OH, bs), 2.67-2.62 (1H, CHH_{2_OCE}, m), 2.60-2.54 (1H, CHH_{2_OCE}, m), 1.85 (3H, CH_3, s).

^{13}C NMR (101 MHz, CD_3CN) δ: 171.2, 140.0, 139.9, 139.8, 139.6, 139.4 (C_q), 129.3x2, 129.2, 129.0, 128.8x2, 128.7x2, 128.6x2, 128.5x3 (C_{arom}), 100.7x2 (C_1), 79.1, 79.0 ($CH_{glycerol}$), 78.3,78.2 (C_3), 77.8, 77.6 ($CH_{glycerol}$), 75.4 (CH_{2_Bn}), 75.0, 74.9 (C_4), 73.8, 73.7 (CH_{2_Bn}), 72.7, 72.6, 72.5, 72.4 (CH_{2_Bn}), 70.9 (C_5), 70.3 (C_6), 69.5 ($CH_{2\_glycerol}$), 68.7-68.6, 68.0-67.9, 63.5-63.4, 61 (3x$CH_{2_glycerol}$, CH_{2_OCE}), 49.9, 49.8 (C_2), 23.3 (CH_3), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.44, -1.37

HRMS: calcd for $C_{52}H_{61}N_2O_{13}P$ + H^+ 953.3894, found 953.3897.

(Protected) (GalNAc-sn3-GroP)(*sn*3-GroP)$_2$ 26



Alcohol **25** (280 µmol) was coupled with phosphoramidite **10** (420 µmol, 1.5 eq) following the general procedure. Compound **26** was obtained after column chromatography (DCM:Acetone, 1:1) in 93% yield (260 µmol).

TLC analysis: Rf = 0.31 (DCM:Acetone; 55:45).

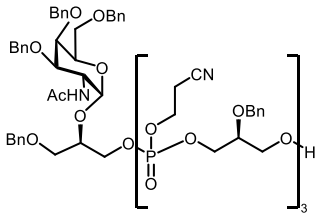
^{1}H NMR (400 MHz, CD_3CN) δ: 7.42 – 7.19 (30H, H_{arom}, m), 7.18 – 7.00 (1H, NH, m), 4.90-4.76 (2H, H_1, CHH_{Bn},m), 4.73-4.33 (12H, 11xCHH_{Bn}, H_2), 4.29 – 3.92 (14H, H_5, H_4, 2xCH_{2_OCE}, 4x$CH_{2_glycerol}$, m), 3.90 – 3.78 (2H, 2x$H_{glycerol}$, m), 3.71 – 3.47 (8H, H_3, $H_{glycerol}$, 2xH_6, 2x$CH_{2_glycerol}$), 3.14 – 3.05 (1H, OH, bs), 2.77-2.62 (4H, 2xCH_{2_OCE}, m), 1.96 (3H, CH_3, s).

^{13}C NMR (101 MHz, CD_3CN) δ: 171.1, 171.0, 139.9x3, 139.8, 139.7, 139.5,139.4, 139.1 (C_q), 129.4, 129.3x3, 129.2, 129.0x2, 128.9, 128.8x2, 128.7x4, 128.6x3, 128.5x3 (CH_{arom}), 100.7x2 (C_1), 79.2-79.1 ($CH_{glycerol}$), 78.4-78.3 (C_3), 77.7-77.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 75.4 (CH_{2_Bn}), 74.9 (C_4), 73.9-73.8, 72.8, 72.7, 72.5, 72.4 (CH_{2_Bn}), 71.0-70.9 (C_5), 70.3 (C_6), 69.6 ($CH_{2\_glycerol}$), 69.5, 68.8,67.8, 67.0, 66.7, 63.6-63.5, 63.4, 61.1 ($CH_{2\_glycerol}$, CH_{2_OCE}), 49.8 (C_2), 23.3 (CH_3), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.72, -1.71, -1.67, -1.65 -1.48, -1.41

HRMS: calcd for $C_{65}H_{77}N_3O_{18}P_2$ + H^+ 1251.2890, found 1251.2889.

(Protected) (GalNAc-sn3-GroP)(*sn*3-GroP)$_3$ 27



Alcohol **26** (130 µmol) was coupled with phosphoramidite **10** (195 µmol, 1.5 eq) following the general procedure. Compound **27** was obtained after column chromatography (DCM:Acetone, 1:1) in 71% yield (92 µmol).

TLC analysis: Rf = 0.27 (DCM:Acetone; 55:45).

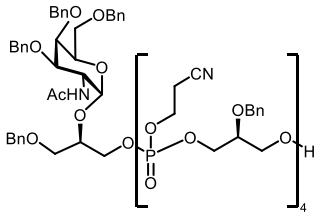
^{1}H NMR (400 MHz, CD_3CN) δ: 7.39 – 7.20 (35H, H_{arom}, m), 7.17 – 6.98 (1H, NH, m), 4.88-4.75 (2H, H_1, CHH_{Bn},m), 4.73-4.33 (14H, 13xCHH_{Bn}, H_2), 4.29 – 3.92 (20H, H_5, H_4, 3xCH_{2_OCE}, 6x$CH_{2_glycerol}$, m), 3.90 – 3.78 (3H, 3x$H_{glycerol}$, m), 3.69 – 3.47 (8H, H_3, $H_{glycerol}$, 2xH_6, 2x$CH_{2_glycerol}$), 3.14 – 3.05 (1H, OH, bs), 2.77-2.62 (6H, 3xCH_{2_OCE}, m), 1.96 (3H, CH_3, s).

^{13}C NMR (101 MHz, CD_3CN) δ: 171.1, 171.0, 139.9x3, 139.8, 139.7, 139.5,139.4, 139.1 (C_q), 129.4x2, 129.3x2, 129.2, 129.0x2, 128.9x2, 128.8x3, 128.7x2, 128.6x3, 128.5x4 (CH_{arom}), 100.8, 100.6 (C_1), 79.1 ($CH_{glycerol}$), 78.3 (C_3), 77.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 75.4 (CH_{2_Bn}), 74.9 (C_4), 73.9-73.8, 72.8, 72.7-72.6, 72.4 (CH_{2_Bn}), 71.0-70.9 (C_5), 70.3 (C_6), 69.6 ($CH_{2\_glycerol}$), 69.5, 68.8,67.8, 67.0, 66.7, 63.6-63.5, 63.4, 61.1 ($CH_{2\_glycerol}$, CH_{2_OCE}), 49.8 (C_2), 23.3 (CH_3), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.72, -1.71, -1.69, -1.68, -1.66, -1.65, -1.64, -1.61, -1.40, -1.38, -1.35

HRMS: calcd for $C_{78}H_{93}N_4O_{23}P_3$+ H^+ 1547.5516, found 1547.5519.

(Protected) (GalNAc-sn3-GroP)(*sn*3-GroP)$_4$ 28



Alcohol **27** (80 µmol) was coupled with phosphoramidite **10** (195 µmol, 1.5 eq) following the general procedure. Compound **28** was obtained after column chromatography (DCM:Acetone, 1:1) in 98% yield (78 µmol).

TLC analysis: Rf = 0.34 (DCM:Acetone; 1:1).

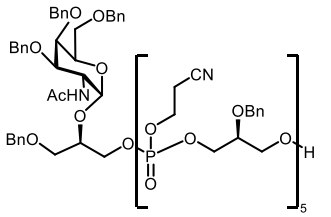
^{1}H NMR (400 MHz, CD_3CN) δ: 7.39 – 7.20 (40H, H_{arom}, m), 7.17 – 7.08 (1H, NH, m), 4.89-4.31 (18H, H_1, 16xCHH_{Bn}, H_2, m), 4.27 – 3.92 (26H, H_5, H_4, 4xCH_{2_OCE}, 8x$CH_{2_glycerol}$, m), 3.90 – 3.78 (4H, 4x$H_{glycerol}$, m), 3.69 – 3.47 (8H, H_3, 2xH_6, $H_{glycerol}$, 2x$CH_{2_glycerol}$), 3.14 – 3.05 (1H, OH, bs), 2.77-2.62 (8H, 4xCH_{2_OCE}, m), 1.96 (3H, CH_3, s).

^{13}C NMR (101 MHz, CD_3CN) δ: 171.1, 171.0, 139.9x3, 139.8, 139.7, 139.5,139.4, 139.1 (C_q), 129.4x2, 129.3x2, 129.2, 129.0x2, 128.9x2, 128.8x3, 128.7x2, 128.6x3, 128.5x4 (CH_{arom}), 100.8, 100.6 (C_1), 79.1 ($CH_{glycerol}$), 78.3 (C_3), 77.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 75.4 (CH_{2_Bn}), 74.9 (C_4), 73.9-73.8, 72.8, 72.7-72.6, 72.4 (CH_{2_Bn}), 71.0-70.9 (C_5), 70.3 (C_6), 69.6 ($CH_{2\_glycerol}$), 69.5, 68.8,67.8, 67.0, 66.7, 63.6-63.5, 63.4, 61.1 ($CH_{2\_glycerol}$, CH_{2_OCE}), 49.8 (C_2), 23.3 (CH_3), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.67, -1.66, -1.64, -1.63, -1.61, -1.59, -1.57, -1.39, -1.37.

HRMS: calcd for $C_{91}H_{109}N_5O_{28}P_4$+ H^+ 1844.6282, found 1844.6285.

(Protected) (GalNAc-sn3-GroP)(*sn*3-GroP)$_5$ 29



Alcohol **28** (68 µmol) was coupled with phosphoramidite **10** (195 µmol, 1.5 eq) following the general procedure. Compound **29** was obtained after column chromatography (DCM:Acetone, 1:1) in 76% yield (52 µmol).

TLC analysis: Rf = 0.28 (DCM:Acetone; 1:1).

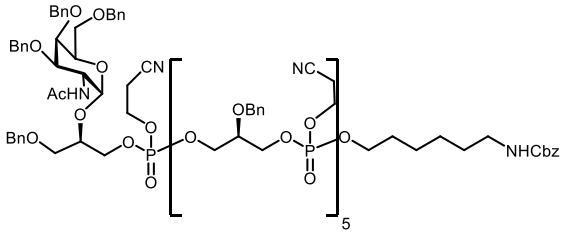
^{1}H NMR (400 MHz, CD_3CN) δ: 7.43 – 7.20 (45H, H_{arom}, m), 7.19 – 7.08 (1H, NH, m), 4.90-4.32 (18H, H_1, 16x$CH\mathit{H}_{Bn}$, H_2, m), 4.27 – 3.92 (26H, H_5, H_4, 4xCH_{2_OCE}, 8x$CH_{2_glycerol}$, m), 3.90 – 3.78 (4H, 4x$H_{glycerol}$, m), 3.69 – 3.47 (8H, H_3, 2xH_6, $H_{glycerol}$, 2x$CH_{2_glycerol}$), 3.14 – 3.05 (1H, OH, bs), 2.77-2.62 (8H, 4xCH_{2_OCE}, m), 1.96 (3H, CH_3, s).

^{13}C NMR (101 MHz, CD_3CN) δ: 171.1, 171.0, 139.9x3, 139.8, 139.7, 139.5,139.4, 139.1 (C_q), 129.4x2, 129.3x2, 129.2, 129.0x2, 128.9x2, 128.8x3, 128.7x2, 128.6x3, 128.5x4 (CH_{arom}), 100.8, 100.6 (C_1), 79.1 ($CH_{glycerol}$), 78.3 (C_3), 77.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 75.4 (CH_{2_Bn}), 74.9 (C_4), 73.9-73.8, 72.8, 72.7-72.6, 72.4 (CH_{2_Bn}), 71.0-70.9 (C_5), 70.3 (C_6), 69.6 ($CH_{2\_glycerol}$), 69.5, 68.8,67.8, 67.0, 66.7, 63.6-63.5, 63.4, 61.1 ($CH_{2\_glycerol}$, CH_{2_OCE}), 49.8 (C_2), 23.3 (CH_3), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.44, -0.43, -0.40, -0.38, -0.34, -0.32, -0.18, -0.15.

HRMS: calcd for $C_{91}H_{109}N_5O_{28}P_4$+ H^+ 1844.6282, found 1844.6285.

(Protected) (GalNAc-sn3-GroP)(*sn*3-GroP)$_5$-Spacer 30



Alcohol **29** (45 µmol) was coupled with phosphoramidite **11P** (135 µmol, 3 eq) following the general procedure. Compound **30** was obtained after column chromatography (DCM:Acetone, 1:1) in 81% yield (36 µmol).

TLC analysis: Rf = 0.33 (DCM:Acetone; 1:1).

^{1}H NMR (400 MHz, CD_3CN) δ: 7.43 – 7.20 (50H, H_{arom}, m), 7.19 – 7.08 (1H, NH, m), 5.68-5.54 (1H, NH, bs), 4.90-4.32 (22H, H_1, 20x$CH\mathit{H}_{Bn}$, H_2, m), 4.27 – 3.92 (40H, H_5, H_4, 4xCH_{2_OCE}, 10x$CH_{2_glycerol}$, m), 3.90 – 3.78 (5H, 5x$H_{glycerol}$, m), 3.69 – 3.47 (4H, H_3, 2xH_6, $H_{glycerol}$, m), 3.14 – 3.05 (2H, CH_{2_Spacer}, m), 2.77-2.62 (12H, 6xCH_{2_OCE}, m), 1.96 (3H, CH_3, s), 1.66 – 1.55 (2H, CH_{2_Spacer}, m), 1.48 – 1.38 (2H, CH_{2_Spacer}, m), 1.38 – 1.28 (4H, 2xCH_{2_Spacer}, m).

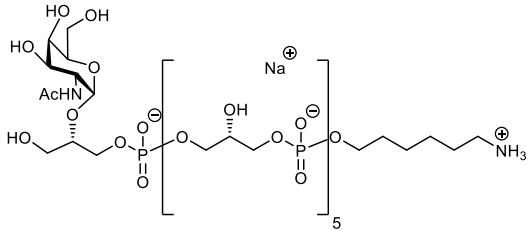
^{13}C NMR (101 MHz, CD_3CN) δ: 171.1, 171.0, 139.9x3, 139.8, 139.7, 139.5,139.4, 139.1 (C_q), 129.4x2, 129.3x2, 129.2, 129.0x2, 128.9x2, 128.8x3, 128.7x2, 128.6x3, 128.5x4 (CH_{arom}), 100.8, 100.6 (C_1), 79.1 ($CH_{glycerol}$), 78.3 (C_3), 77.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 75.4 (CH_{2_Bn}), 74.9 (C_4), 73.9-73.8, 72.8, 72.7-72.6, 72.4 (CH_{2_Bn}), 71.0-70.9 (C_5), 70.3 (C_6), 69.6 ($CH_{2\_glycerol}$), 69.5, 68.8,67.8, 67.0, 66.7, 63.6-63.5, 63.4, 61.1 ($CH_{2\_glycerol}$, CH_{2_OCE}), 49.8 (C_2), 41.4 ($CH_{2\_Nspacer}$), 30.7, 30.4, 26.8, 25.7 (CH_{2_spacer}), 23.3 (CH_{3Ac}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.48, -1.47, -1.43, -1.42, -1.41, -1.23, -1.21, -1.19.
HRMS: calcd for $C_{91}H_{109}N_5O_{28}P_4$+ H^+ 1844.6282, found 1844.6285.

Final deprotection

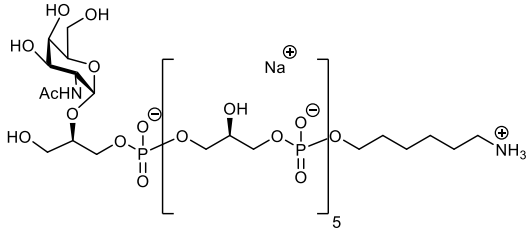
The oligomer is dissolved in dioxane (2mM) and upon the addition of ammonia solution in H_2O (33%) the reaction mixture turns turbid. Once the solution becomes transparent (1-3 hours) the reaction mixture is concentrated *in vacuo*. After checking the disappearing of the cyanoethyl group by ^{1}H-NMR, the residue is flushed over a Dowex Na^+ cation-exchange resin (type 50WX4-200, stored in 0.5M NaOH in MilliQ, flushed with MeOH and MilliQ before use) column. After evaporation, the residue is dissolved in MilliQ (2mM) and 2 drops of AcOH are added. Ar is bubbled in the reaction mixture for 20 minutes while sonicating, Pd-black (≈10 mg) is added and after an additional 10 minutes of Ar bubbling, the solution is left stirring under H_2 atmosphere for 1 week. After filtration over Celite®, the reaction mixture is concentrated *in vacuo*. The final compound is purified by sixe-exclusion chromatography (HW40, dimensions: 16/60 mm, eluent: 0.15M NH_4OAc). After several co-evaporation with MilliQ, the product is eluted through a small column containing Dowex Na^+ cation-exchange resin (type 50WX4-200, stored in 0.5M NaOH in MilliQ, flushed with MeOH and MilliQ before use).

(GalNAc-sn1-GroP)(sn1-GroP)$_5$-Spacer 6



Compound **24** (20 µmol) was deprotected following the general procedure. The final product **6** was obtained in 75% yield (15 µmol).
^{1}H-NMR (850 MHz, D_2O), δ: 5.03 (1H, H_1, J=3.8 Hz, d), 4.18-4.12 (2H, 4.05-3.95, H_5, H_2, m), 4.02-3.80 (31H, 6 x $CH_{glycerol}$, H_4, H_3, 11x$CH_{2_glycerol}$, $CH_{2\_Ospacer}$, m), 3.74-3.63 (4H, $CH_{2\_glycererol,}$ 2 x H_6, m), 2.98-2.94 (2H, $CH_{2\_Nspacer}$, m), 2.00 (3H, CH_3, s), 1.67-1.59 (4H, CH_{2_spacer}, m), 1.42-1.35 (4H, CH_{2_spacer}, m).
^{13}C-NMR(214 MHz, D_2O) δ: 174.6 (C_q), 96.8 (C_1), 76.9 ($CH_{glycerol}$), 71.0 (CH_5), 69.3 ($CH_{glycerol}$) 68.4 (C_4), 67.7 (C_3), 66.1-66.0 ($CH_{2\_glycerol}$), 65.9 ($CH_{2\_OSpacer}$), 65.1 ($CH_{2\_glycerol}$), 61.1 (CH_6), 60.1 ($CH_{2\_glycerol}$), 49.8 (C_2), 39.3 ($CH_{2\_Nspacer}$), 29.3, 26.5, 25.0, 24.3, ($CH_{2spacer}$), 21.8 (CH_3).
^{31}P-NMR(162 MHz, D_2O), δ: 1.62, 1.84, 1.94, 2.04.
HRMS: calcd for $C_{35}H_{66}N_2Na_6O_{35}P_4$ + Na^+ required 1359.1675, found 1359.1679

(GalNAc-sn3-GroP)(sn3-GroP)$_5$-Spacer 7



Compound **30** (10 µmol) was deprotected following the general procedure. The final product **7** was obtained in 78% yield (7.8 µmol).

^{1}H-NMR (850 MHz, D_2O), δ: 5.05 (1H, H_1, J=3.6 Hz, d), 4.16 (1H, H_2, J=3.8, J=11.1, dd),4.09-4.05 (1H, H_5, m), 4.04-3.79 (32H, 6 x $CH_{glycerol}$, H_4, H_3, 11x$CH2_{_glycerol}$, $CH_{2\_Ospacer}$, m), 3.76-3.68 (2H, $CH_{2\_glycererol,}$ m), 3.66-3.63 (1H, H_6, m), 3.58-3.54 (1H, H_6, m), 2.98-2.94 (2H, $CH_{2\_Nspacer}$, m), 2.03 (3H, CH_3, s), 1.67-1.59 (4H, CH_{2_spacer}, m), 1.42-1.35 (4H, CH_{2_spacer}, m).

^{13}C-NMR(214 MHz, D_2O) δ: 174.7 (C_q), 98.3 (C_1), 72.1 ($CH_{glycerol}$), 71.6 (C_5), 70.4 ($CH_{glycerol}$), 69.5 (C_4), 68.6 (C_3), 67.2-66.9 ($CH_{2\_glycerol}$), 66.9 ($CH_{2\_OSpacer}$), 66.5 ($CH_{2\_glycerol}$), 62.9 (CH_6), 62.1 ($CH_{2\_glycerol}$), 50.8 (C_2), 40.3 ($CH_{2\_Nspacer}$), 30.3, 27.5, 26.0, 25.4, ($CH_{2spacer}$), 23.0 (CH_3).

^{31}P-NMR(162 MHz, CD_3CN), δ: 1.78, 1.89, 1.93, 2.04.

HRMS: calcd for $C_{35}H_{66}N_2Na_6O_{35}P_4$ + Na^+ required 1359.1675, found 1359.1681

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