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

Berni, F.

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Francesca Berni

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Promotor:

Prof.dr. J.D.C. Codée

Co-promotor:

Prof.dr. G.A. van der Marel

Promotiecommissie:

Prof.dr. M. Ubbink

Prof.dr. H.S. Overkleeft

Dr. D.V. Filippov

Prof.dr. N. van Sorge (AMC – UvA)

Prof.dr. J. Jiménez Barbero (CICbioGUNE)

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List of Abbreviations

Ac_2O	*Acetic anhydride*
AcCl	*Acetyl chloride*
ACN	*Acetonitrile*
AcOH	*Acetic acid*
AcSH	*Thioacetic acid*
AgOTf	*Silver trifluoromethanesulfonate*
Ala	*Alanine*
BAIB	*(Diacetoxyiodo)benzene*
Bn	*Benzyl*
bs	*broad singlet*
BSA	*Bovine serum albumine*
BSP	*1-Benzene-sulfonyl piperidine*
Bu	*Butyl*
CAN	*Ammonium Cerium(IV) Nitrate*
CPG	*Controlled pore glass*
CPs	*Capsular polysaccharides*
CSO	*(10-Camphorsulfonyl)oxaziridine*
d	*doublet*
DBU	*1,8-Diazabicyclo[5.4.0]undec-7-ene*
DCA	*Dichloroacetic acid*
DCC	*N,N'-Dicyclohexylcarbodiimide*
DCI	*4,5-Dicyanoimidazole*
DCM	*Dichloromethane*
DDQ	*2,3-Dichloro-5,6-dicyano-1,4-benzoquinone*
DIPEA	*N,N-Diisopropylethylamine*
DMAP	*4-Dimethylaminopyridine*
Me	*Methyl*
MeOH	*Methanol*
MP	*Methoxyphenyl*
MRSA	*Methicillin resistant S. aureus*
NaOMe	*Sodium methoxide*
Nap	*Naphthyl*
N-Cbz	*N-carboxybenzyl*
NIS	*N-Iodosuccinimide*
NMR	*Nuclear Magnetic Resonance*
OPA	*Opsonophagocytic assay*
OPIA	*Opsonophagocytic inhibition assay*
PBS	*Phosphate-buffered saline*
PMB	*p-Methoxybenzyl*
PNAG	*Poly-β(1-6)-N-acetylglucosamine*
PPh_3O	*Triphenylphosphine oxide*
Py	*Piridine*
quant.	*quantitative*
RboP	*Ribitolphosphate*
rEPA	*Replication initiation proteins*
s	*singlet*
Sbox	*S-Benzoxazolyl*
t	*triplet*
TBAB	*Tetrabutylammonium bromide*
TBAF	*Tetrabutylammonium fluoride*
TBAI	*Tetrabutylammonium iodide*
$TBAN_3$	*Tetrabutylammonium azide*

DMF	*Dimethylformamide*
DMTr	*4,4'-Dimethoxytrityl*
dPNAG	*Deacetylated PNAG*
DPS	*Diphenylsulfoxide*
EDCI	*1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide*
ELISA	*Enzyme-linked immunosorbent assay*
Et	*Ethyl*
TEA	*Triehtylamine*
EtOH	*Ethanol*
GalNAc	*N-acetyl-galactosamine*
GalNAcA	*N-acetyl-D-galacturonic acid*
GlcNAc	*N-acetyl-glucosamine*
GroP	*Glycerolphosphate*
Hla	*S.aureus alpha toxin*
Lev	*Levulinoyl*
LTA	*Lipoteichoic Acids*
m	*multiplet*
TBDMS	*tert-Butyldimethylsilyl*
TBDPS	*tert-Butyldiphenylsilyl*
TBSOTf	*tert-Butyldimethylsilyl trifluoromethanesulfonate*
t-BuOH	*tert-Butyl alcohol*
TCEP	*tris(2-carboxyethyl)phosphine hydrochloride*
TEMPO	*(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl*
Tf_2O	*Trifluoromethanesulfonic anhydride*
TFA	*Trifluoroacetic acid*
TfOH	*Triflic acid*
THF	*Tetrahydrofuran*
TMSI	*Trimethylsilyl iodide*
TMSOTf	*Trimethylsilyl trifluoromethanesulfonate*
Troc	*2,2,2-Trichloroethoxycarbonyl chloride*
TT	*Tetanus Toxoid*
TTBP	*2,4,6-Tri-tert-butylphenol*
WHO	*World Health Organization*
WTA	*Wall Teichoic Acids*

Chapter 1

Synthetic carbohydrate-based cell wall components from *Staphylococcus aureus*

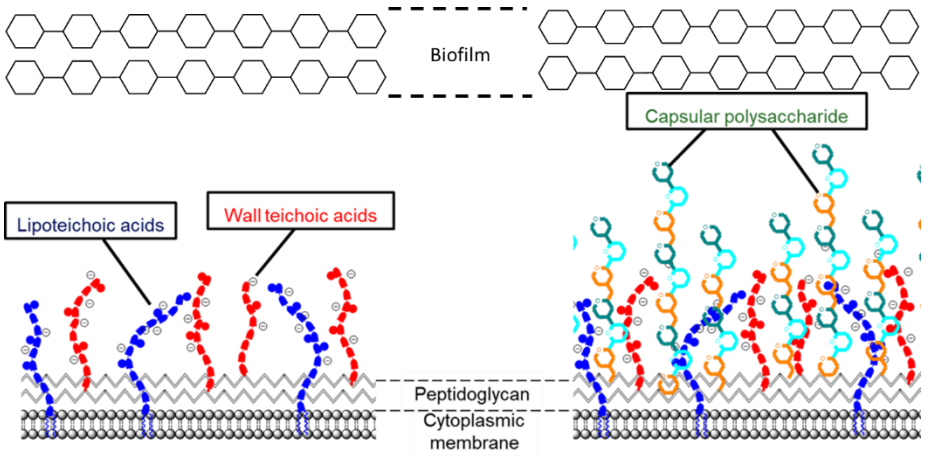
F. Berni, J. Enotarpi, T. Voskuilen, S. Li, G. A. van der Marel, J. D. C. Codée; *Drug Discovery Today: Technology,* **2020**, 38: 35-43.

INTRODUCTION

Staphylococcus aureus is a commensal ubiquitous Gram-positive bacterium associated with a range of severe infections (skin and soft tissue infections, sepsis, arthritis, pneumonia, bacteraemia and others).[1] Although *S. aureus* can be present as asymptomatic colonizer, most of the infections are hospital acquired and affect mainly young children, elderly, immunocompromised and post-surgical patients, leading to high health-care costs and higher risk of in-hospital deaths.[2] The rise of highly antibiotic resistant strains, such as Methicillin resistant *S. aureus* (MRSA), urges the development of new treatments.[3] In the last decade much attention has been focused on the development of active or passive immunization strategies.[4]

The structurally complex cell envelope of *S. aureus* is composed of peptidoglycan, cell wall glycopolymers and proteins. [5] All of these are involved in several physiological processes and they play a key role in staphylococcal virulence, making them promising antigen candidates. Figure 1 shows a schematic representation of *S. aureus* cell wall, highlighting the three major classes of glyco-based cell wall components that have been found to be promising antigen candidates: 1) capsular polysaccharides[6], the structure of which varies between strain types and which may also be absent; 2) wall teichoic acids (WTAs) and lipoteichoic acids (LTAs),[7] which are anionic glycopolymers either covalently attached to the peptidoglycan or anchored to the lipid bilayer through hydrophobic interactions, respectively; 3) the thick peptidoglycan layer. Herein an overview is presented on the major *S. aureus* carbohydrate-based antigen candidates for which organic synthesis efforts have delivered well-defined fragments to delineate clear structure-activity relationships. These fragments are attractive tools not only for vaccine applications but also for diagnostics as well as other interaction studies (such as lectin binding) and biosynthesis studies.

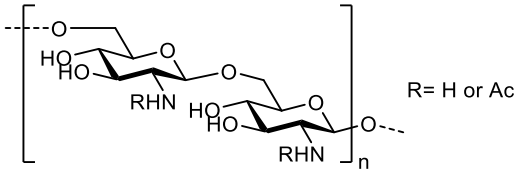
Figure 1: *Schematic representation of the cell wall from S. aureus*



PNAG

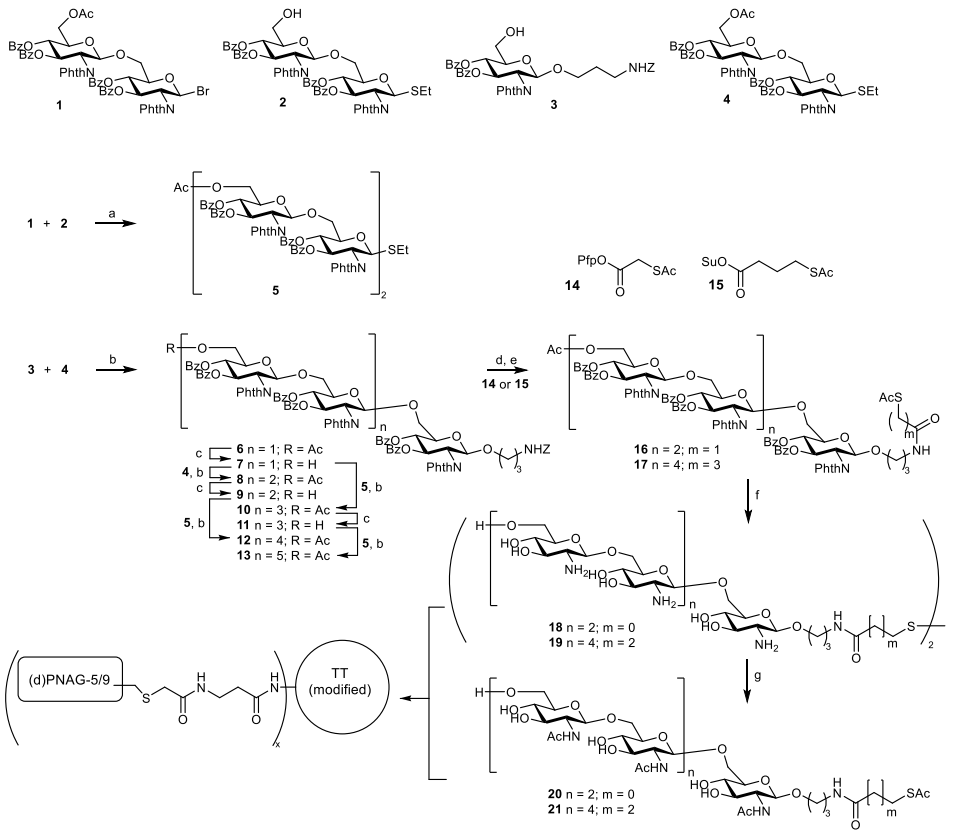
Biofilm formation represents one of the bacterial defence mechanisms against both host immune response and antibiotics. Poly-β(1-6)-*N*-acetylglucosamine (PNAG) is a cell surface polysaccharide produced not only by many bacterial species, including S. *aureus*, but also fungi and protozoal parasites.[8] Thus, in the last decades much attention has been addressed to the immunological properties of PNAG and its potential application in vaccine development.[9] The structure of PNAG is shown in Figure 2 and it has been reported that about 10-20% of the amino groups are not *N*-acetylated (dPNAG).[10]

Figure 2: *Structure of PNAG and dPNAG*



Native PNAG and chemically deacetylated PNAG (dPNAG) have been conjugated to diphtheria toxoid to generate model vaccines that were evaluated in different animal models and it was observed that antibodies against dPNAG were more effective in mediating opsonophagocytic killing than the ones raised against the PNAG conjugate.[11] Native polysaccharides are generally obtained as a heterogeneous mixture of oligosaccharides, varying in length and substitution pattern with different immunological activity. Chemically synthesized, well-defined fragments, on the other hand, can be used to define the structure-immunogenicity relationships. In 2007, Nifantiev and co-workers reported the synthesis of well-defined PNAG and dPNAG fragments up to the undecamer level, equipped with an aminopropyl linker for further functionalization.[12] The strategy relied on four key building blocks **1**, **2**, **3** and **4** (Scheme 1), the synthesis of which was reported earlier.[13] In their strategy, the C-6-OH was temporarily protected with an acetyl group, while the C-3 and C-4 hydroxyl groups were protected as benzoyl esters. The presence of a phthalimide group at the C-2-nitrogen allows to direct the glycosylation reactions stereoselectively via neighbouring participation group. Tetrasaccharide **5** was obtained in 76% yield by a coupling between disaccharide bromide donor **1** and disaccharide acceptor **2** under Helferich conditions. Coupling of disaccharide donor **4** with acceptor **3** using NIS and catalytic amount of TfOH afforded linker equipped trisaccharide **6** in 94%. Selective removal of the acetyl group using acetyl chloride in MeOH then afforded acceptor trisaccharide **7** in quantitative yield and the subsequent condensation with disaccharide donor **4** delivered pentasaccharide **8** in excellent yield. After acetyl cleavage, tetrasaccharide donor **5** was coupled with either trisaccharide acceptor **7** or pentasaccharide acceptor **9** to obtain an heptamer (**10**, 74%) and a nonamer (**12**, 60%) respectively. Final acetyl removal from the heptasaccharide gave **11** and subsequent coupling with tetrasaccharide donor **5** furnished fully protected undecamer **13** in 50% yield. In order to define structural requirements for

immunogenicity, pentasaccharide **8** and nonasaccharide **12** were selected to be further functionalized and conjugated to a carrier protein for immunization experiments.[14] Selective deprotection of the amino group of the spacer, followed by amide formation using **14** or **15**, afforded thiol functionalized pentasaccharide **16** and nonasaccharide **17**. Global deprotection was achieved using hydrazine hydrate in boiling EtOH to afford compounds **18** and **19**. *N*-acetylated derivatives **20** and **21** were generated using dithiothreitol and acetic anhydride. The oligosaccharide thiol derivatives were obtained by treatment of compounds **18**, **19**, **20** and **21** with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in a 7% solution of ammonia in water. The so-generated thiols were coupled to modified tetanus toxoid (TT) protein generating four glycoconjugates. Mice were immunized with the four synthetic glycoconjugates, after which the opsonic activity of the generated sera was evaluated. These studies confirmed that antibodies raised against the dPNAG conjugates from **18** or **19** had greater opsonic activity than the ones raised against PNAG conjugates derived from **20** or **21**. Interestingly, the former antibodies were cross-reactive towards native PNAG and dPNAG, while dPNAG was not recognized by antibodies raised against fully *N*-acetylated conjugates.



***Scheme 1**: Synthesis of well-defined PNAG, dPNAG fragments and its corresponding glycoconjugates. a) $HgBr_2$, $Hg(CN)_2$, CH_3CN, 76%. b) NIS, TfOH, MS 4Å, CH_2Cl_2, **6**, 94%; **8**, 90%; **10**, 74%; **12**, 60%; **13**, 51%. c) AcCl, CH_3OH, **7**, 95%; **9**, 93%; **11**, 96%. d) H_2, $Pd(OH)_2$, 1M HCl, CH_3OH*

/THF (1/2, v/v). e) ***14*** *or* ***15****, Et_3N, CH_2Cl_2/DMF (4/1, v/v),* ***16****, 95%;* ***17****, 90%. f) NH_2 NH_2•H_2O, EtOH,* ***18****, 80%;* ***19****, 86%. g) dithiothreitol (DTT), Ac_2O,* ***20****, 90%;* ***21****, 95%.*

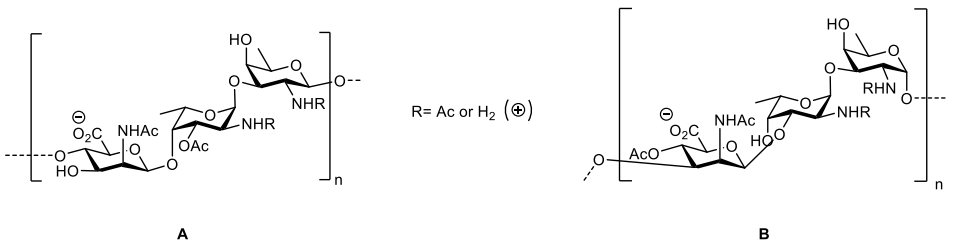
CAPSULAR POLYSACCHARIDES (CPs)

Capsular polysaccharides (CPs) are extracellular cell-wall components comprising long polysaccharide chains covalently attached to the peptidoglycan layer. They represent a first line of defence for bacteria by shielding important cell-wall constituents and providing a mechanism to evade phagocytic uptake and killing by the host immune system.[15] They also contribute to host colonization and biofilm formation and thereby in the progression of invasive diseases. To date, 13 serotypes have been identified among the clinical *S. aureus* isolates, carrying different CPs.[16]

CP5 and CP8

S. aureus CP5 and CP8 are the most abundant among the clinical isolates, and they account for the 25%-50% of clinically encountered MRSA.[17] Most of the vaccine candidates that reached an advanced stage in clinical trials, contained either of these two CPs as antigenic component.[18] CP5 and CP8 are structurally very similar.[19] The first is constituted by a trisaccharide repeating unit composed of an *N*-acetyl mannuronic acid, β-(1,4) linked to an *N*-acetyl-L-fucose moiety, bearing an acetyl group at the C-3-alcohol, which is α-(1,3)-linked to an *N*-acetyl-D-fucose. The repeating units are linked through an β-(1,4)-linkage between the latter fucose and the mannuronic of the next repeating unit (Figure 3A). The trisaccharide unit from CP8 is composed of the same monosaccharides, but the mannuronic acid is β-(1,3) linked to an *N*-acetyl-L-fucose and the acetyl substituent is placed on the C-4-hydroxy of the mannuronic acid unit and the *N*-acetyl-D-fucose is linked to the C-3 of the mannuronic acid through an α-linkage (Figure 3B). These structures have been shown to possess zwitterionic character originating from the presence of a negative charge from the mannuronic acid and a positive charge, resulting from (random) deacetylation of one of the two fucose residues. Currently it is not known how many positive charges are found in these structures and where exactly they reside in the polysaccharides.[20]

Figure 3: *Structures of trisaccharide repeating units of S. aureus CPs type 5 and type 8*



A **B**

The synthesis of these two CP trisaccharide units represents a great challenge due to the presence of several 1,2-*cis* glycosidic linkages, the anionic charges and the *O*-acetyl substituents. The first synthesis of the CP5 repeating unit was reported by Adamo and co-workers (Scheme 2A).[21] In their strategy, a benzyl glucuronate imidate donor **22** was used to introduce the β-linkage to the C-4 of L-fucosyl **23**, through a TMSOTf-mediated glycosylation affording the desired disaccharide **24** in 61% yield. The conversion to the *manno*-configured disaccharide derivative was achieved by removal of the levulinoyl protecting group, installation of the triflate and finally nucleophilic substitution using tetrabutylammonium azide. After deallylation and introduction of the imidate leaving group, disaccharide **26** was coupled with acceptor **27**, bearing an *N*-Cbz protected amino propyl spacer for ligation purposes, achieving trisaccharide **28** as a mixture of α/β anomers (2.4/1). Final hydrogenolysis and chemoselective *N*-acetylation with acetic anhydride in methanol deliver the final trisaccharide **29**. Several attempts were carried out to selectively reduce the azide groups over the Cbz protected amine of the linker, but unfortunately, either Staudinger conditions or treatment with H_2S led to (partial) lactamization of the mannuronic unit. From the immunological evaluation by competitive ELISA and immunodot blot experiments, it has become clear that longer fragments are needed to be sufficiently antigenic and effectively mimic the native CP structures.

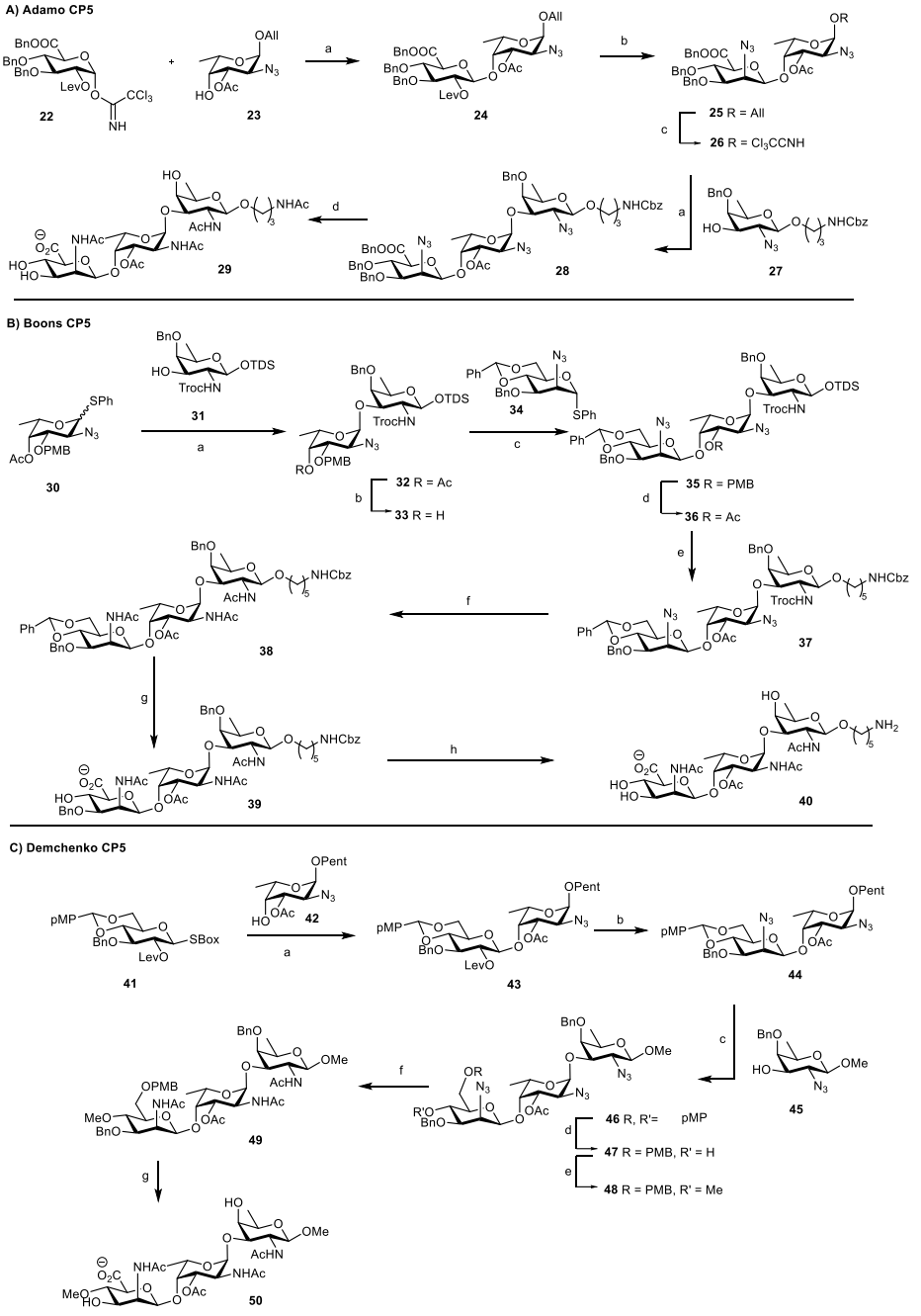
In 2015, Boons and co-workers reported a different synthetic strategy towards the synthesis of the CP5 trisaccharide unit (Scheme 2B).[22] First L-fucose donor **30** was coupled to D-fucose acceptor **31** and different glycosylation conditions were explored. Pre-activation of donor **30**, using either 1-benzene-sulfonyl piperidine (BSP) or diphenylsulfoxide (DPS) in the presence of Tf_2O and 2,4,6-tri-*tert*-butylpyrimidine as acid scavenger, stereoselectively afforded disaccharide **32** in 30% yield. Instead, activation of donor **30** with NIS in the presence of TMSOTf as promotor delivered the desired disaccharide **32** in much higher yield (72%) but as a 4:1 α/β-mixture. After treatment of the disaccharide with NaOMe in MeOH to effectuate the removal of the acetyl ester, disaccharide acceptor **33** was coupled with benzylidene protected azido-mannose donor **34**. Via formation of anomeric α- triflate at low temperature using DPS/Tf_2O as promoter, the β-mannoside trisaccharide **35** was formed as the major anomer and isolated in 72% yield. Subsequently, the PMB protecting group was replaced by an acetyl to deliver fully protected trisaccharide **36** in excellent yield over two steps. At the reducing end the TBS group was removed to introduce an *N*-Cbz protected aminopentanol spacer as ligation handle via the intermediate *N*-phenyl trifluoroacetimidate. In order to avoid the possible

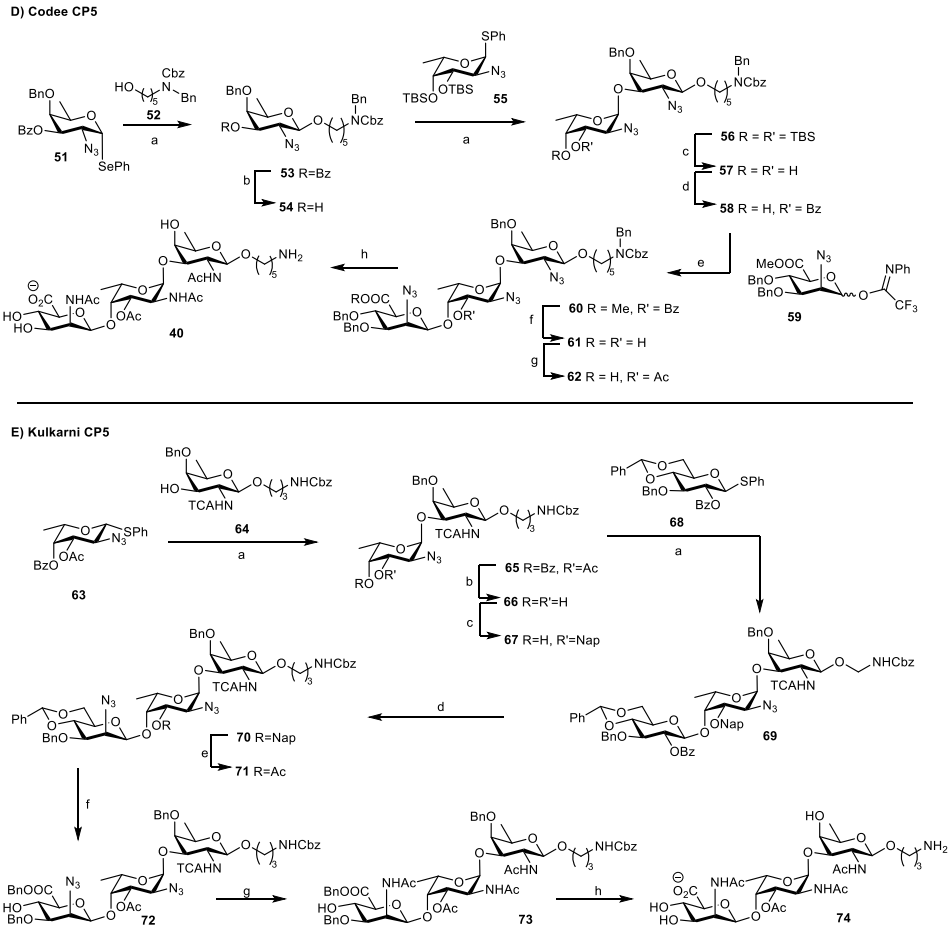
formation of the lactamized by-product, the azide and Troc-groups were first converted to the corresponding acetamide moieties using zinc-mediated reductions in the presence of AcOH and Ac_2O, affording compound **38** in 63% yield. After acidic hydrolysis of the benzylidene group, the primary alcohol of the mannose sugar was selectively oxidized using a one-pot TEMPO/NaOCl-$NaClO_2$ procedure yielding compound **39** in good yield over two steps. At last, all the benzyl type groups were removed by hydrogenolysis using $Pd(OH)_2$ in MeOH. Completely deprotected trisaccharide **40** was isolated in quantitative yield.

In 2016, Demchenko and co-workers described the synthesis of a CP5 trisaccharide unit bearing methyl groups at the sides of propagation of the polysaccharide sequences (Scheme 2C).[23] First glucosyl donor **41**, bearing a Lev-protecting group at the C-2-hydroxy was coupled by selective activation of the SBox leaving group using AgOTf to L-fucose acceptor **42** delivering disaccharide **43** in 78% yield as a single anomer. Epimerization of the glucosyl C-2 stereocenter was achieved by a three-step sequence, similar to the one described previously by Adamo, affording mannosyl disaccharide **44** in 70% yield overall. Subsequently, disaccharide **44** was coupled to D-fucose acceptor **45** by activation of the *O*-pentenyl group with NIS and TfOH, affording the fully protected trisaccharide **46** as a single anomer. The differences in stereochemical outcome of this glycosylation and the condensation between donor **26** and acceptor **27** as reported by Adamo, described above, are at present difficult to rationalize. Next the 4'',6''-*p*-methoxybenzylidene was regioselectively opened using $NaCNBH_3$ in the presence of 2M HCl after which the C-4''-hydoxy was capped with a methyl group affording **48** in 74% yield. All azide groups were reduced using propane 1,3-dithiol and triethylamine in pyridine, followed by acetylation, obtaining the derivative **49** in 91% yield. The last steps involved deprotection of the PMB group, followed by selective oxidation of the primary alcohol using TEMPO/BAIB and finally a hydrogenolysis event, affording the target compound **50** in 73% over the three steps.

One year later, another synthetic route towards the CP5 trisaccharide unit was published by Codée and co-workers (Scheme 2D).[24] Their strategy relied on a [1+2] coupling similar to the one applied by Boons. First D-fucosyl selenophenyl donor **51** was coupled with aminopentanol spacer derivative **52** under pre-activation conditions using Ph_2SO, Tf_2O and TTBP in a mixture of DCM and Et_2O. It was observed that the presence of Et_2O as co-solvent increased the stereoselectivity of this glycosylation reaction and product **53** was isolated in 80% yield as a 1:7 α/β-mixture. Removal of the benzoyl moiety under Zémplen conditions afforded acceptor **54** in 95% yield, which was next coupled to L-fucose donor **55**. In an extensive study on the reactivity of azidofucose donors, it was observed that 'arming' protecting groups (such as di-*tert*-butyldimethylsilyl ethers) on the C-3 and C-4-hydroxy groups enhances the α-stereoselectivity of the glycosylation reaction. Indeed, α-linked disaccharide **56** was isolated as a single anomer in 76% yield. Both silyl groups were next removed and the C-3'-OH was selectively benzoylated using Taylor's boron catalyst. Different than the strategies shown above, the synthesis of the fully protected trisaccharide **60** was achieved by employing a mannuronic acid donor (**59**), which enables the stereoselective formation of the required β-glycosidic linkage. The use of a pre-oxidized donor for the assembly of the trisaccharide unit reduces the functionalization and deprotection steps at a late stage of the synthesis. Using a large excess of donor **59**

and TBSOTf as promoter, β-linked trisaccharide **60** was isolated in 75% as a single anomer. Subsequently, the ester group were removed in a KOH/H_2O_2 mediated saponification, followed by the introduction of the acetyl group on the L-fucosyl C-3-alcohol and conversion of the azides into the corresponding acetamides using AcSH in pyridine. Final hydrogenolysis delivered target trisaccharide **40** in 37% overall yield.
Recently, one additional synthesis of the CP5 trisaccharide unit was reported by Kulkarni and co-workers.[25] First a condensation between L-fucose donor **63** and D-fucose acceptor **64** using NIS/TMSOTf as promotor delivered α-linked disaccharide **65** in 94% yield. Zémplen conditions were used to simultaneously remove all esters and subsequently the tin-mediated regioselective protection delivered disaccharide **67**. Compound **67** was used as acceptor for the glycosylation reaction with glucosyl donor **68** in the presence of NIS and TMSOTf delivering trisaccharide **69** in 84% yield. The mannosyl trisaccharide derivative **70** was then obtained by employing a similar epimerization strategy discussed previously. Functionalization and protecting group manipulations to deliver the final trisaccharide **74**, started with a selective oxidative cleavage of the Nap-ether, followed by acetylation, delivering intermediate **71**. Subsequent benzylidene hydrolysis, selective oxidation of the primary alcohol to the carboxylic acid and protection with a benzyl group afforded trisaccharide **72** in 69% overall yield. The azides were converted to acetamide groups by a zinc-mediated reduction, followed by acetylation and finally a hydrogenolysis reaction to deliver the target compound **74** in 72% over the three steps.


A) Adamo CP5
22
23
24
25 R = All
26 R = Cl3CCNH
27
28
29
B) Boons CP5
30
31
32 R = Ac
33 R = H
34
35 R = PMB
36 R = Ac
37
38
39
40
C) Demchenko CP5
41
42
43
44
45
46 R, R'= pMP
47 R = PMB, R' = H
48 R = PMB, R' = Me
49
50

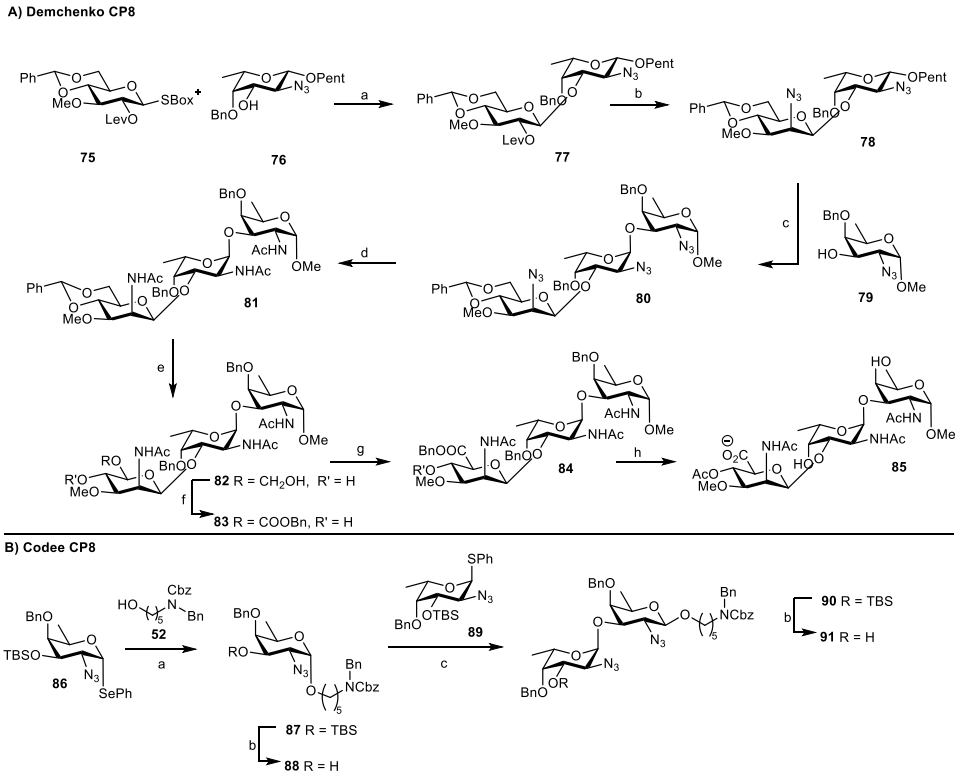


Scheme 2: *Chemical synthesis of trisaccharide repeating units of S. aureus CPS type 5. A) a) TMSOTf, CH_2Cl_2, -10 °C,* ***24****, 61%;* ***28****, 65%. b) (i) NH_2NH_2•AcOH, CH_2Cl_2/CH_3OH, 86%; (ii) Tf_2O, Py, CH_2Cl_2; $TBAN_3$, Toluene, 70%. c) (i) $PdCl_2$, CH_3OH, 78%; (ii) CCl_3CN, DBU, CH_2Cl_2, 98%. d) (i) 10% Pd/C, CH_3OH/H_2O, AcOH; (ii) Ac_2O, CH_3OH/H_2O, 40%. B) a) NIS, TMSOTf, CH_2Cl_2, -60 °C, 73%, α/β = 4/1. b) Na(cat.), CH_3OH, guanidine•HCl, rt, 18 h, quant. c) DSP, Tf_2O, TTBP, CH_2Cl_2, -60 °C to -30 °C, 1 h, 72%. d) (i) DDQ, CH_2Cl_2/H_2O (9/1, v/v), rt, 4 h; (ii) Ac_2O, Py, DMAP, rt, 2 h, 85% over 2 steps. e) (i) HF/Py, THF, rt, 18 h, 90%; (ii) CF_3(=NPh)CCl, Cs_2CO_3, CH_2Cl_2, rt, 2 h; (iii) $HO(CH_2)_5NHCbz$, TMSOTf, CH_2Cl_2/CH_3CN (1/1, v/v), -78 °C, 10 min, 72% over 2 steps. f) Zn, THF, AcOH, Ac_2O, rt, 1 h, 63%. g) (i) 80% aq. AcOH, 90 °C, 2 h; (ii) TEMPO, BAIB, CH_2Cl_2/H_2O (4/1, v/v), rt, 3 h, $NaClO_2$, 2-methyl-2-butene, t-BuOH, rt, 1 h, 61% over 2 steps. h) $Pd(OH)_2$, CH_3OH, AcOH, 2 h, quant. C) a) AgOTf, 3Å MS, 1, 2-dichloroethane, 1 h, 78%, β only. b) NH_2NH_2•AcOH, CH_2Cl_2/CH_3OH (20/1, v/v), rt, 16 h, 86%; Tf_2O, Py, CH_2Cl_2, 0 °C, 4 h; NaN_3, DMF, 60 °C, 3 h, 81%. c) NIS, TfOH, 3Å MS, 1, 2-dichloroethane, 0 °C, 1 h, 79%, α only. d) $NaCNBH_3$, 2 M HCl/ether, THF, rt, 1 h, 94%. e) MeI, Ag_2O, DMF, 16 h, rt, 79%. f) $HS(CH_2)_3SH$, Et_3N, Py, H_2O, rt, 3 h; then Ac_2O, CH_3OH, rt, 2 h, 91% over 2 steps. g) (i) DDQ, H_2O, CH_2Cl_2, rt, 6 h, 84%; (ii) TEMPO, BAIB, aq. CH_2Cl_2, rt, 16 h; H_2, 10% Pd/C, aq. EtOH, rt, 16 h, 73% over 2 steps. D) a) Ph_2SO, Tf_2O, TTBP, 3Å MS, -80 °C to -40 °C, for* ***53:*** *acceptor* ***52****, CH_2Cl_2/Et_2O (1/1, v/v), 80%, α/β = 1/7; for* ***56:*** *donor* ***55****, CH_2Cl_2, 76%, α only. b) Na, CH_3OH, 95%. c) TBAF, THF, quant. d) Taylor's catalyst, BzCl, DIPEA, CH_3CN, 67%. e) TBSOTf, 3Å MS, CH_2Cl_2, -80 °C to -55 °C, 5 h, 75%. f)*

*H_2O_2, KOH, THF, H_2O. g) Ac_2O, Py. h) (i) AcSH, Py; (ii) $Pd(OH)_2/C$, H_2, AcOH, THF, t-BuOH, H_2O, 57% over 3 steps. E) a) NIS, TMSOTf, 3Å MS, CH_2Cl_2, for **65:** 0 °C, 30 min, 94%; for **69:** -60 °C to -10 °C, 4 h, 84%; b) CH_3ONa, CH_3OH, rt, 82%. c) Bu_2SnO, toluene, 110 °C, TBAB, NapBr, 60 °C, 79%. d) NaOMe, CH_3OH, rt, 2 h, 90%; Tf_2O, Py, CH_2Cl_2, 0 °C, 30 min; $TBAN_3$, toluene, 60 °C, 3 h, 71% over 2 steps. e) (i) DDQ, CH_2Cl_2/H_2O (9/1, v/v), rt, 1 h; (ii) AcCl, Py, CH_2Cl_2, 0 °C - rt, 4 h, 92% over 2 steps. f) (i) 80% aq. AcOH, 80 °C, 1 h; (ii) TEMPO, BAIB, CH_2Cl_2/H_2O (4/1, v/v), rt, 14 h, (iii) BnBr, $NaHCO_3$, DMF, 6 h, 69% over 3 steps. g) (i) Zn, AcOH, THF, rt, 12 h; (ii) Ac_2O, Et_3N, CH_3OH. h) $Pd(OH)_2/C$, EtOH, rt,10 h, 72% over 3 steps.*

Up to now only one synthetic route towards the CP8 trisaccharide unit has been described.[23] In 2015 Demchenko and co-workers reported the synthesis of the trisaccharide bearing methyl groups at the sites of propagation similar to the CP5 fragment **50** shown previously (Scheme 3A). First, coupling of glucosyl donor **75** to L-fucosyl acceptor **76** delivered disaccharide **77** in 73%. Subsequent C-2'-epimerization afforded mannosyl disaccharide derivative **78** in 65% overall yield. Glycosylation of donor **78** and acceptor **79** was performed using NIS and TfOH, delivering fully protected trisaccharide **80** in 87% yield as a single α-anomer. Subsequently, the azide groups were reduced using propane-1,3-dithiol and acetylation afforded the triacetamido derivative **81**. Hydrolysis of the benzylidene group, followed by TEMPO/BAIB mediated oxidation and subsequent protection of the newly formed carboxylic acid with a benzyl group delivered compound **83** in 44% overall yield. Finally, acetylation on the C-4''-OH of the mannuronate moiety and a last hydrogenolysis step afforded target compound **85** in 96% over the last two steps.

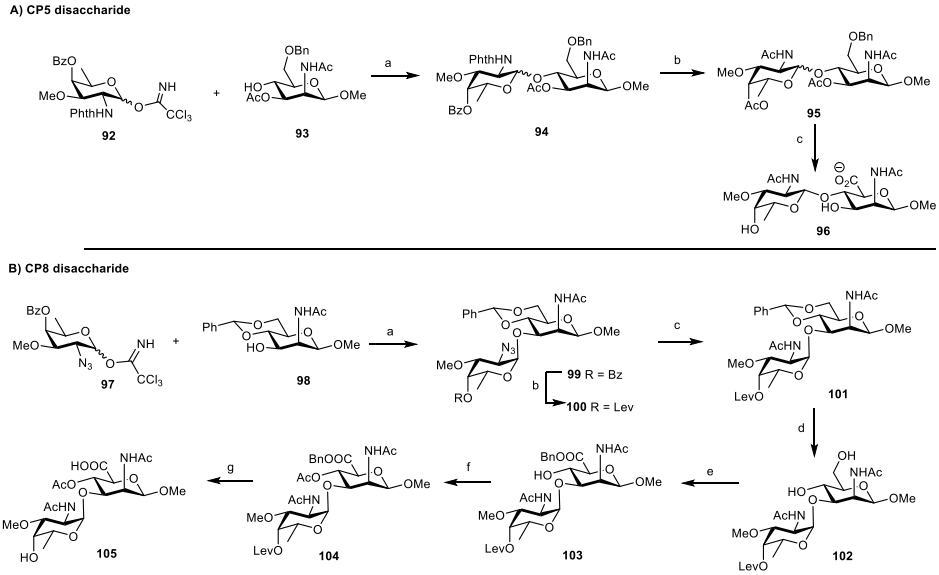
Together with the synthesis of the CP5 trisaccharide unit,[24] Codée and co-workers reported the synthesis of a semi-protected CP8-disaccharide core, built up from the two enantiomeric fucosyl moieties, that can be used to achieve the complete trisaccharide fragment in the future (Scheme 3B). First D-fucose donor **86** was coupled to aminopentanol linker **52**. In order to enhance the stereoselectivity a protocol, previously described by Bennett and co-workers, was explored. Donor **86** was pre-activated using Ph_2SO/Tf_2O at -78°C, followed by the addition of acceptor and tetrabutylammonium iodide (TBAI), to enable the *in situ* formation of the anomeric iodide, and *N*-methylmaleimide as an electrophilic scavenger. This protocol delivered D-fucose **87** in 85% yield as α/β mixture (7:1). After removal of the silyl-group, D-fucose acceptor **88** was coupled with L-fucose donor **89** delivering fully protected disaccharide **90** in 73% as α/β mixture (7/1). Subsequent deprotection of the silyl group with TBAF afforded the pure α-linked disaccharide **91** in 71% yield.



Scheme 3*: Synthesis of trisaccharide repeating units and disaccharide fragment of S. aureus CPS type 8. A) a) AgOTf, 3Å MS, 1, 2-dichloroethane, rt, 1 h, 73%, β only. b) (i) NH_2NH_2•AcOH, CH_2Cl_2/CH_3OH (20/1, v/v), rt, 3 h, 72%; (ii) Tf_2O, Py, CH_2Cl_2, 0 °C, 3 h; (iii) NaN_3, DMF, 60 °C, 16 h, 90%. c) NIS, TfOH, 3Å MS, 1, 2-dichloroethane, 0 °C, 1 h, 87%, α only. d) (i) $HS(CH_2)_3SH$, Et_3N, Py, H_2O, rt, 6 h; (ii) Ac_2O, CH_3OH, rt, 16 h, 94% over 2 steps. e) aq. TFA, CH_2Cl_2, rt , 16 h, 92%. f) (i) TEMPO, BAIB, aq. CH_2Cl_2, rt, 4 h; (ii) BnBr, $NaHCO_3$, DMF, rt, 16 h, 61% over 2 steps. g) Ac_2O, Py, rt, 16 h, 99%. h) H_2, 10% Pd/C, aq. EtOH, rt, 24 h, 97%. B) a) Ph_2SO, Tf_2O, TTBP, N-methylmaleimide, 3Å MS, -80°C to -70°C; TBAI, -80°C to r.t., 1,4 dioxane,* ***52****, 85%, α/β = 7/1. b) TBAF, THF, 65% for* ***88****, 71% for* ***91****. c) Ph_2SO, Tf_2O, TTBP, CH_2Cl_2, -80°C to -70°C,* ***87****, -80°C to -50°C, 73%, α/β = 7/1.*

Recently Demchenko and co-workers reported the synthesis of two disaccharides (**96** and **105**) from CP5 and CP8 to develop chemistry that would allow the interconnection of the repeating units.[26] In the case of the CP5 fragment (Scheme 4A), D-fucose trichloroacetimidate donor **92** was coupled to mannose acceptor **93** using a catalytic amount of TMSOTf affording disaccharide **94** in 63% as an 1:5 α/β mixture. Treatment of the dimer with hydrazine monohydrate and subsequent acetylation provided disaccharide intermediate **95** in 81% yield. Hydrogenolysis, TEMPO/BAIB mediated oxidation to carboxylic acid of the primary alcohol and final hydrolysis of acetyl esters delivered disaccharide derivative **96** in 44% yield. In the case of the CP8 disaccharide fragment (Scheme 4B), D-fucose donor **97** was coupled with acceptor **98** using TfOH in DCM affording disaccharide **99** in 65% yield as a single anomer, likely as the result of the presence of the C-4 fucosyl benzoyl protecting group, capable of performing long-range

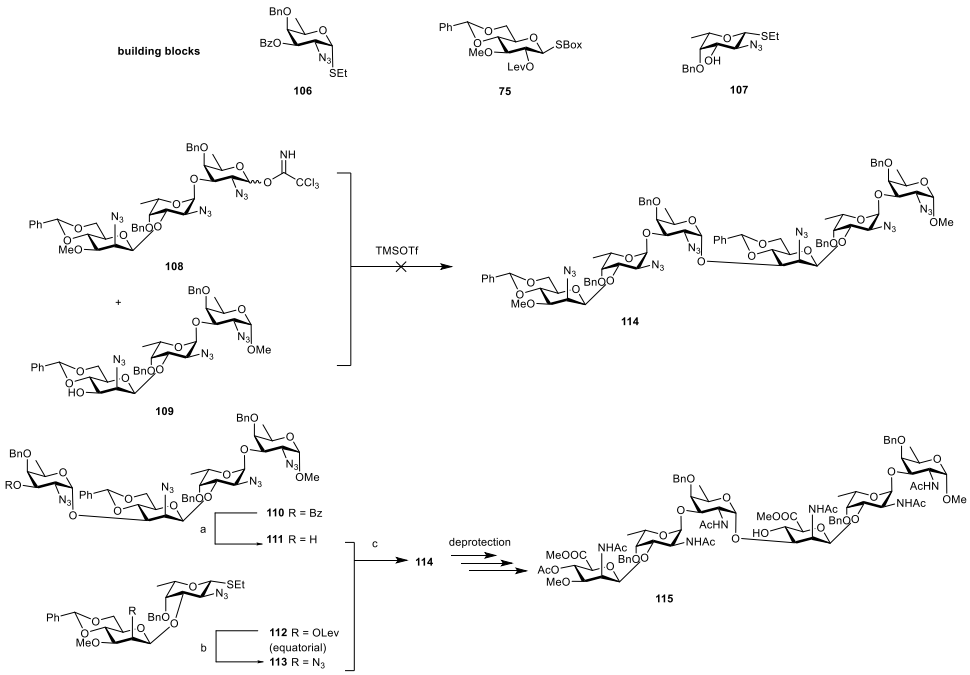
participation[27]. Next, the benzoyl group was replaced by a levulinoyl ester, obtaining disaccharide intermediate **100** in 89% over two steps. Reduction and subsequent acetylation of the azide delivered disaccharide derivative **101** in excellent yield, after which hydrolysis of the benzylidene acetal, selective oxidation of the primary alcohol, benzyl ester formation and acetylation delivered disaccharide **104** in 60% yield overall. Finally, the selective removal of the levulinoyl group with hydrazine acetate and hydrogenolysis afforded target disaccharide **105** in 93% over the two steps.



***Scheme 4**: Synthesis of interconnection disaccharides of S. aureus CPS type 5 and type 8. A) a) TMSOTf, 4Å MS, CH_2Cl_2, α/β = 1/5. b) (i) $NH_2NH_2•H_2O$, CH_3OH, 65 °C; (ii) Ac_2O, Py, 81%. c) (i) H_2, Pd/C, EtOH, H_2O, 75%; (ii) TEMPO, BAIB, CH_2Cl_2, H_2O, 70%; (iii) aq. NaOH, CH_3OH, 84%. B) a) TfOH, 4Å MS, CH_2Cl_2, 65%. b) (i) NaOMe, CH_3OH; (ii) LevOH, DIC, DMAP, CH_2Cl_2, 89%. c) (i) $HS(CH_2)_3SH$, Et_3N, Py, H_2O, rt; (ii) Ac_2O, CH_3OH, rt, 96% over 2 steps. d) TFA, wet CH_2Cl_2, 81%. e) (i) TEMPO, BAIB, CH_2Cl_2, H_2O; (ii) BnBr, $NaHCO_3$, DMF, 76%. f) Ac_2O, Py, 99%. g) (i) $NH_2NH_2•AcOH$, CH_3OH, CH_2Cl_2; (ii) H_2, Pd/C, EtOH, H_2O, 93%.*

The first synthesis of a fully protective CP8 hexamer has been achieved recently by Demchenko's group (Scheme 5).[28] In line with their previous studies, they capped the propagation sites with methyl groups. At first a [3+3] strategy was explored and to this end trisaccharides **108** and **109** were generated via a synthetic route similar to the one described above. Unfortunately, the TMSOTf-mediated glycosylation didn't deliver any desired product and acceptor **109** was fully recovered. The authors attributed this failure to steric hindrance from both the benzylidene group of **109** and the bulkiness of donor **108**. Therefore, a different synthetic approach was adopted based on a [2+1+3] strategy. To this end, donor **106** was synthesized and coupled to trisaccharide acceptor **109** using NIS and TfOH as promoter. Tetrasaccharide **110** was isolated in 81% yield as a single anomer. After removal of the benzoyl protecting group using Zémplen conditions, tetrasaccharide acceptor **111** was coupled with disaccharide thioethyl donor **113**,

activated by the NIS/TfOH couple, affording completely protected hexamer **114** in 85%. Subsequently, this hexasaccharide was converted to the acetamide derivative using an unusual double reduction/acetylation protocol. First, 1,3-propandithiol and TEA were used in wet pyridine at 70°C, which was followed by acetylation, after which the product was treated with zinc dust in acetic acid and acetic anhydride. After deprotection of the benzylidene acetals, the primary alcohols were oxidized using Huang's one-pot TEMPO/NaOCl–$NaClO_2$ procedure. After protection of the carboxylic acids with benzyl bromide in the presence of NaH and DMF, the C-4-OH of the mannuronate sugars were acetylated. By NMR and mass spectroscopy, it was found, however, that compound **115** was formed due to probably a transesterification while quenching the acetylation with methanol. No further deprotection steps were reported.



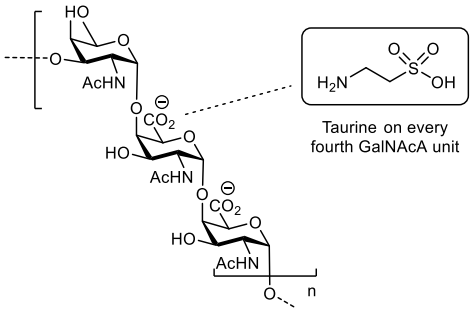
Scheme 5: *Synthesis of hexasaccharides of S. aureus CPS type 8. For synthesis of tetrasaccharide* ***110****: donor* ***106****, acceptor* ***109****, NIS, TfOH, CH_2Cl_2, 0 °C, 81%; For synthesis of disaccharide* ***112****: donor* ***75****, acceptor* ***107****, AgOTf, CH_2Cl_2, 0 °C, 85%. a) CH_3ONa, CH_3OH, 75%. b) (i) NH_2NH_2•AcOH, (ii) 85%; Tf_2O, Py 0 °C; (iii) NaN_3, DMF, 70 °C, 59%. c) NIS, TfOH, 1,2-dichloroethane, 0 °C, 4 h, 85%.*

Up to now, no extensive biological studies have been reported using the synthetic CP5 or CP8 trisaccharides. However, as described above, it can be concluded from the competitive ELISA assay and immunodot blot analysis performed by Adamo, that longer fragments might be necessary to understand the key structural immunogenic elements of these saccharides.

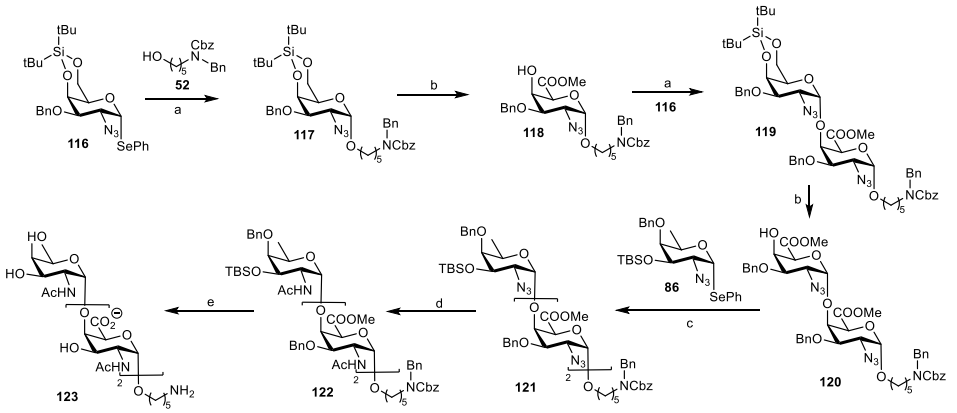
STRAIN M (CP1)

The isolation of the capsular polysaccharide from S. *aureus* stain M was reported in 1962 by Smith,[29] and the complete molecular structure was later elucidated by Murthy et al. in 1983 (Figure 4).[30] The repeating unit consists of an *N*-acetyl-D-fucose α(1,4)-linked to an *N*-acetyl-D-galacturonic acid, which is α(1,4) linked to a second *N*-acetyl-D-galacturonic acid, connecting to the next fucose moiety of the next trisaccharide repeating unit through an α-(1,3)-linkage. It has been described that a taurine unit is incorporated in one out of four GalNAcA sugars through an amide bond.

Figure 4: *Structure of the CPS from S. aureus strain M*



In 2017 Codée and co-workers reported the synthesis of the trisaccharide unit featuring an aminopentanyl linker for ligation purposes (Scheme 6).[31] The synthetic strategy relied on a [1+2] coupling strategy and a post-glycosylation oxidation procedure to introduce the galacturonic acid moieties Using galactosamine donors, bearing a di-*tert*-butylsilylidene ketal ensured the formation of the desired α-linkages, as shown originally by Kiso and co-workers.[32] Thus, donor **116** was first coupled to amino-protected pentanol spacer **52** affording product **117** in 82% yield. Subsequently, the silylidene protecting group was removed using HF*pyridine, after which the primary alcohol was selectively oxidized and methylated, delivering compound **118** in 85% yield. Acceptor **118** was coupled with donor **116** using NIS and catalytic amount of TMSOTf and disaccharide **119** was isolated in 88% as a single anomer. The same sequential steps of silyl deprotection, oxidation and methylation of the newly carboxylic acid afforded disaccharide acceptor **120** in 84% yield. A modification of the oxidation protocol was used since cleavage of glycosidic bond was observed when using the 'normal' TEMPO/BAIB conditions. In this new oxidation protocol, the aldehyde was first formed using TEMPO and BAIB under anhydrous conditions, after which a Pinnick oxidation was employed to provide the desired carboxylic acid. Final glycosylation between 'armed' D-fucose donor **86** and acceptor **120** was performed using Ph_2SO/Tf_2O activation system stereoselectively delivering fully protected trisaccharide **121** in 79% yield. Conversion of the azides into acetamides using AcSH in pyridine afforded intermediate **122** after which desilylation, saponification and hydrogenolysis delivered the target trisaccharide **123**.

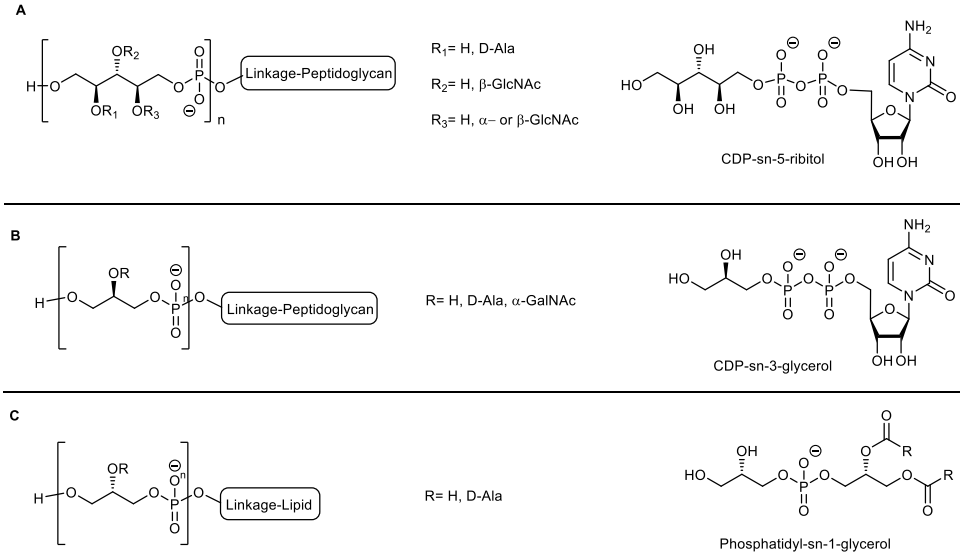


Scheme 6: *Synthesis of trisaccharides repeating unit of S. aureus CPS strain M. a) NIS, TMSOTf, CH_2Cl_2, 0 °C, 2 h, for* ***117****, 82%; for* ***119****, 88%. b) i) HF•Py, THF, rt, 2 h, quant; ii) then TEMPO, BAIB, AcOH, CH_2Cl_2, H_2O, 4 °C, 16 h; iii) MeI, K_2CO_3, DMF, rt, 16 h, for* ***118****, 85%; for* ***120****, 84%. c) i) Ph_2SO, TTBP, CH_2Cl_2, Tf_2O, -80 °C to -70 °C, ii)* ***86****, -80 °C to -40 °C, 6 h, 79%. d) AcSH, Py, 9 d, rt, 47%. e) HF•Py, THF, rt, 2 d; 30% aq. H_2O_2, KOH, THF, t-BuOH, H_2O, rt, 2 d; then H_2, $Pd(OH)_2$/C, AcOH, THF, t-BuOH, H_2O, 3 d, 34% over 3 steps.*

TEICHOIC ACIDS (TAs)

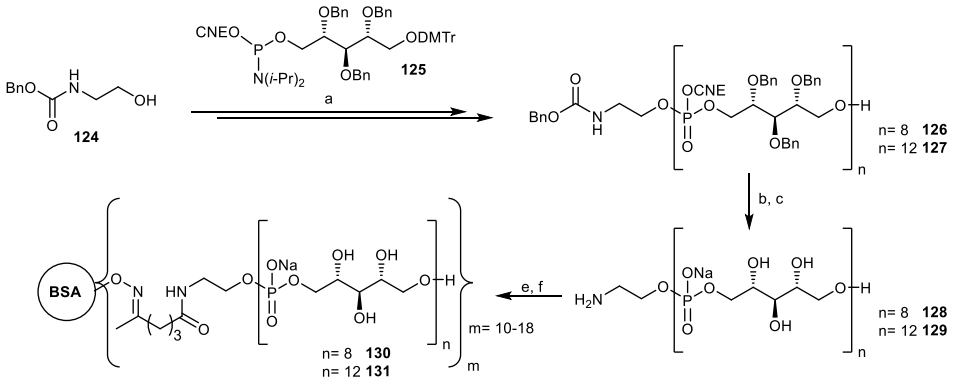
Teichoic acids (from the Greek word τείχος, fortified wall) are structurally diverse anionic carbohydrate-based polymers that can be found in the cell wall of the majority of Gram-positive bacteria. They are divided in two main classes: wall teichoic acids (WTAs), which are covalently attached to the peptidoglycan matrix, while lipoteichoic acids (LTAs) are inserted in the lipid bilayer through a diacyl glycerol lipid anchor. They are involved in several important biological processes contributing to bacterial fitness and virulence.[33] Since TAs are exposed to the extracellular milieu, they constitute possible recognition sites for host cell surface lectins, antibodies of the host immune system and phage binding.[34] TAs have been considered to be suitable antigen candidates for vaccine development.[35] The WTA from S. *aureus* is generally constituted by 1→5-linked ribitol phosphate (RboP) units, which can be modified with D-alanine substituents at the C-2 and α- or β-*N*-acetylglucosamine appendages at C3 or C4 (Figure 5A).[31] However, some strains carry structurally different WTAs. For example, the WTA from S. *aureus* ST395 is composed of 3→1 linked glycerol phosphate (GroP) chains, where the C-2 position can be decorated with either D-Ala or α-GalNAc substituents (Figure 5B).[36] In contrast, S. *aureus* LTAs feature 1→3 linked GroP-oligomers having D-Ala or α-GlcNAc substituents.[37] Notably, the GroP-backbone of WTA and LTA are enantiomeric structures, whose stereochemistry has been assigned based on their biosynthetic pathways. While GroP-WTA is built by oligomerization of CDP-*sn*-3-glycerol, LTAs are constructed using phosphatidyl-*sn*-1-glycerol (Figure 5C).[38]

Figure 5: *General structures and biosynthetic precursors A) RboP-WTA from S. aureus and CDP-sn-5-ribitol. B) GroP-WTA from S. aureus ST395 and CDP-sn-3-glycerol. C) GroP-LTA from S. aureus and phosphatidyl-sn-1-glycerol.*



WALL TEICHOIC ACIDS

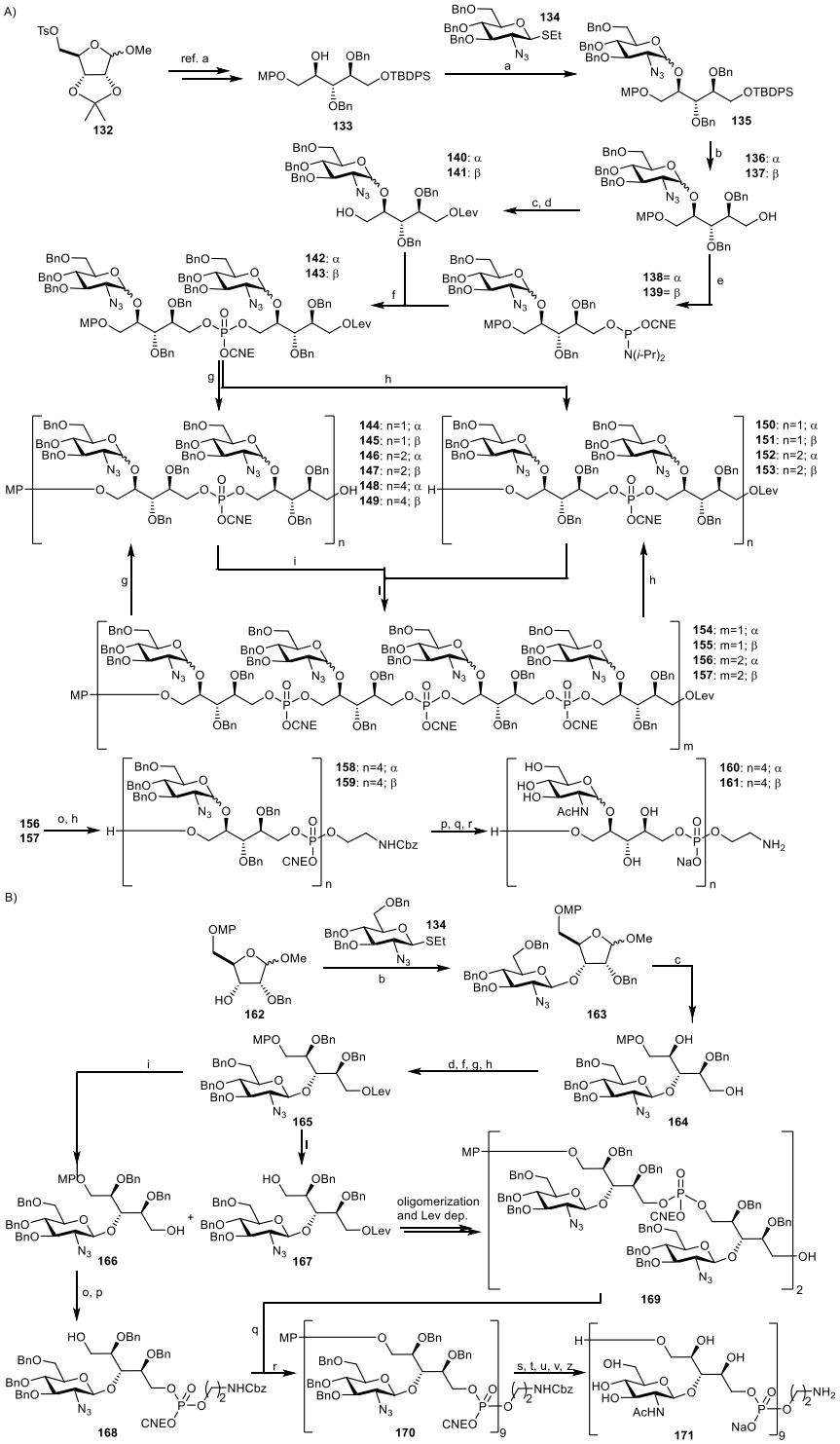
In 2006 Pozsgay and co-workers reported the synthesis of an RboP-octamer and dodecamer, equipped with an amino spacer for further conjugation to BSA as carrier protein.[39] Their synthetic strategy relied on the introduction of the phosphodiester linkage using the phosphoramidite approach, developed for solid-phase nucleotide assembly. In scheme 7 the synthetic route is shown, where phosphoramidite derivative **125** was used as key building block, bearing a dimethoxytrityl (DMTr) group at the hydroxyls to be elongated. Phosphoramidite **125** was coupled with amino ethanol derivative **124** using tetrazole in ACN, followed by oxidation using I_2 in a mixture of THF and water. The DMTr group was removed using AcOH in DCM and water. After 8 and 12 cycles of coupling/oxidation/deprotection, fully protected octamer **126** and dodecamer **127** were obtained, respectively. Final compounds **128** and **129** were obtained after treatment of **126** and **127** with ammonium hydroxide in MeOH to remove the cyanoethyl protecting group, followed by hydrogenolysis using Pd/C. Conjugation to BSA of compound **128** and **129** was performed using a procedure developed by Kubler-Kielb and Pozsgay, involving the functionalization of the oligomers with a levulinoyl group and connection to the carrier protein through the formation of an oxime linkage. No evaluation of the WTA-BSA conjugates **130** and **131** has been reported to date.



Scheme 7: *Pozsgay's synthesis of a ribitol 12-mer-BSA conjugate. a) (i) 10 eq. 0.45 M tetrazole in CH_3CN, CH_3CN, r.t., 1 h; (ii) 0.5 M I_2 in 2:1 THF/H_2O; (iii) 85:10:5 AcOH/CH_2Cl_2/H_2O n=1, 88%; b) (i) 10eq. 0.45 M tetrazole in CH_3CN, CH_3CN, r.t., 1 h; (ii) 0.5 M I_2 in 2:1 THF/H_2O, n=8; 9.9%, n=12; 2.4%; c) (i) CH_3OH, conc. NH_4OH, 50 °C, 8 h; (ii) H_2, 10% Pd/C, 2:1 tBuOOH/H_2O, n=8; 66%, n=12; 80%; e) 5-ketohexanoic anhydride, Et_3N, CH_3OH, H_2O; f) aminooxy-BSA, PBS (pH=7.4), EDTA, glycerol.*

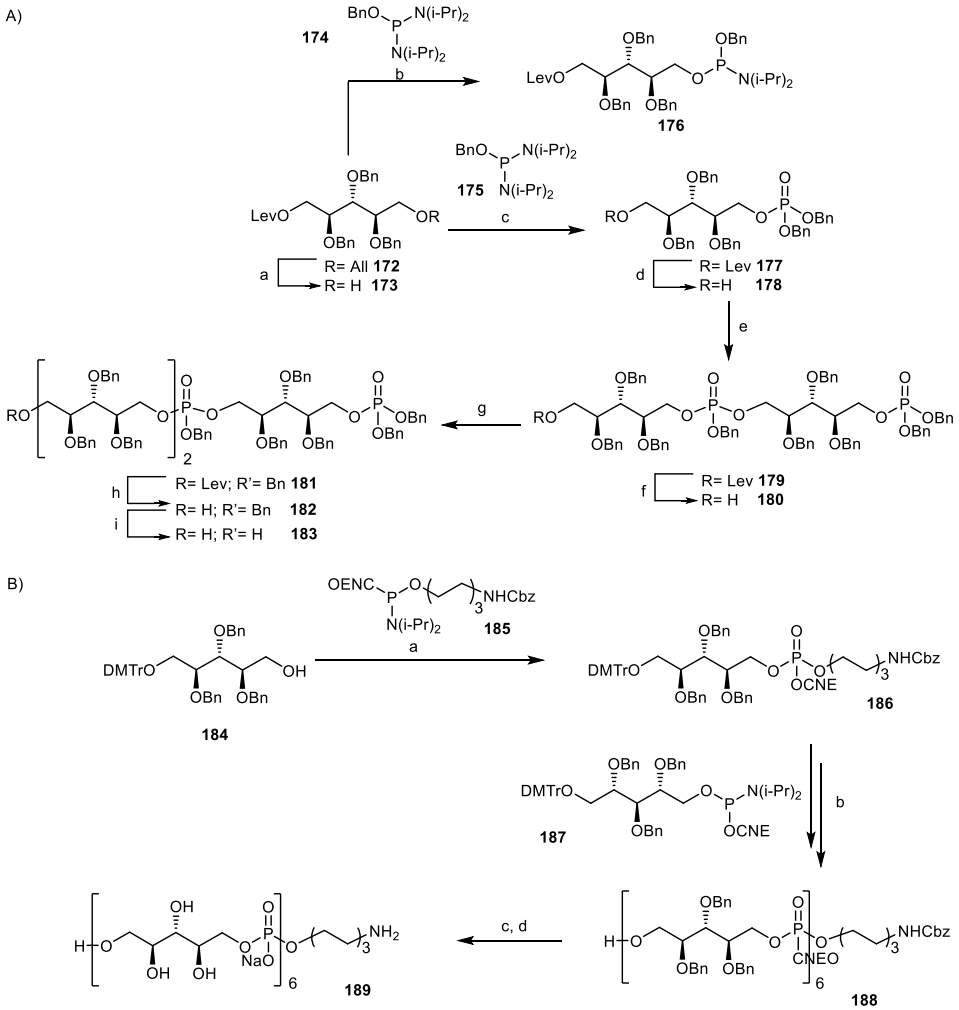
Three RboP oligomers, bearing different GlcNAc appendages, were recently synthesized by a team at Sanofi Pasteur, featuring either α-D-GlcNAc or β-D-GlcNAc at all C-4 hydroxyls of a RboP octamer (**160** and **161**) or a β-D-GlcNAc at all C-3 hydroxyls of a RboP nonamer (**171**).[40] In order to introduce a glucosyl moiety at the C4 of a ribitol synthon (Scheme 8A), acceptor **133** was synthesized in 6 steps from compound **132**. Glucosyl thiodonor **134** was then coupled to acceptor **133** under activation of NIS/TfOH yielding **135** in 94% as a 52/48 of α/β-mixture. The two anomers were separated after TBDPS removal. In order to access RboP dimers **142/143**, a phosphoramidite group was introduced and *in situ* coupled with compound **140/141**, obtained from **136**/**137** by protection of the primary alcohol with a levulinoyl group and subsequently removal of the methoxyphenyl (MP) group. The coupling step was performed using 5-ethiolthio-1H-tetrazole, while the oxidation was accomplished using I_2/pyridine/H_2O. Further elongation was accomplished by either removal of the Lev or MP group, delivering two tetramers **154** and **155** that were coupled using the same *in situ* condensation approach as described. The same strategy was then used to obtain octamers **156** and **157**. Next these octamers were functionalized with an alcohol spacer. Of note, the spacer was attached to these WTA oligomers at the opposite side of the peptidoglycan attachment. The final deprotection was accomplished by removal of the cyanoethyl groups, transformation of the azides into the corresponding acetamides and Birch-type reduction of all benzyl groups. In the case of 1,3-β-D-GlcNAc nonamer **171**, the glycosyl substituent was introduced by glycosylation of acceptor **162** and donor **134** using NIS/TfOH as activating system at low temperature (-70°C), affording disaccharide **163** in 84% yield (Scheme 8B). Subsequent hydrolysis and reduction delivered ribitol derivative **164** in 73% yield and after three protecting groups manipulation steps, compound **165** was obtained in excellent yield. Similar to the strategy described before, compounds **166** and **167** were used to build up a fully protected nonamer **170**. Deprotection of **170** using the same conditions as described above afforded target **171**.

In order to access the immunological properties of the different glycosylated RboP oligomers, synthetic fragments **160**, **161** and **171**, together with native RboP isolated from different strains, were conjugated to rEPA or S. *aureus* alpha toxin (Hla). The conjugation was performed via carbodiimide condensation reaction using hydrazide linker derivatives. Immunization in mice was performed using conjugated and as well nonconjugated fragments, with or without adjuvant. Subsequently IgG1 and IgG2 titers were determined after 0, 21, 35 and 42 days. A strong and robust immune response was elicited when the conjugates were used in combination with adjuvant. No differences were detected between synthetic and native TAs, but it was observed that C-4-β-GlcNAc RboP was able to induce antibodies that were cross-reactive towards different S. *aureus* strains, carrying either C-4-β-GlcNAc or C-3-β-GlcNAc substituents, suggesting that this antigen can be used for the development of a broad-spectrum S. *aureus* vaccine.



Scheme 8: *Synthesis of glycosylated RboP fragments by Sanofi Pasteur. A) a) CH_2Cl_2/Et_2O, NIS and TfOH at -20°C for 10 min, 94%, (52:48 α/β). b) TBAF 1M/AcOH, THF, 0°C to rt 17h, 40% β, 46%α. c) Levulinic acid, DMAP, EDCI, CH_2Cl_2. d) CAN, CH_3CN/H_2O, 0°C to rt 2h, 81% β. e) (i) Alcohol in ACN, cooled to 0°C, DIPEA and chloro-2-cyanoethyl-N,N-diisopropylamidite added at 0°C for 0.5 h, (ii) cool to -10 °C, add alcohol then 5-ethiolthio-1H-tetrazole 1h at 0°C, m=1, β, 80%, m=2, β, 72%. f) step e, 69% β. g) $NH_2NH_2•H_2O$, pyridine/AcOH, 83% β. h) step d, 79% β. i) step e,* ***150*** *(80%);* ***151*** *(72%). j) $NH_2NH_2•H_2O$, pyridine/AcOH, 80%β. k) step e, benzyl N-(2-hydroxyethyl) carbamate, 67%. l) CAN, CH_3CN/H_2O, 0°C to rt 2h,* ***159*** *(76%). m) thioacetic acid, pyridine, 3.5 days, β 97%. n) (i) Na, THF, NH_3, -78°C, 30 min; (ii) sat. aq. $NHCl_4$, -78°C, 1h,* ***161*** *(96%). B) a) TBAB, BnBr, 10% aq. NaOH, CH_2Cl_2, 34%. b) CH_3CN/propionitrile/CH_2Cl_2 (2:1:1), NIS and TfOH, at -70°C 10 min, 84%. c) (i) 3M HCl/dioxane, reflux 7h, 73%; (ii) $NaBH_4$, CH_3OH, 0°C. d) TBDMSCl, DMAP, TEA, CH_2Cl_2. e) BnBr, DMF, NaH. f) THF, AcOH, TBAF, 0°C to rt, 94%. g) Levulinic acid, DMAP, EDCI, CH_2Cl_2/dioxane 1:10 (0.08M). h) CAN, CH_3CN/H_2O, 0°C to rt 2h, 71%. i) (i) Alcohol in CH_3CN, cooled to 0°C, DIPEA and chloro-2-cyanoethyl-N,N-diisopropylamidite added at 0°C for 0.5 h; (ii) cool to -10°C, add second alcohol then 5-ethiolthio-1H-tetrazole 1h at 0°C, n=1 (82%), n=2 (79%). j) $NH_2NH_2•H_2O$, pyridine/AcOH, n=1 (86%), n=2 (82%). k) step i, benzyl N-(2-hydroxyethyl) carbamate, 65%. l) CAN, CH_3CN/H_2O, 0°C to rt 2h, 85%. m) step I, n=5, 77%. n) CAN, CH_3CN/H_2O, 0°C to rt, 2h, n=5, (74%). o) step i, n=9, 27%; p) CAN, CH_3CN/H_2O, 0°C to rt 2h, n=9, (80%). q) thioacetic acid, pyridine, 3.5 days, 90%. r) NH_4OH, CH_3OH, reflux 5h, 97%. s) (i) Na, THF, NH_3, -78°C, 30 min; (ii) sat. aq. $NHCl_4$, -78°C, 1h, 50%.*

The role of the different glycosylation patterns on *S. aureus* WTA is still under investigation. Recently it has been suggested that changing Rbo-GlcNAc glycosylation might contribute to *S. aureus* escape from host immune surveillance.[41] In a study by Peschel and coworkers, two synthetic RboP fragments were used to unravel the activity of a glycosyl transferases that had unknown activity. For this study, a trimer and a hexamer were synthesized by Seeberger's and Codée's groups respectively. Trimer **181** was synthesized starting from key building block **173** (Scheme 9A). Compound **173** was coupled with either reagent **174**, using diisopropylammonium tetrazolide, or **175**, using 1H-tetrazole, delivering, after oxidation with *t*BuOOH, compound **176** and compound **177**. After removal of the Lev group from compound **177**, the coupling between **177** and **176** was performed using the same conditions as for the synthesis of **177**. After another cycle of Lev removal, coupling and oxidation, the fully protected trimer was generated. Final deprotection was performed by treating compound **181** with hydrazine acetate, followed by hydrogenolysis. For the synthesis of the hexamer **189** the strategy reported by Pozsgay was applied.

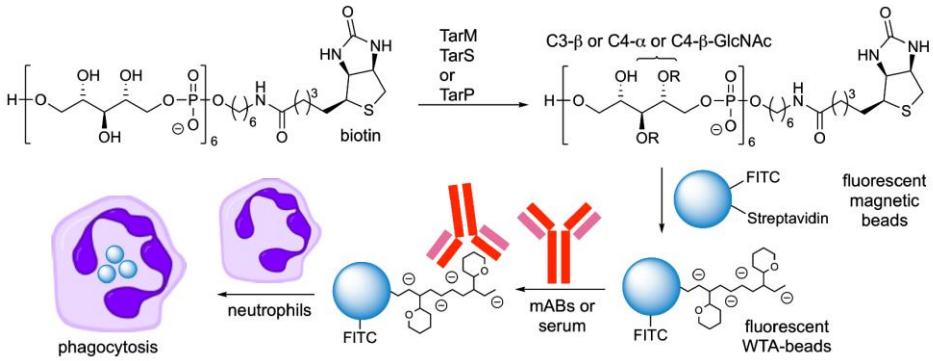


Scheme 9*: Assembly of a S. aureus WTA RboP trimer. a) LevOH, DMAP, DCC, CH_2Cl_2, 3 h, 98%. b) $Pd(PPh_3)4$, 1,3-dimethylbarbituric acid, CH_3OH, 40°C, 24 h, 77%. c) diisopropylammonium tetrazolide, CH_2Cl_2, 2 h, 98%. d) (i) 1H-tetrazole, CH_3CN, 2 h; (ii) tBuOOH, 1 h, 85%. e) $NH_2NH_2•H_2O$, pyridine, AcOH, CH_2Cl_2, 4 h, 93%. f) (i) 29, 1H-tetrazole, CH_3CN, 2 h; (ii) tBuOOH, 1 h, 86%. g) $NH_2NH_2•H_2O$, pyridine, AcOH, CH_2Cl_2, 4 h, 94%. h) (i) 29, 1H-tetrazole, CH_3CN, 2 h; (ii) tBuOOH, 1 h, 94%. i) $NH_2NH_2•H_2O$, pyridine, AcOH, CH_2Cl_2, 4 h, 98%. j) Pd-C, H_2, EtOAc/ CH_3OH/H_2O, 24 h, quant.*

In the study by Peschel's group,[38] a screen on S. *aureus* genomes was performed with the intend of identifying paralogues of WTA biosynthesis genes. It was found that three S. *aureus* prophages were able to encode a glycosyl transferase, TarP, which was shown to be 27% identical to the well-known TarS enzyme, that transfers the β-GlcNAc to the RboP units. While TarS places the carbohydrate on the C4 of the ribitol backbone, TarP was shown to place the β-GlcNAc on the C3 position, as deduced from NMR studies and crystal structures using synthetic trimer **183**. In order to probe the immunogenicity of these subtle differences in glycosylation activity, sera from healthy human donors were

evaluated for the binding towards the different β-GlcNAc-WTA types. The relatively low IgG titer towards TarP glycosylated Rbo-WTA as compared to the TarS homologue led to the hypothesis that the former enzyme can be used by *S. aureus* for immune evasion. Different results were obtained by van Sorge's group, where the synthetic hexamer **189** was used as precursor for the generation of semi-synthetic Rbo-WTA structures.[42] In this study compound **189** was first biotinylated and subsequently enzymatically glycosylated either with TarS (delivering C4-βGlcNAc), TarM (leading to C4-αGlcNAc) or TarP (yielding C3-GlcNAc). The semisynthetic fragments were immobilized on streptavidin coated beads and used to probe binding of IgG antibodies present in pooled sera of healthy human donors. It was observed that the titer of IgGs against the TarS glycosylated WTA was higher than the titer against the TarP glycosylated WTA, but the level of antibodies against the latter type were still significant. The designed protocol (Figure 6) proved to be much more sensitive than the assay used by Gerlach *et al.* The WTA-beads have also been used to probe antibody mediated phagocytosis, showing effective uptake of the TarS and TarP modified WTA-beads.

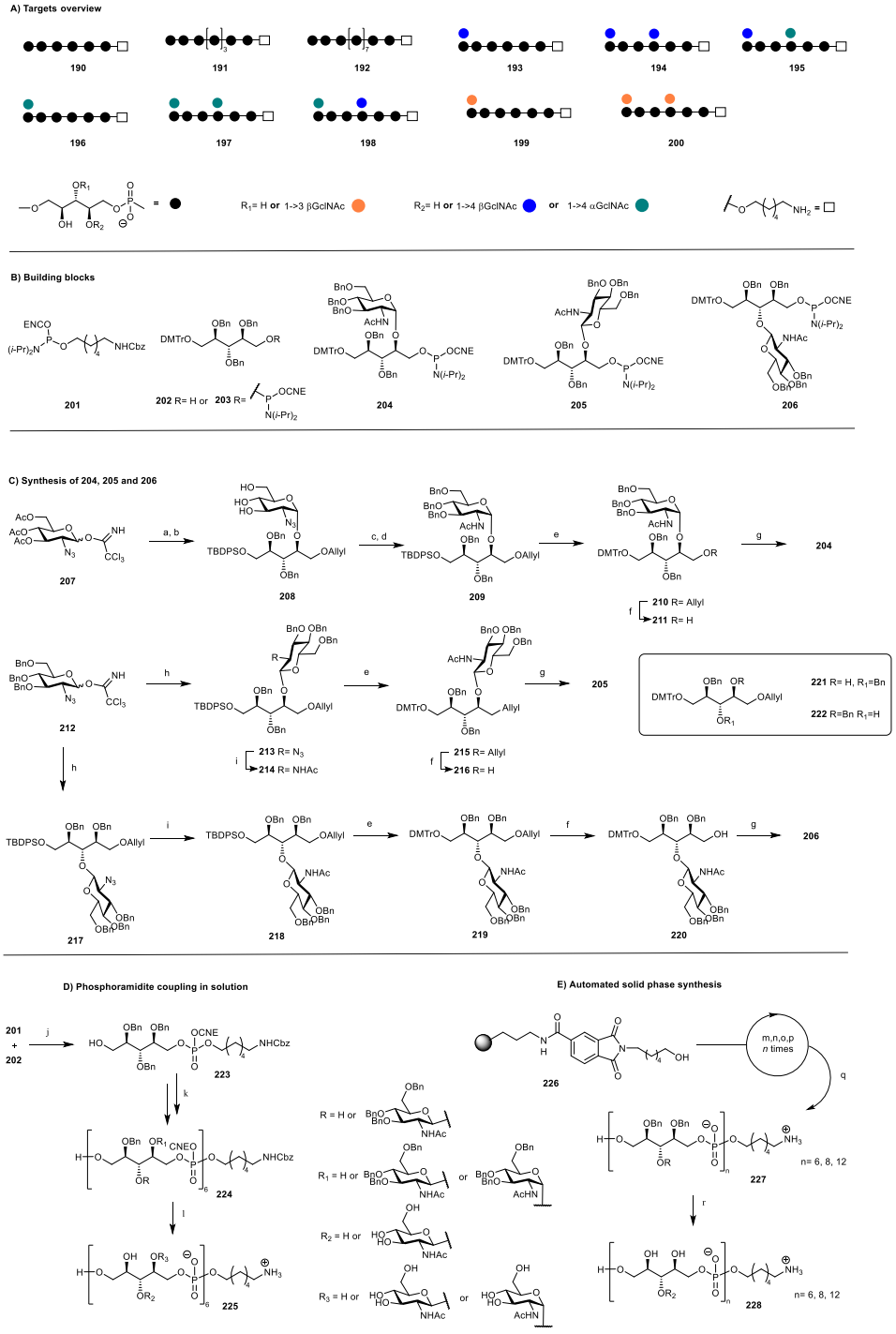
Figure 6: *Schematic overview of antibody mediated phagocytosis using enzymatically modified synthetic biotinylated (RboP) hexamer immobilized on streptavidin coated beads.*



More recently, both automated and in solid phase strategies were developed to deliver different unsubstituted or site-specific mono- or di- glycosylated RboP fragments (**190**-**200**, Scheme 10A).[43] Compare to the synthetic route undertaken by the team at Sanofi Pasteur, a stereoselective glycosylation methodology was employed by Codeé's group to deliver C-4-αGlcNAc RboP (**204**), C-4-βGlcNAc RboP (**205**) and C-3-βGlcNAc RboP (**206**) building blocks in a highly efficient manner (Scheme 10B). In order to introduce the α glycosylic bond (Scheme 10C), glucosazide donor **207** was coupled with acceptor **221** using TMSOTf as promoting agent, delivering glycosylated product in 92% as 7:1 α/β ratio. After deacetylation, the α isomer **208** was isolated in 70% yield and all free hydroxyl were protected with benzyl groups. Subsequently the azido moiety was reduced by Staudinger conditions and acetylated affording **209** in excellent yield. Final protecting groups manipulation delivered key phosphoramidite derivative **204**. For the assembly of building blocks **205** and **206**, glycosylation reaction between donor **212** and acceptors

221 or **222** respectively was performed using acetonitrile as β-directing solvent at low temperature to deliver compounds **213** and **217** in high yields. Similar protecting groups manipulation, as previously, was then followed to obtain phosphoramidite derivatives **205** and **206**. With all building blocks in hand (Scheme 10B), oligomerization was investigated via phosphoramidite approach at first in solution (Scheme 10D). Differently than Pozsgay's RboP synthesis, 4,5-dicyanoimidazole (DCI) was used as activating agent for the generation of a phosphite intermediate upon attack by primary alcohol and subsequently oxidized using (10-camphorsulfonyl)oxaziridine (CSO). The temporary DMTr group placed at the site of propagation was removed using dichloroacetic acid (DCA, 3% in DCM) and the elongated intermediate purified. All couplings proceeded with high efficiency and final targets **190-200** obtained after deprotection of the cyanoethyl protecting groups in basic conditions and hydrogenolysis to remove benzyl groups. Contrarily than Sanofi Pasteur RboP library, the aminohexanol linker was placed at the site where naturally occurring peptidoglycan is attached to the RboP-WTA. In order to streamline the oligomerization of RboP based fragments, automated solid phase synthesis was investigated (Scheme 10E). Previously, Pozsgay and co-workers attempted this approach for the synthesis of compounds **128** and **129** (Scheme 7). Unfortunately, after cleavage from the solid support, a complex mixture was obtained and the failure was attributed to the use of trichloroacetic acid for the DMTr removal. Using the same conditions as reported by Pozsgay but replacing TCA with the milder acid DCA, as proved in solution, Codeé and co-workers obtained semi-protected octamer and dodecamer in 15% and 11% yield. The same conditions were then applied to obtained final targets **199** and **200**.

The synthesized compounds were biotinylated in order to be evaluated for antibody binding using the aforementioned magnetic beads assay. Monoclonal antibodies directed towards α-1,4-GlcNAc WTA were able to recognized also monosubstituted fragments. In the case of the β isomers, two monoclonals were tested. Interestingly one of the antibodies could recognize both regioisomer 1,4 and 1,3 GlcNAc RboP hexamers with a preference for the former and as previously observed even one substituent was sufficient for antibody deposition. In the other case, the monoclonal was able to recognized only the fragment enzymatically glycosylated due to a higher degree of substitution or multiple displacement of the epitope recognized.



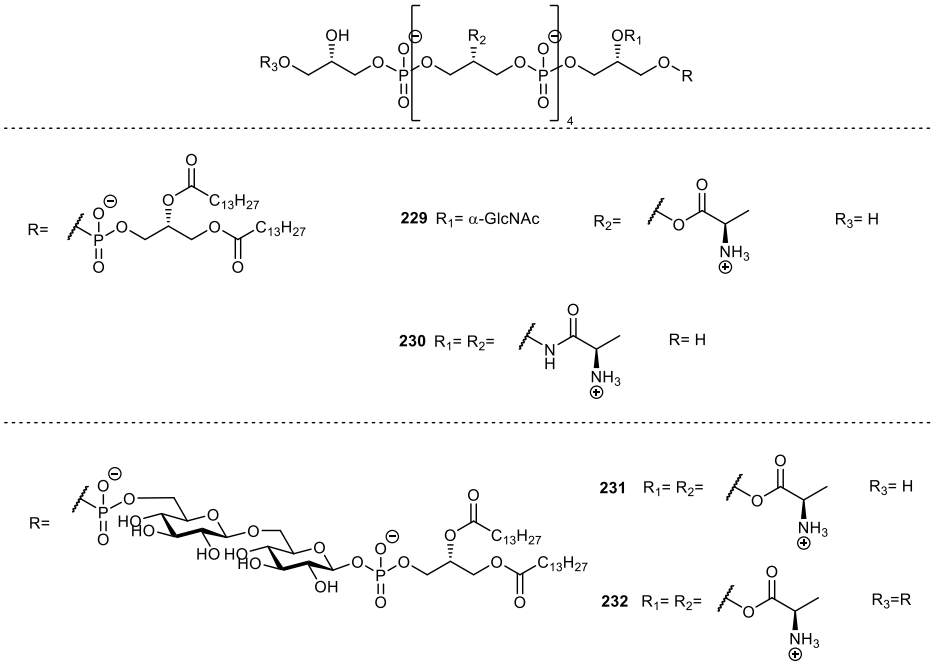
***Scheme 10**: (Automated) synthesis of unsubstituted and glycosylated RboP well-defined fragments. A) Schematic overview of the target compounds **190-200**; ribitol phosphate repeating unit = black*

circle, β-1,3-GlcNAc = orange circle, β-1,4-GlcNAc = blue circle, α-1,4-GlcNAc = green circle. B) Building blocks ***201-206****. C) Synthesis of building blocks* ***204, 205*** *and* ***206;*** *a)* ***221****, TMSOTf, CH_2Cl_2, rt, 92 %, 7:1 α/β; b) NaOMe, CH_3OH, rt, α-anomer* ***208*** *70%; c) BnBr, NaH, THF/DMF (7:1), 0°C to rt, 73 %; d) (i) PMe_3, KOH, THF; (ii) Ac_2O, pyridine,* ***209*** *89 % over 2 steps; e) (i). TBAF, THF, rt; (ii) DMTrCl, TEA, CH_2Cl_2,* ***210****: 67 %;* ***215****: 36 %;* ***219****: 60%; k) (i). Ir(COD)$(Ph_2MeP)_2PF_6$, H_2, THF, (ii). I_2, sat. aq. $NaHCO_3$, THF,* ***211****: 88 %;* ***216****: 79%;* ***220****: 94 %; g) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2,* ***204****: 81 %;* ***205****: 78%;* ***206****: 85 %; h)* ***222****, TMSOTf, CH_3CN, - 40°C to 0°C,* ***213****: 85 %;* ***217****: 80 %; i) (i) propanedithiol, pyridine, H_2O, TEA, rt; (ii). Ac_2O, pyridine,* ***214****: 59 %;* ***218****: 86 % 2 steps. D) Oligomerization in solution; j) (i) DCI, CH_3CN, phosphoramidite; (ii) CSO; (iii) 3 % DCA in DCM. 85%. k) (i) DCI, CH_3CN, phosphoramidite* ***203****,* ***204****,* ***205*** *or* ***206****; (ii) CSO; (iii) 3 % DCA in CH_2Cl_2. 53%-quant. l) (i) NH_3(30-33% aqueous solution), dioxane; (ii) Pd black, H_2, AcOH, H_2O/dioxane,* ***190****: 87 %;* ***193****: 96 %;* ***194****: 55%;* ***195****: 16 %;* ***196****: 68 %;* ***197****: 78%;* ***198****: 88 %;* ***200****: 70 %. E) Automated solid phase assembly. m) 3% DCA in toluene (3 min); n)* ***203*** *or* ***20,*** *BTT, CH_3CN (5 min); o) I_2, Pyridine/H_2O(1 min); p) Ac_2O, N-methylimidazole, 2,6-lutidine, CH_3CN (0.2 min); q) 25 % NH_3(aq) (60 min); 11%-20%; r) Pd black, H_2, dioxane H_2O,AcOH,* ***191****: 100 %;* ***192****: 100 %;* ***199****: 87%;* ***200****: 100%.*

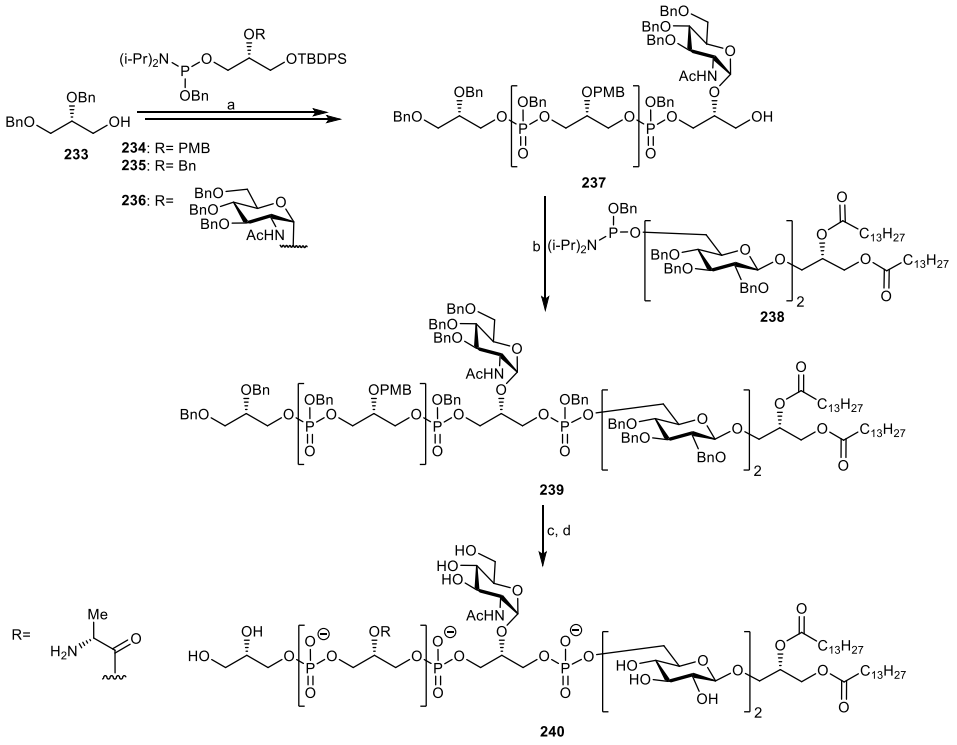
LIPOTEICHOIC ACIDS

A large set of S. *aureus* type LTA-oligomers has been synthesized by Schmidt and co-workers. Figure 7 shows a selection of these fragments, in which a lipid anchor as well as α-GlcNAc and D-Ala substituents were incorporated.

Figure 7: *A selection of GroP fragments from LTA of S. aureus synthesized by Schmidt's group*

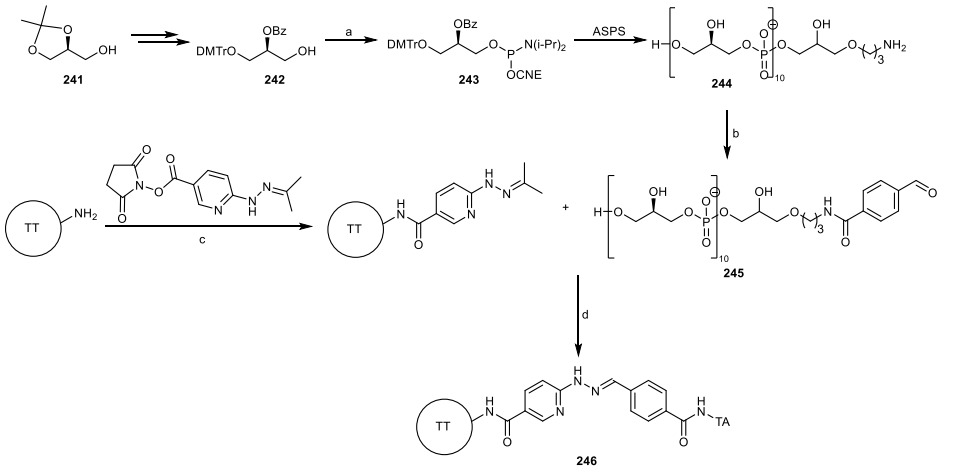


Scheme 10 shows a representative synthetic strategy used to build this kind of structures.[44] The strategy relied on a phosphoramidite approach, where glycerol derivatives **234**, **235** and **236** carried a temporarily TBDPS protecting group at the site of elongation. Notably, due to the lability of D-Ala moieties, glycerol **235** carried a PMB protecting group to allow the introduction of these labile substituents at a later stage. Thus, elongation proceeded using tetrazole as activating agent, *t*BuOOH for oxidation and subsequent TBDPS removal using TBAF. After 5 cycles, compound **237** was coupled to gentibiose diacyl lipid anchor derivative **238**. Before final hydrogenolysis, the PMB groups were removed by oxidative cleavage and D-Ala substituents were introduced. The set of oligomers were used to establish the structural requirements needed to elicit an innate immune response. As assessed by cytokine production in a whole blood assay, it was observed that both lipid anchor and positively charged D-Ala substituents were important structural features for the innate immune stimulating activity.[45]



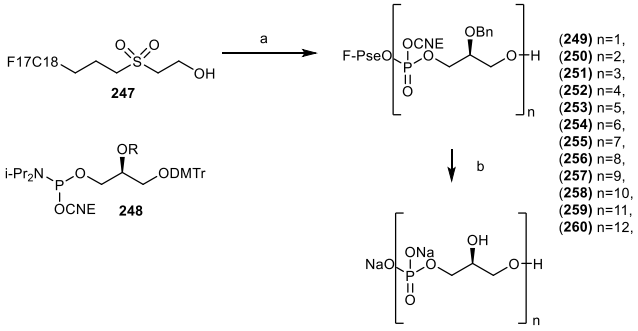
Scheme 11: *Synthesis of S. aureus LTA fragments by Schmidt and co-workers. a) (i)* ***234, 235*** *or* ***236****, tetrazole, CH_2Cl_2, (ii) tBuOOH; (iii) TBAF. b) (i) tetrazole, CH_2Cl_2; (ii) tBuOOH, 75%. c) CAN, CH_3CN/toluene/H_2O, 67%. d) PyBOP, N-methylimidazole, Z-protected alanine, CH_2Cl_2. e) $Pd(OH)_2$, H_2, CH_2Cl_2/ CH_3OH/H_2O, 9:1:1, 25% over 2 steps, 10: 33% over 2 steps.*

A solid phase approach for the construction of unsubstituted GroP oligomers was used by Snapper and co-workers (Scheme 11).[46] The key building block **243** was designed based on the contemporary nucleic acid chemistry, having a DMTr as orthogonal protecting group at the site of elongation. Different than other strategies (*vide infra*), the secondary alcohol was protected with a benzoyl group, even though it is known that migration to the primary position readily takes place. For the solid support amino spacer functionalised glycerol-controlled pore glass (CPG)-resin was used. A GroP decamer was synthesized using 30-40 equivalent of phosphoramidite building block **244** per cycle and upon treatment with ammonia, the final compound **245** was released from the resin and simultaneously all protecting groups were removed. After desalting, the crude product (no purification or characterisation of the material has been reported) was conjugated to tetanus toxoid (TT) via the formalbenzoate hydrazinonicotinamide conjugation couple. Mice serum raised against glycoconjugate **246** showed to mediate opsonophagocytic killing of S. *aureus in vitro* and protection in a bacteremia model *in vivo*.



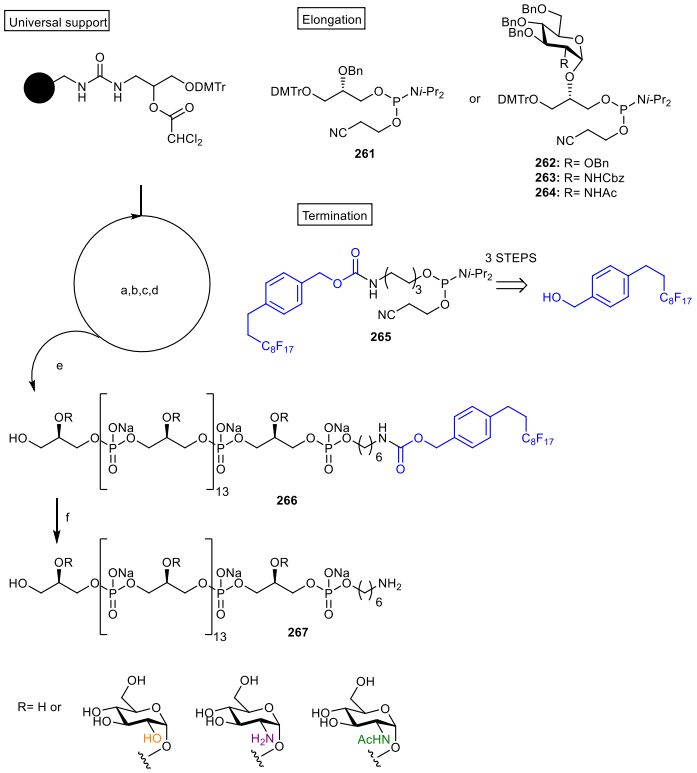
Scheme 12: *Automated solid phase synthesis of a S. aureus LTA GroP oligomer. a) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. b) 4FB-OSu; c) PBS, aniline.*

The automated solid phase approach is an efficient method that can reduce synthesis time and labour, but it also requires large amounts of building blocks per cycle and has scale limitations. As an alternative, Codée and co-workers reported the use of a fluorous soluble support for the construction of unsubstituted GroP oligomers up to the 12-mer (Scheme 12).[47] This methodology allows the rapid and efficient isolation of the synthetic intermediates, with the possibility to scale up and performing the coupling cycles using a relatively small excess of reagent. As shown in Scheme 13, (perfluorooctyl)succinyl spacer **247** was used to deliver TA oligomers featuring a terminal hydroxyl group. Based on the phosphoramidite approach, as previously described, building block **248** was used and it was noticed that a larger amount of phosphoramidite reagent was needed with growing chain length of the oligomers. The usual deprotection steps delivered target fragments **249-260**.



Scheme 13: *Automated solid phase synthesis of GroP based fragments. a) (i)* ***248****, DCI, ACN (ii),* I_2*, pyridine, THF (iii) DCA, TES-H,* CH_2Cl_2*. b) (i)* ***248****, DCI, ACN (ii)* I_2*, pyridine, THF (iii) DCA, TES-H,* CH_2Cl_2*.*

More recently, the same group reported the synthesis of a large set of GroP-based TA fragments using automated solid phase synthesis (Scheme 14).[48] A universal solid support was used in order to avoid the need of linker functionalized building blocks and at the end a fluorous aminospacer phosphoramidite **265** was used to facilitate purification of the long fragments. Notably, several pentadecamers were synthesized having different carbohydrate substituents, including α-GlcNAc from S. *aureus* LTA, in different position of the chain.



Scheme 14: *"Second-generation" automated solid-phase assembly of long GroP-TA fragments. (a) 3% DCA, toluene. (b) 5-BTT, ACN; (c) I_2, pyridine, H_2O/CAN. (d) Ac_2O, N-methylimidazole, 2,6-lutidine, CAN. (e) (i) NH_3, CH_3OH;(ii) NH_4OH, H_2O. (f) H_2, Pd_0, H_2O.*

CONCLUSION

Since the arising of *S. aureus* antibiotic resistant strains, over the last two decades, much more efforts have been focussed on the development of a vaccine against it. Although glyco-based active immunization strategies proved to be promising in preclinical trial settings, understanding on how this pathogen interacts with human immune system has to be still carried out. Therefore, structure activity/immunogenicity

relationship studies can represent a suitable methodology to unravel the basis for developing effective *S. aureus* treatments.
In the last decade, much more efforts have been focused on the achievement of well-defined glyco-based structures. A panoramic overview is here presented, dealings with synthetic strategies for the achievement of fragments, based on surface and capsular polysaccharides, as well different glycosyl substituted teichoic acids oligomers.
The achievement of carefully designed and variegate libraries can be applied to evaluate at molecular level not only the minimal structural elements required for the immunogenic properties, but also to deepen the in and out of their biological role. This review also stresses the need of a close collaboration among different scientific fields, from organic chemistry to immunobiology and medical expertise. By sharing the cutting-edge on different scientific skills, the hard challenge against *S. aureus* might be overcome in the near future.

OUTLINE OF THIS THESIS

The work described in this thesis aimed to understand the interaction between antibodies and glyco-based cell wall components from both staphylococci and enterococci species at molecular level. To this end, well defined fragments were synthesized and therefore new or already established organic chemical methodologies were applied. In **Chapter 2**, the first synthesis of CP5 hexamer is described and in particular a strategy was developed in order to selectively introduced the acetyl substituent on the C3 of the L-Fucosyl moiety. With the described approach both oligomers with and without substituents can be achieved in order to understand the role of this decoration in the future for both antibody binding and virulence.
As it has been described in this introduction, in Jeroen Codeé's lab a large library of GroP and RboP based fragments has been generated. In order to evaluate it in a rapid and efficient way, a TA-microarray tool was developed (**Chapter 3**). The assessment was performed using monoclonal antibodies, the specificity of which was already known. The tool was then applied for antibody profiling using polyclonal sera from immunized rabbits or even healthy human volunteers. All the GroP and RboP compounds differ for the length (number of repeating units), presence and degree of glycosyl substituent, the kind of glycosyl substituent as well the position of it along the chain. One structural feature that has not been evaluated was the stereochemistry of the backbone. In **Chapter 4** the synthesis of sn-1-GroP fragments differing for the position of a glucosyl substituent along the chain is described as diastereomeric counterpart of the already synthesized sn-3-GroP library. During the generation of this fragments, it has been observed that the stereoselectivity of the glycosylation reaction, when PPh_3O (triphenylphosphinoxide) was used as modulating agent in the presence of TMSI (trimethylsilyl iodide), was affected upon the GroP chirality especially if the acceptor was bearing a bulky protecting group. The polyclonal sera from rabbit immunized with either native LTA from *E. faecalis*

or a previously generated fully synthetic TA-conjugate showed binding preference for one or the other stereochemistry.
Based on previous results, where a GroP hexamer bearing a glucosyl substituent at the terminal part of the chain was able to induce protective and opsonic antibodies against enterococci and staphylococci species, the fully synthetic conjugate was used to generate a monoclonal antibody via hybridoma technology. The monoclonal was then employed to characterize the interaction with synthetic TA-fragments using a variety of techniques. In order to understand the structural feature of the epitope recognized, the synthetic fragments were evaluated using TA-microarray, ELISA, SPR and STD-NMR.
A summery of the overall work described in this thesis is provided in **Chapter 6**, addressing possible future prospects. The synthesis of GroP based fragments bearing *N*-acetyl-galactosamine at the C2 is provided as this kind of biomolecules have been found in other *S. aureus* species as well as an extended application of the TA-microarray for the evaluation of glycan binding protein such as DC-SIGN and Langerin.

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Chapter 2

Chemical synthesis of fragments of the capsular polysaccharide of Staphylococcus aureus serotype 5

INTRODUCTION

Capsular polysaccharides (CPs) are found as the protective outer layer of many bacterial species. They are built up from monosaccharides leading to linear or branched, homo- or heteropolymers, that are covalently attached to the cell membrane. Interfacing with their surroundings, their main biological roles are communication with and protection from the environment. The diversity of CPs structures is driven not only by the nature of the constituting monosaccharides and the way these are interconnected, but also by the presence of cationic or anionic charges, substituents such *N*- or *O*- acetyls, pyruvic acid ketals, lactic acids among the most abundant.[1]

The first use of CP as immunogenic component in vaccines were reported at the beginning of the last century,[2] but it is only in the last decades that glycoconjugate based vaccines have been effectively applied for controlling and preventing infectious diseases caused by for example *H. influenzae* type b (Hib), *N. meningitidis*, *S. pneumoniae* or group *B Streptococcus*.[3] However, their potential has not yet been fully exploited and better understanding of their molecular composition can help to design more effective glycoconjugate vaccines in the future.[4]

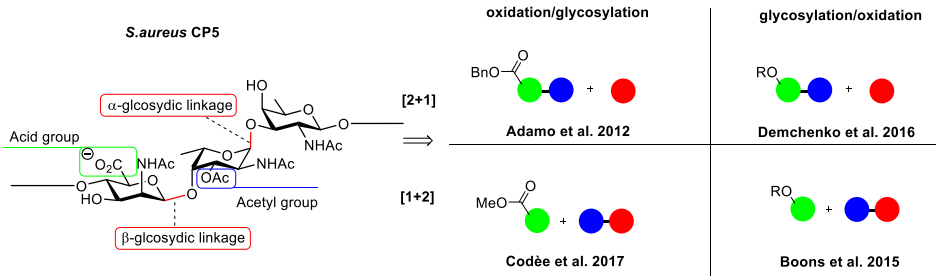
Methicillin resistant *S. aureus* (MRSA) strains have been appointed by the WHO as one of those pathogens (ESKAPE), for which new treatments are urgently needed.[5] Serotype 5 and 8 are the most abundant among the clinical isolates and they are characterized by the presence of a capsular polysaccharide referred to as respectively CP5 and CP8. Vaccine candidates based on both native CP serotypes have shown promising results in preclinical trial settings, but discordancy in efficacy has been observed during advanced clinical phase studies.[6] The negative outcome has been related to various strategies of the bacterium to evade the innate and adaptive host immune system,[7] but the failure has also been attributed to possible manufacturing inconsistencies.

The use of native extracted glycopolymers, and the method for protein-conjugation, can lead to heterogeneous molecular composition and variability of the glycoconjugate vaccines.[8] Organic chemistry can deliver structurally defined glycopolymer fragments, equipped with a conjugation handle, which can be used to generate more homogeneous vaccine modalities, and these well-defined molecules can be employed for the determination of structure-immunogenicity relationships. In addition, they can be used as tools for analytical and diagnostic assessment.[9] Therefore several syntheses of *S. aureus* CP-fragments have been reported as described previously in Chapter 1.

As depicted in Figure 1, the repeating unit of CP5 is a trisaccharide unit, consisting of *N*-acetyl-D-mannuronic acid, *N*-acetyl-L-fucose and *N*-acetyl-D-fucose. Several challenging structural features have to be overcome in order to achieve the synthesis of well-defined fragments such as the introduction of 1,2 *cis*-glycosidic bonds, the presence of the carboxylic acids and the regioselective installation of the *O*-acetyl groups. Moreover, a zwitterionic charge motif can be present due to partial *N*-deacetylation of the fucosyl residues, the position and the degree of which can vary depending on the strain.[10] The synthesis of a trisaccharide repeating unit has been achieved by several groups via different routes, but longer fragments have not been assembled to date.[11-15] A schematic overview is depicted in Figure 1, with four strategically different pathways, based on the

order of glycosylation [2+1] or [1+2] and the pre- or post-oxidation of the primary alcohol of the mannose residue.

Figure 1: *Chemical structure of CP5 and schematic overview of the synthetic strategies for the trisaccharide unit.*

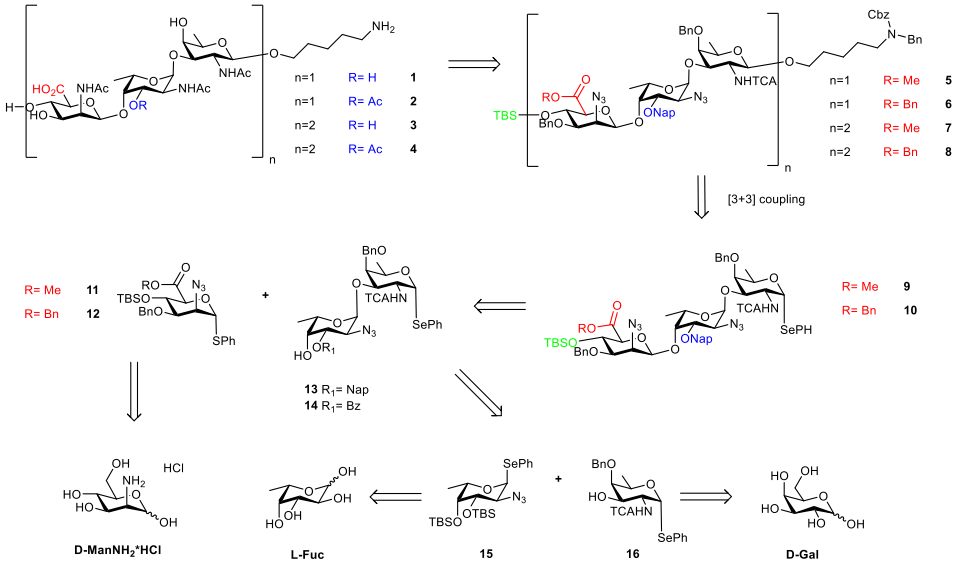


The only immunological evaluation of a synthetic CP5-fragment was reported by Adamo and co-workers, who used the synthetic trisaccharide fragment in a competitive ELISA and immunodot blot assay. The inefficient binding of this trimer by IgG antibodies raised against the native polysaccharide suggests that longer oligomers are needed.[11] Therefore, this chapter is directed to the development a convergent synthetic route to generate well-defined CP5 oligosaccharide fragments longer than a trimer. Because the importance of the presence of *O*-acetyl substituent for the immunogenicity has been assessed using only native CP sources,[16] fragments with and without *O*-acetyl substituents have been targeted as well.

RESULTS AND DISCUSSION

To develop a convergent synthetic route to fragments of CP5, two trimers (**1** and **2**) and two hexamers (**3** and **4**) were selected as targets. Both the trimers and the hexamers differ in the presence or absence of an acetyl substituent on C-3 of the L-fucosyl moiety. As shown in the retrosynthetic analysis (Figure 2) the feasibility of a 3+3 block coupling strategy was investigated.

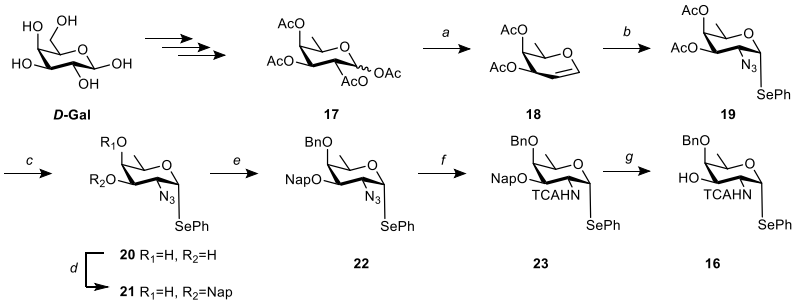
Figure 2: *Retrosynthetic analysis of S. aureus CP5*



Before the removal of all protecting groups, the final operation to obtain target oligomers **2** and **4** is the introduction of the acetyl group in fully protected oligomers (**5**-**8**) that without this operation also can lead to targets **1**, **3**. Oligomers **5**-**8** are derived from trisaccharide donors **9** or **10** having three orthogonally protecting groups: a phenylselenyl group on the anomeric position, a TBS masking the hydroxy group that is to be elongated and an oxidatively cleavable Naphthyl group for the installation of the *O*-acetyl substituent. The *N*-acetyl groups on both D-mannose and L-fucose residues were masked as azides, while an *N*-trichloroacetyl protecting group on the D-fucosyl moiety allows a 1,2-*trans* glycosylation. Two groups were explored for the protection of the mannuronate ester, a methyl and a benzyl ester. While the first one has been employed by Hagen *et al.*[14] in a previous synthesis of the CP5 trimer, the second one would reduce the number of final deprotection steps. Although different synthetic strategies are feasible for the synthesis of trisaccharide donors **9** and **10** (see Figure 1) a [1+2] pre-oxidation glycosylation strategy was chosen, since this requires the least number of reaction steps. Moreover, the *cis*-stereoselectivity of mannuronate donors is usually very high.[17,18] Disaccharide acceptors **13** and **14** can be obtained by glycosylation of an L-fucosyl donor **15** and D-fucosyl acceptor **16**, the protecting groups of which were chosen based on a previous extensive study on the reactivity of fucosazide building blocks.[14]

The synthesis of D-fucosyl acceptor **16** commenced with D-galactose as starting compound (Scheme 1). Peracetylated fucal intermediate **17** was obtained in five steps

Scheme 1: *Synthesis of D-FucNTCA acceptor **16***

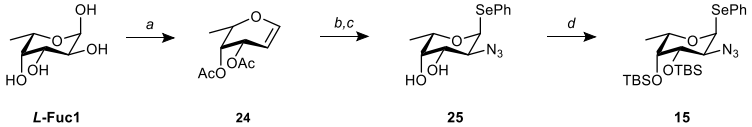


Reagents and conditions: *a) (i) HBr 33% in AcOH, DCM, 0° C to r.t.; (ii) Zn, NH_4Cl, EtOAc, 70° C, 77% (over two steps); b) $(PhSe)_2$, Me_3SiN_3, DCM, -8°C (±2°C), 70%; c) $Na_{(s)}$, MeOH, quant.; d) (i) Bu_2SnO, Toluene, 120° C; (ii) NapBr, CsF, Bu_4NBr, 120° C, 77%; e) BnBr, NaH, DMF, 0° C to r.t., 88%; f) (i) PPh_3, THF, 40° C (3h), H_2O, 60° C (o.n.); (ii) trichloroacetyl chloride, Pyridine, quant (over two steps) or (i) Pd/C, THF, tBuOH, AcOH; (ii) trichloroacetyl chloride, Pyridine, 96% (over two steps); g) DDQ, DCM:H_2O (4:1), 72%.*

following a reported procedure.[19] After bromination and zinc mediated elimination, fucal derivative **18** was obtained in 77% yield over the two steps. A regio- and stereoselective azidophenylselenation (APS) was performed using a protocol developed by Nifantiev and co-workers,[20] in which the more soluble $TMSN_3$ is used as azide source instead of NaN_3.[21] Keeping the temperature constant at -8°C (±2°C), the desired product **19** was isolated in 70%. Deacetylation under Zémplen conditions was followed by regioselective introduction of a naphthyl protecting group via the formation of a tin acetal intermediate. After benzylation of the remaining free hydroxyl group, the azide was converted into a trichloroacetimidoyl group by reduction and subsequent acetylation using trichloroacetyl chloride in pyridine. Both Staudinger conditions and hydrogenolysis were employed for the reduction step, affording compound **23** in similar yields. For the latter procedure, it might be speculated that the phenylselenyl group is responsible for the chemoselective reduction of the azide in the presence of the benzyl and the naphthyl group, since selenium containing compounds can deactivate the Pd/C catalyst in hydrogenation reaction.[23] Indeed, no formation of any related byproduct was observed by TLC even after 24h of stirring at room temperature.[24] Finally, acceptor **16** was obtained in good yield by DDQ treatment to remove the naphthyl group.

Synthesis of L-fucosyl donor **15** was accomplished by following a similar procedure to the one of D-fucosyl acceptor **16** (Scheme 2). Donor **15** was obtained in quantitative yield by silylation of intermediate **25**, using TBSOTf and catalytic amount of DMAP in pyridine at 70° C.

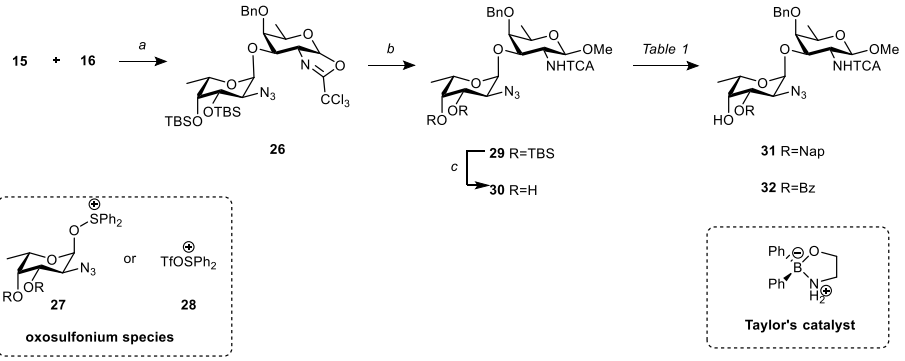
Scheme 2: *Synthesis of L-FucN_3 donor* ***15***



Reagents and conditions: *a) (i) Ac_2O, Pyridine, 4°C; (ii) HBr 33% in AcOH, DCM, 0° C to r.t.; (ii) Zn, NH_4Cl, EtOAc, 70° C, 79% (over three steps); b) $(PhSe)_2$, Me_3SiN_3, DCM, -8°C (±2°C), 73%; c) $Na_{(s)}$, MeOH, quant.; d) TBSOTf, DMAP (cat.), Pyridine, 70° C quant.*

Next, the coupling of D-fucosyl donor **15** with L-fucosyl acceptor **16** and the ensuing processing of the disaccharide product was investigated (Scheme 3). The Ph_2SO/Tf_2O mediated preactivation protocol was chosen as it was observed that highly reactive L-FucN_3 donors favourably form 1,2-*cis*-glycosidic linkages.[14] Moreover, this glycosylation procedure, in combination with anomeric phenylselenyl group in acceptor **16** has the potential for an iterative one-pot glycosylation event.[26]

Scheme 3*: Glycosylation reaction between donor* ***15*** *and acceptor* ***16*** *under preactivation conditions*



Reagents and conditions: *a) Ph_2SO, TTBP, 3Å MS, DCM; Tf_2O, -80° C to -70° C;* ***16****, -80° C to -50° C, 71% (****26****); b) after purification by size exclusion gel chromatography (DCM:MeOH); c) TBAF, THF, quant.*

A first attempt was carried out using 1.3 equivalents of donor **15**, Ph_2SO and Tf_2O with respect to acceptor **16** (Scheme 3). After a reaction time of 30 minutes at -50°C and purification by column chromatography, oxazoline **26** was isolated in 71% yield. The formation of the oxazoline moiety in **26** likely originates from intramolecular cyclization of the trichloroacetoimidoyl group, upon activation of the selenylphenyl group by oxosulfonium species **27** or **28**, as the presence of the former has been previously observed by NMR studies.[14] When oxazoline dimer **26** was purified by size exclusion chromatography (DCM:MeOH=1:1), β-O-methyl difucoside **29** was isolated. This made it possible to use this dimer as model for the intended functionalization. For the removal of the silyl groups, an excess of 8 equivalents of TBAF was required, while the regioselective protection of the 3'-OH needed optimization, the results of which are summarized in Table 1. Tin ketal mediated installation of the naphthyl group afforded

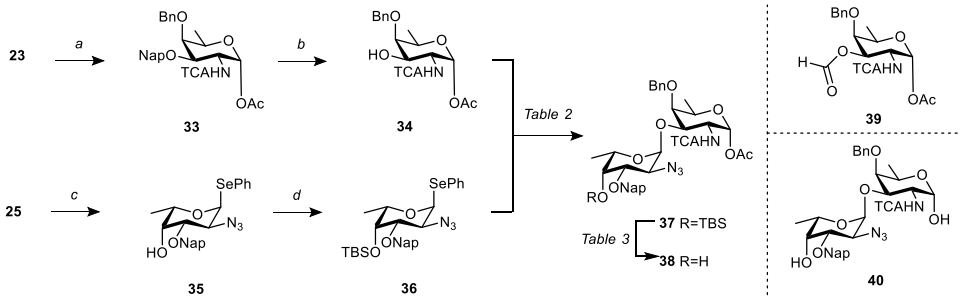
compound **31** in 22% (entry 1) or 35% (entry 2) yield. In both cases no starting material could be recovered but several side products were detected by TLC analysis. The introduction of the benzoyl group using Taylor's catalyst (B cat.) in acetonitrile failed, even when the reaction was run for 24 hours at 60° C (entry 3 and 4).[27] Solubility issues of the starting material **30** were reduced by the addition of dichloromethane and after 3 days of stirring compound **32** was isolated in 41% yield.

Table 1**: Regioselective protection of C-3' from disaccharide **30

Entry	Conditions	Time	Yield	30
1	(i) Bu_2SnO, Toluene, 110° C (ii) NapBr, DMF, Bu_4NBr, CeF, 90° C	(i) 2 h (ii) 5 h	22% (**31**)	-
2	(i) Bu_2SnO, Toluene, 60° C, 100 mbar (ii) NapBr, DMF, Bu_4NBr, CeF, 90° C	(i) 30 min. (ii) 3 h	35% (**31**)	-
3	B cat., DIPEA, BzCl, ACN	24 h	0% (**32**)	94%
4	B cat., DIPEA, BzCl, ACN, 60° C	24 h	0% (**32**	87%
5	B cat., DIPEA, BzCl, ACN:DCM (2:1), 60° C	3 days	41% (**32**)	55%

Based on the outcome of the study above, the protecting group strategy was adapted and **38** was chosen as new target dimer, requiring the syntheses of D-fucosyl acceptor **34** and L-fucosyl donor **36** (Scheme 4). To achieve this, phenylselenyl fucoside **23** was hydrolyzed and subsequently acetylated, leading to the predominant formation of α-acetyl fucoside **33**. Oxidative cleavage of the napthyl group in **33** gave D-fucosyl acceptor **34** in 69% yield. Donor **36** was prepared by a tin-mediated regioselective introduction of the naphthyl group, followed by protection of the hydroxyl group at C-3 with a TBS group using the procedure described above. Testing of the glycosylation methods started with the exploration of preactivation conditions (Table 2 entry 1). Acceptor **34** proved to be poorly soluble in dichloromethane, explaining the isolation of dimer **37** in 47% yield. The yield was slightly improved using the NIS/TBSOTf activator combination but still acceptor was recovered in 38% (entry 2). Improvement of the solubility by the use of a mixture of DCM and DMF (8:2) (entry 3) led to the isolation of target **37** and by-product **39** in a yield of 33% and 62%, respectively. Fortunately, the yield towards **37** could be improved considerably (entry 4) when a more diluted (0.05 M) mixture of donor and acceptor in dichloromethane was sonicated for 10 minutes before cooling the temperature to -78° C.

Scheme 4**: Synthesis of disaccharides **37** and **38



***Reagents and conditions:** a) (i) NIS, THF:H_2O (4:1); (ii) Ac_2O, Pyridine, 92% over two steps; b) DDQ, DCM:H_2O (4:1), 71%; c) (i) Bu_2SnO, Toluene, 120° C; (ii) NapBr, CsF, Bu_4NBr, 120° C, 82%; d) TBSOTf, DMAP (cat.), Pyridine, 70° C quant.*

Table 2:** Glycosylation conditions to achieve disaccharide **37

Entry	Conditions	Temp (° C)	Yield	Notes
1	(i) Ph_2S, TTBP, 3Å MS, DCM (ii) Tf_2O (iii) **34**	(i) r.t. (ii) -80 to -60 (iii) -80 to -40	47%	41% **34**
2	NIS, TBSOTf, DCM	-78° C to r.t.	56%	38% **34**
3	NIS, TBSOTf, DCM:DMF (8:2)	-80 to-30	33%	62% **39**
4	NIS, TBSOTf, DCM (0,05 M)	-80 to -50	96%	-

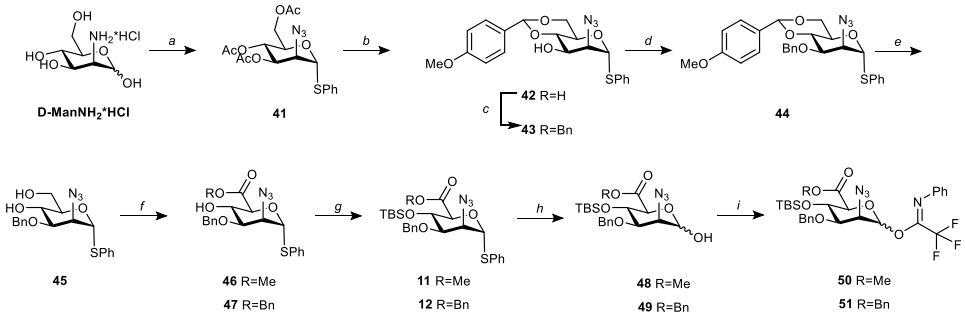
The removal of the TBS group in **37** to give target dimer **38** proved to be challenging and several conditions were probed (Table 3). Using 2 equivalents of TBAF, either at room temperature or at 66°C (entry 1 and 2), no product formation was observed and the starting material was fully recovered. Compound **38** was achieved by increasing the number of equivalents of the fluorine reagent. However, under these conditions the acetyl group was also cleaved (**40**) and the best yield was obtained with a large excess of TBAF and a relatively short reaction time (45 minutes, entry 3). Unfortunately, these conditions led to inconsistent yields when the reaction was performed on a larger scale. Addition of AcOH (entry 4) or the use of HF* pyridine (entry 5) didn't lead to any desired product. Finally, a quantitative yield was achieved when a large excess of 3HF*TEA was used in boiling THF (entry 6).

Table 3:** Attempts towards disaccharide acceptor **38

Entry	Conditions	Temp (° C)	Time	38 (%)	37 (%)
1	TBAF (2 eq), THF	r.t.	24 h	0%	quant
2	TBAF (2 eq), THF	66	24 h	0%	quant
3	TBAF (15 eq), THF	r.t.	45 min	75%	-
4	TBAF (5 eq), AcOH (5 eq), THF	66	24 h	0%	quant
5	HF*Py (20 eq), THF	66	24 h	0%	quant
6	3HF*TEA (20 eq), THF	66	24 h	quant	-

For the synthesis of mannuronate donors **11**, **12**, **50** and **51**, mannosamine hydrochloride was chosen as starting material. First a diazo transfer reaction was performed using freshly prepared TfN_3 in pyridine and $CuSO_4$ as catalyst. The choice of pyridine allowed the subsequent *in situ* acetylation, to avoid the formation of the glucose epimer by-product as previously observed.[14] The crude product thus obtained was used directly for the introduction of the anomeric thiophenyl group, achieving intermediate **41** over three steps in 76% yield.[28] The subsequent protective group manipulation involved deacetylation via Zèmplen conditions, introduction of the 4,6-*O*-pOMe-benzylidene group, benzylation of the remaining free hydroxyl and removal of the benzylidene group with catalytic amounts of TfOH in MeOH, delivering diol **45** in excellent yield. The primary alcohol in **45** was selectively oxidized using TEMPO/BAIB system and the crude carboxylic acid was alkylated to give methyl and benzyl mannuronate esters (**46** and **47**). Silylation of the hydroxyl at C-4 using TBSOTf in pyridine and catalytic amount of DMAP proceeded quantitatively to furnish donors **11** and **12.** Finally, the corresponding imidoyl donors **50** and **51** were obtained in high yields after NBS-mediated hydrolysis of the thiophenyl functionality, followed by treatment with *N*-phenyl-2,2,2-trifluoroimidoyl chloride and Cs_2CO_3.

Scheme 5: *Synthesis of mannuronate donors **11/12** and **50/51***

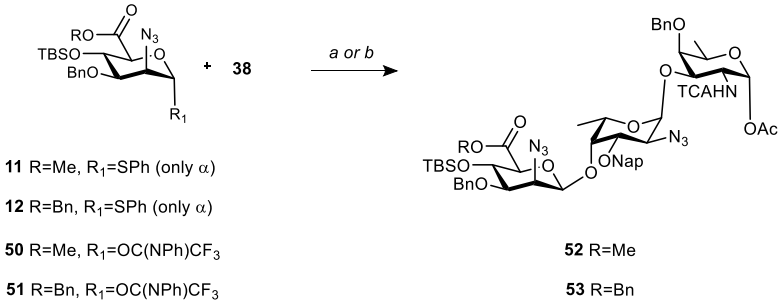


Reagents and conditions: *a) (i) TfN_3, $CuSO_4$, TEA, Pyridine, H_2O; (ii) Ac_2O; (iii) PhSH, BF_3*OEt_2, DCM 76% (over 3 steps); b) $Na_{(s)}$, MeOH, quant.; c) p-MeO-PhCH(OMe)$_2$, Cu(OTf), ACN, 60° C, 82%; d) BnBr, NaH, DMF, 0° C to r.t., 88%; e) TfOH, MeOH, quant.; f) (i) TEMPO, BAIB, AcOH, tBuOH, H_2O, DCM; (ii) BnBr or MeI, K_2CO_3, DMF, 72% (**46**), 75% (**47**); g) TBSOTf, DMAP (cat.), Pyridine, quant.(**11** and **12**); h) NBS, acetone, H_2O, quant (**48** and **49**); i) CF_3(Cl)CNPh, Cs_2CO_3, DCM, 88% (**50**), 84% (**51**).*

Now the stage was set to investigate the assembly of orthogonally protected trisaccharides **52** and **53**, using the prepared thiophenyl donors **11** and **12** and *N*-phenyl trifluoroacetimidate donors **50** and **51** in combination with dimer acceptor **38**. With donor **50,** three different promoters (TfOH, TMSOTf and TBSOTf) were tested. A molar ratio of 0.1:2.5:1 between these promoters, the donor and acceptor was chosen since mannuronate donors have relatively low reactivity. The addition of promoters happened at -78° C, after which the temperature was raised to -40° C and the reaction was left stirring at this temperature for 12 hours. After quenching with TEA and aqueous work

up, the trisaccharide **52** was isolated in respectively 15%, 38% and 45% yield. Therefore, TBSOTf was chosen as promoter while the number of equivalents was increased, keeping the temperature of reaction at -40° C, since decomposition of the α-triflate-mannuronate intermediate at higher temperature has been reported.[29] TBSOTf also proved to be a suitable promotor for the glycosylations not only with donor **51**, but also in combination with NIS to activate thiodonors **11** and **12,** producing the trisaccharides **52** and **53** in around 70% yield (Scheme 6).

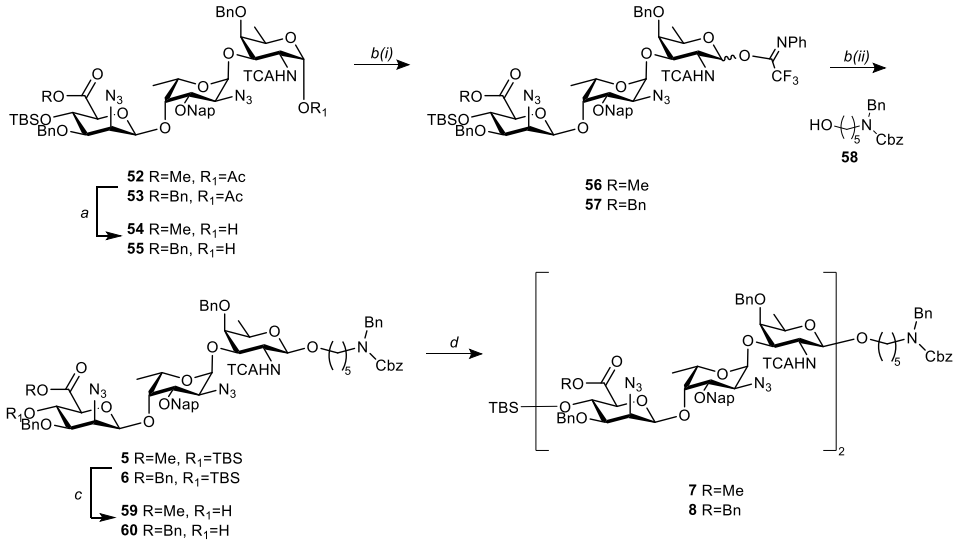
Scheme 6: *Synthesis of orthogonally protected trisaccharides **52** and **53***



Reagents and conditions: *a) Donor **11** or **12**, **38**, NIS, TBSOTf, DCM, -78° C to -40° C, 67% (**52**), 70% (**53**); b) Donor **50** or **51**, TBSOTf, DCM, -78° C to -40° C, 70% (**52**), 69% (**53**).*

A prerequisite to construct a hexamer by a 3+3 block coupling is the conversion of trimer **52** or **53** in a suitable donor (Scheme 7). The first step to provide imidates **56** and **57** is anomeric deacetylation, a conversion for which different conditions were tested. With one equivalent hydrazine acetate in DMF or ACN the reaction proceeded very slowly but with ten equivalents completion was reached within 18 h to give **54** in 82% yield. Alternatively sub stoichiometric amounts of Bu_3SnOMe (0.5 equivalents) in MeOH at reflux could deliver **54** in 81% yield. It was later noticed that when a new fresh pot of hydrazine acetate was employed, the desired products **54** and **55** could be obtained in quantitively yields in less than one hour. Treatment of **54** and **55** with 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride in the presence of Cs_2CO_3 led to the formation of **56** and **57**. Trisaccharide donors **56** and **57** could not withstand purification with column chromatography and were directly used in the ensuing glycosylation. Coupling of donor **56** or **57** with pentenyl acceptor **58** (2 eq) in DCM under influence of TBSOTf (0.2 equivalents) with increasing the temperature from -78° C to -35° C, resulted in complete consumption of the donors after one hour and trimers **5** and **6** were isolated in 68% and 71% yield, respectively. Removal of the TBS group in **5** and **6**, using the aforementioned conditions, gave the spacer containing acceptors **59** and **60** for the final key [3+3] coupling toward hexamers **7** and **8**. TBSOTf (0.02 eq) mediated coupling of donor **56** with 1.3 equivalent acceptor **59** (and similarly donor **57** with **60**) in DCM with a temperature increase from -78°C to -50°C resulted after one hour in the formation of hexasaccharides **7** and **8** that were isolated in excellent yields.

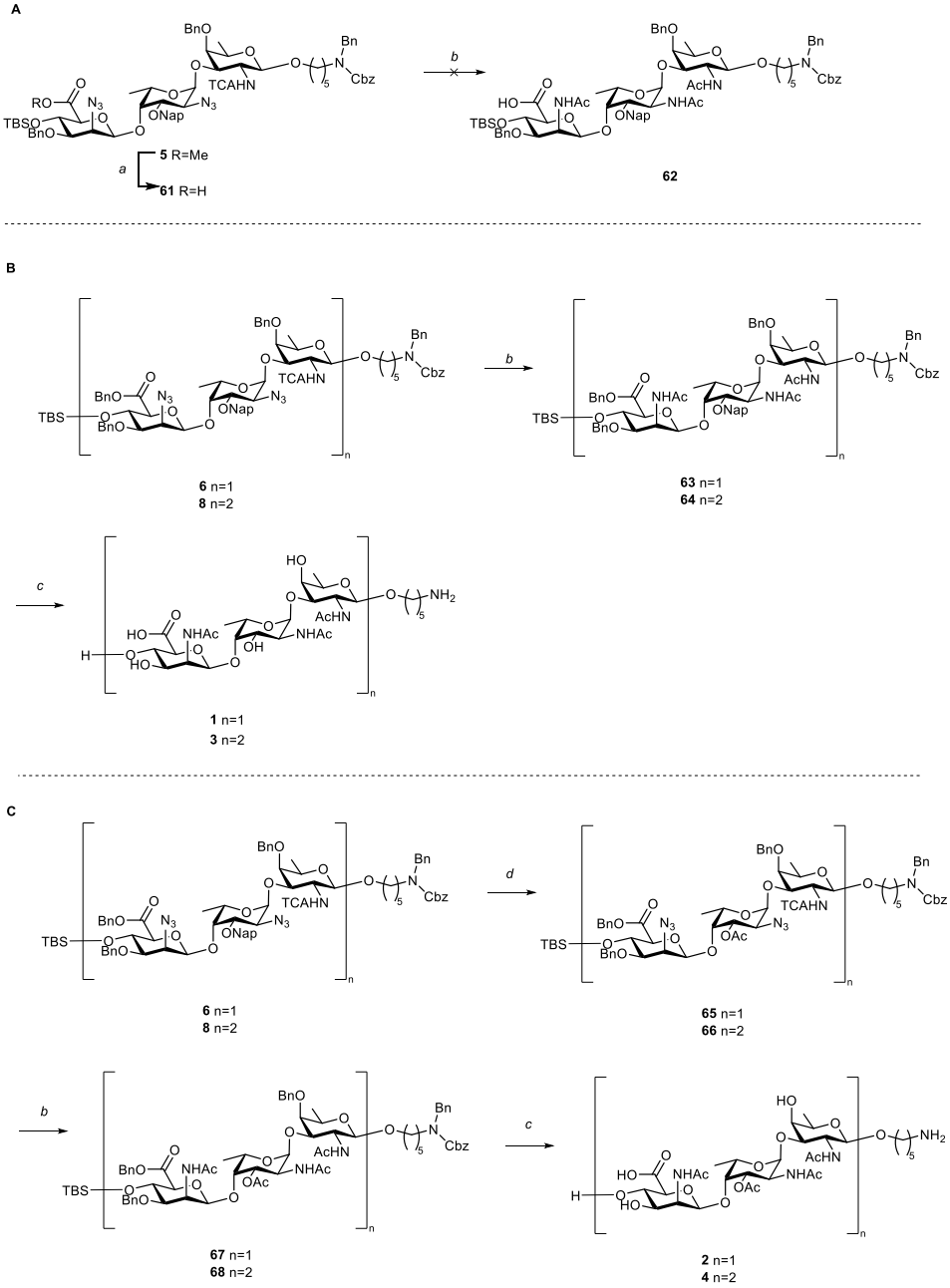
Scheme 7: *Synthesis of fully protected trimers **5** and **6** and hexamers **7** and **8***



Reagents and conditions: *a) NH_2NH_2*AcOH, DMF, quant. (**54** and **55**); b) (i) $CF_3(Cl)CNPh$, Cs_2CO_3, DCM, 88%; (ii) TBSOTf, DCM, -78° C to -35° C, 68% (**5**), 71% (**6**); c) TEA*HF, THF, 66° C, quant (**59** and **60**); d) TBSOTf, DCM, -78° C to -50° C, 88% (**7**), 96% (**8**)*

With the availability of the protected progenitors of the target tri- and hexamers, attention was directed to explore the most efficient deprotection strategy. To obtain trisaccharide **1**, lacking the acetyl ester, protected trimer **5** was subjected to $LiOH/H_2O_2$ mediated saponification of the methyl mannuronate ester in THF/H_2O (4:1) (Scheme 8). However, after 2 days 93% of starting material **5** was recovered. Observed solubility issues were an incentive to replace LiOH by TBAOH and together with raising the temperature to 40° C, intermediate **61** could be isolated in quantitative yield. The subsequent simultaneous conversion of azide and TCA groups in to acetamido groups, with Zn in $THF/AcOH/Ac_2O$ [12] led to a complex mixture, in which a lactamized by-product prevailed. Subjection of benzyl ester **6** to the same reaction conditions afforded trimer **63** in 67% yield. Complete removal of the TBS group in **63** and hydrogenolysis of the crude product, using Pearlman's catalyst in a mixture of tBuOH and H_2O (4:1) and purification by size exclusion chromatography (HW40) afforded target trimer **1** in 96% yield. Application of the same deprotection protocol to protected progenitor **8** afforded target hexamer **3** in 55% overall yield. To obtain the acetyl substituted trimer **2** and hexamer **4**, the naphthyl group in **6** and **8** was removed using DDQ, followed by acetylation to give trimer **65** and hexamer **66** in 98% and 88% yield, respectively. The acetamido derivatives **67** and **68** were obtained in similar yield using the aforementioned procedure, followed by removal of TBS group and hydrogenolysis. Final size exclusion chromatography delivered final targets **2** and **4** in 88% and 62% yield.

Scheme 8: *Final deprotections towards final trimer **1/2** and hexamer **3/4** targets; (**A**) starting from compound **54**; (**B**) non acetylated fragments and (**C**) acetylated fragments from compounds **55** and **59***



Reagents and conditions*: a) TBAOH (40% w in H_2O), H_2O_2, THF, quant.; b) Zn, AcOH, Ac_2O, THF, 40° C, 67% (**63**), 62% (**64**), 65% (**67**), 64% (**68**); c) (i) TEA*HF, THF, 40° C, (ii) Pd/C, AcOH, H_2, tBuOH:H_2O*

*(4:1), 96% (**1**), 55% (**3**), 88% (**2**), 62% (**4**); d) (i) DDQ, DCM:H_2O (4:1); (ii) Ac_2O, Pyridine, 98% (**65**), 88% (**66**).*

CONCLUSION

This chapter deals with the development of an efficient synthetic route to fragments of the capsular polysaccharide of *S. aureus* type 5, with and without a C3 acetyl ester in the *N*-acetyl-L-fucose residue. A fully protected trisaccharide building block, corresponding to the repeating unit of the capsular polysaccharide was constructed that gave access not only to an acetylated and a non-acetylated trimer with an anomeric amino spacer but also, by means of a [3+3] block coupling, towards the corresponding hexamers. Two trisaccharide building blocks with either a methyl or benzyl ester mannuronate residue were synthesized using a [1+2] glycosylation assembly. Although no differences were found at the coupling step, the benzyl protection proved to be superior during the deprotection procedure towards the target oligomers. Other notable findings concern the replacement of the anomeric selenophenyl by an acetyl group in the D-FucNHTCA acceptor to prevent oxazoline formation during the glycosylation toward the difucoside intermediate and the favorable use of the naphthyl protecting group to allow the selective introduction of the acetyl substituent in the final stage of the assembly. This chapter has reported the first successful synthesis of CP5 fragments longer that the trimer repeating unit. The fragments will be evaluated for their capacity to bind to antibodies raised against the native CP5 polysaccharide and in the generation of conjugate vaccine modalities.

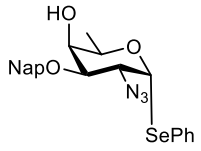
EXPERIMENTAL SECTION

General procedures

All reactions were carried out in oven-dried glassware (85 °C). Prior to reactions, traces of water and solvent were removed by co-evaporation with toluene where appropriate. Reactions sensitive to air or moisture were carried out under an atmosphere of argon (balloon). Solvents for reactions were of reagent grade and stored over 4Å molecular sieves (3Å for DCM, MeOH and ACN), except pyridine and DMF. TEA was stored over KOH pellets. Tf_2O used in glycosylations was dried over P_2O_5 (~3 hours), followed by distillation, and stored in a Schlenk flask at -20 °C. All other chemicals were used as received. Reaction progress was monitored using aluminium-supported silica gel TLC plates (Merck, Kieselgel 60, with fluorescent indicator); visualization was carried out by irradiation with UV light (λ: 254 nm), followed by spraying with 20% H_2SO_4 in EtOH (w/v) or Hanessian's stain ($(NH_4)_6Mo_7O_2$*$4H_2O$, 25 g/L; $(NH_4)_4Ce(SO_4)$*$2H_2O$, 10 g/L; in 10% aq. H_2SO_4). Column chromatography was carried out using silica gel (Screening Devices, 0.040-0.063 mm). Size-exclusion chromatography was carried out using Sephadex WH-40 (GE Healthcare). NMR spectra were recorded on Bruker AV-400, AV-500 or AV 850 instruments. Chemical shifts (δ) are reported in ppm relative to Me_4Si (δ: 0.00 ppm) or residual solvent signals. NMR spectra were recorded at ambient temperature, and samples were prepared in $CDCl_3$ unless noted otherwise. ^{13}C-APT spectra are ^{1}H

decoupled. Structural assignment was achieved using HH-COSY and HSQC 2D experiments. Coupling constants of anomeric carbon atoms (JH_1,C_1) were determined using HMBC-GATED experiments. LC-MS analyses were performed on a Thermo Finnigan Surveyor HPLC system equipped with a Gemini C-18 column (250 x 10 mm), connected to a Thermo Finnigan LCQ Advantage Max Ion-trap mass spectrometer with (ESI+). Eluents used were ACN, H_2O with addition of TFA (0.1%). HRMS spectra were recorded on a Thermo Finnigan LCQ Orbitrap instrument.

Phenyl 2-azido-2-deoxy-3-*O*-naphtyl-1-seleno-α-D-fucopyranoside (21)



Diol **20** (40 mmol) was dissolved in toluene (200 mL, 0.2 M) in a two-necked flask, equipped with a stopcock and a Dean-Stark trap. Bu_2SnO (41 mmol, 1.02 eq) was added to the mixture, which was heated to 120°C in an oil bath for 2 hours. After cooling to 60°C, Bu_4NBr (41 mmol, 1.02 eq), CeF (41 mmol, 1.02 eq) and NapBr (41 mmol, 1.02 eq) were added and the mixture was heated to 120°C and stirred for 1 hour after which TLC (Pentane:EtOAc, 6:4) indicated complete conversion of the starting material. The mixture was cooled to room temperature and a 10% aq solution of KF was added. After 30 minutes of vigorous stirring, the layers were separated, the aqueous phase extracted with EtOAc and the combined organic fractions were washed with Brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The desired product **21** was isolated by column chromatography (Pentane:EtOAc, 9:1 → 7:3) in 77% yield as yellow oil.

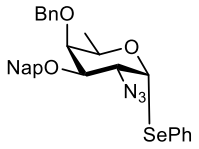
TLC analysis: R_f=0.32 (Pentane:EtOAc; 8:2)

^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.91-7.79 (4H, H_{arom}, m), 7.60-7.45 (5H, H_{arom}, m), 7.32-7.20 (3H, H_{arom}, m), 5.89 (1H, H_1, $J_{1\text{-}2}$=5.4 Hz, d), 4.90 (1H, CH*H*$_{Nap}$, J=11.5 Hz, d), 4.85 (1H, CH*H*$_{Nap}$, d), 4.28 (1H, H_5, $J_{5\text{-}6}$=6.6 Hz, q), 4.20 (1H, H_2, $J_{2\text{-}3}$=10.2 Hz, dd), 3.89 (1H, H_4, $J_{4\text{-}3}$=3.2 Hz, d), 3.74 (1H, H_3, dd), 1.24 (3H, 3 x H_6, d).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 134.6 (CH_{arom}), 134.5, 133.3 x2 (C_q), 129.2, 128.8 (CH_{arom}), 128.6 (C_q), 128.1, 127.9 x 2, 127.1, 126.5, 126.4, 125.8 (CH_{arom}), 85.3 (C_1), 79.4 (C_3), 72.4 (CH_{2_Nap}), 68.7 (C_5, C_4), 60.4 (C_2), 16.2 (C_6)

HRMS: $C_{23}H_{23}N_3O_3Se$ + H^+ required 469.0905, found 470.0978

Phenyl 2-azido-2-deoxy-3-*O*-naphtyl-4-*O*-benzyl-1-seleno-α-D-fucopyranoside (22)

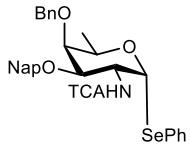


Compound **21** (21 mmol) was dissolved in DMF. At 0°C NaH (23 mmol, 1.1 eq, as 60% dispersion in mineral oil) and BnBr (23 mmol, 1.1 eq) were added slowly to the reaction mixture. The solution was allowed to warm to room temperature and after 2 hours TLC analysis (Pentane:EtOAc, 8:2) showed complete consumption of starting material. The reaction was quenched by slow addition of cold MeOH, followed by partitioning of the mixture between Et_2O and water. After extraction of the aqueous phase (Et_2O x 2), the combined organic phases were washed with brine, dried over $Na_2S_2O_4$, filtered and concentrated *in vacuo*. The final product was isolated by column chromatography (Pentane:EtOAc, 97:3 → 8:2) yielding 88% of **22**.

TLC analysis: R_f= 0.48 (Pentane:EtOAc; 9:1)

^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.91-7.80 (4H, H_{arom}, m), 7.60-7.45 (5H, H_{arom}, m), 7.35-7.20 (8H, H_{arom}, m), 5.94 (1H, H_1, J_{1-2}=5.3 Hz, d), 5.00-4.86 (3H,3 x CH*H*, m), 4.65 (1H, CH*H*, J=11.4 Hz, d), 4.39 (1H, H_2, J_{3-2}=10.3 Hz, dd), 4.22 (1H, H_5, J_{5-6}=6.7 Hz, q), 3.78 (1H, H_3, J_{4-3}=2.7 Hz, dd), 3.73 (1H, H_4, d), 1.13 (3H, 3 x H_6, d).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 138.2, 135.1 (C_q) 134.5 (CH_{arom}), 133.4, 133.2 (C_q), 129.2 (CH_{arom}), 128.8 (C_q), 128.5 x 2, 128.3, 128.1, 128.0, 127.9, 127.8, 126.8, 126.4, 126.3, 125.8 (CH_{arom}), 85.7 (C_1), 80.8 (C_3), 76.0 (C_4), 75.2 (CH_2), 72.8 (CH_2), 69.6 (C_5), 61.2 (C_2), 16.7 (C_6)
HRMS: $C_{30}H_{29}N_3O_3Se$ + Na^+ required 582.1266, found 582.1269

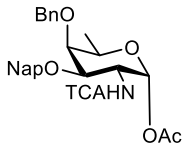
Phenyl 2-trichloroacetylamido-2-deoxy-3-*O*-naphtyl-4-*O*-benzyl-1-seleno-α-D-fucopyranoside (23)



Method a: Compound **22** (1 mmol) was dissolved in a mixture of THF:*t*BuOH (4:1, 5 mL, 0.2 M) and 1 drop of acetic acid was added. After purging $Ar_{(g)}$ for 20 minutes, Pd/C was added and the reaction was stirred overnight under $H_{2(g)}$ atmosphere. The reaction mixture was filtered through Celite® and concentrated *in vacuo*. After co-evaporation with toluene (x3), the residual crude was dissolved in pyridine (10 mL, 0.1 M) and at 0°C trichloroacetylchloride (1.1 mmol, 1.1 eq) was added dropwise. The reaction was let to warm up to room temperature and after 1 hour TLC analysis showed complete consumption of starting material (DCM:MeOH:AcOH, 95:4.5:0.5). After quenching with cold water, the reaction mixture was diluted with EtOAc, washed (HCl 1M, H_2O, $NaHCO_{3(aq)}$, H_2O, brine), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The final compound **23** was isolated by column chromatography in 96% yield as white foam.
Method b: Compound **22** (1 mmol) was dissolved in THF (10 mL, 0.1M) and upon addition of PPh_3 the resulting mixture was heated at 45°C for 4 hours. H_2O was added and the reaction was stirred at reflux overnight. The solution was concentrated *in vacuo*, co-evaporated with toluene (x3) and the residual crude was dissolved in pyridine (10 mL, 0.1 M) and at 0°C trichloroacetylchloride (1.1 mmol, 1.1 eq) was added dropwise. The reaction was let to warm up to room temperature and after 1 hour TLC analysis (DCM:MeOH:AcOH, 95:4.5:0.5) showed complete consumption of starting material. After quenching with cold water, the reaction mixture was diluted with EtOAc, washed (HCl 1M, H_2O, $NaHCO_{3(aq)}$, H_2O, brine), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The final compound **23** was isolated by column chromatography (Pentane:EtOAc, 95:5 → 8:2) in 96% yield as white foam.
TLC analysis: R_f= 0.31 (Pentane:EtOAc; 9:1)
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.90-7.76 (4H, H_{arom}, m), 7.55-7.43 (5H, H_{arom}, m), 7.41-7.18 (8H, H_{arom}, m), 6.86 (1H, NH, J=7.5 Hz, d), 6.04 (1H, H_1, J_{1-2}=4.8 Hz, d), 5.01 (1H, CH*H*, J=11.6 Hz, d), 4.90 (1H, CH*H*, J=12.1 Hz, d), 4.83-4.75 (1H, H_2, m), 4.74-4.65 (2H,2 x CH*H*, m), 4.22 (1H, H_5, J_{5-6}=6.5 Hz, q), 3.86 (1H, H_4, J_{4-3}=2.6 Hz, d), 3.65 (1H, H_3, J_{3-2}=11 Hz, dd), 1.28 (3H, 3 x H_6, d).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 161.6, 138.1, 134.6 (C_q) 134.2 (CH_{arom}), 133.4, 133.2 (C_q), 129.4 (CH_{arom}), 128.9 (C_q), 128.8, 128.5, 128.3, 128.0 x 2, 127.9 x 2, 126.7, 126.5, 126.4,

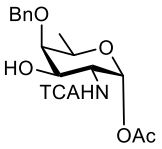
125.5 (CH_{arom}), 89.2 (C_1), 78.8 (C_3), 74.9 (CH_2), 74.6 (C_4), 71.7 (CH_2), 70.6 (C_5), 52.0 (C_2), 16.9 (C_6)
HRMS: $C_{32}H_{30}Cl_3NO_4Se + NH_4^+$ required 695.0744, found 695.0740

2-trichloroacetylamido-2-deoxy-3-*O*-naphtyl-4-*O*-benzyl-1-*O*-acetyl-α-D-fucopyranoside (33)



Compound **23** (12 mmol) was dissolved in THF (120 mL, 0.1 M) and NIS (14.4 mmol, 1.2 eq) and H_2O (2.2 mL, 10 eq) were added. After 1 hour TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of starting material. The reaction mixture was diluted with DCM and washed with $Na_2S_2O_{3(sat.aq.)}$ (x2), H_2O and brine. After drying over $MgSO_4$ and filtration, the organic phase was concentrated *in vacuo*. The residual crude was dissolved in pyridine (120 mL, 0.1 M) and at 0°C Ac_2O (3.4 mL, 3 eq) and DMAP (0.02 mmol, 2%) were added. The reaction was let to warm up to room temperature and after 1 hour TLC analysis (Pentane:EtOAc, 1:1) showed complete consumption of starting material. After quenching with cold water, the reaction mixture was diluted with EtOAc, washed (HCl 1M, H_2O, $NaHCO_{3(aq)}$, H_2O, brine), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The final compound **33** was isolated by column chromatography (Pentane:EtOAc, 8:2→6:4) in 92% yield as white foam and as a mixture of α/β 5.3:1.
TLC analysis: R_f= 0.41 (Pentane:EtOAc; 7:3)
Only the signals relative to the α anomer are reported
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.90-7.76 (4H, H_{arom}, m), 7.55-7.43 (3H, H_{arom}, m), 7.41-7.26 (5H, H_{arom}, m), 6.41 (1H, NH_{TCA}, J=8.1 Hz, d), 6.33 (1H, H_1, J_{1-2}=3.8 Hz, d), 5.00 (1H, CH*H*, J=11.6 Hz, d), 4.90 (1H, CH*H*, J=12.1 Hz, d), 4.79-4.65 (3H,2 x CH*H*, H_2, m), 3.95 (1H, H_5, J_{5-6}=6.5 Hz, q), 3.88-3.81 (2H, H_4, H_3, m), 2.02 (3H, CH_{3_OAc}, s), 1.28-1.19 (3H, 3 x H_6, m).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.0, 161.8, 138.0, 134.8, 133.4, 133.3 (C_q), 128.8, 128.6, 128.5 x 3, 128.4, 128.0 x 2, 127.9, 126.7, 126.6, 126.4, 125.6 (CH_{arom}), 91.2 (C_1), 76.7 (C_3), 74.9 (CH_2), 74.5 (C_4), 71.5 (CH_2), 69.4 (C_5), 50.2 (C_2), 21.0 (CH_{3_OAc}), 17.1 (C_6).
HRMS: $C_{28}H_{22}Cl_3NO_6 + NH_4^+$ required 597.1315, found 597.1321

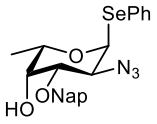
2-trichloroacetylamido-2-deoxy-4-*O*-benzyl-1-*O*-acetyl-α-D-fucopyranoside (34)



Compound **33** (10 mmol) was dissolved in a mixture of DCM:H_2O (4:1, 100 mL, 0.1 M). DDQ (20 mmol, 2 eq) was added and after 2.5 hours of vigorous stirring TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of starting material. After dilution with DCM, the reaction mixture was washed with a 10% aq solution of $Na_2S_2O_3$. The aqueous layer was extracted with DCM and the combined organic phases were washed with saturated aq solution of $NaHCO_3$, H_2O and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound **34** was isolated by column chromatography (Pentane:EtOAc, 75:25→1:1) in 71% yield as a white foam.
TLC analysis: R_f= 0.29 (Pentane:EtOAc; 7:3)
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.44-7.29 (5H, H_{arom}, m), 6.62 (1H, NH_{TCA}, J=8.3 Hz, d), 6.27 (1H, H_1, J_{1-2}=3.6 Hz, d), 4.87 (1H, CH*H*, J=11.4 Hz, d), 4.67 (1H, CH*H*, d), 4.44-3.31 (1H, H_2,

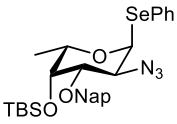
m), 4.03 (1H, H_5, $J_{5\text{-}6}$=6.6 Hz, q), 3.73 (1H, H_3, $J_{3\text{-}2}$=10.6, $J_{3\text{-}4}$=3.4, dd), 1.32 (1H, H_4, d), 2.12 (3H, CH_{3_OAc}, s), 1.32 (3H, 3 x H_6, d).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.1, 162.8, 137.6 (C_q), 128.9, 128.4, 128.1 (CH_{arom}), 114.2 (C_q), 91.1 (C_1), 79.6 (C_3), 76.4 (CH_2), 69.4 (C_4), 69.3 (CH_2), 52.0 (C_2), 20.9 (CH_{3_OAc}), 17.0 (C_6).
HRMS: $C_{17}H_{20}Cl_3NO_6$ + NH_4^+ required 457.0654, found 457.0636

Phenyl 2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-1-seleno-α-L-fucopyranoside (35)



Diol **25** (22 mmol) was dissolved in toluene (110 mL, 0.2 M) in a two-necked flask, equipped with a stopcock and a Dean-Stark trap. Bu_2SnO (22 mmol, 1eq) was added to the mixture, which was heated to 140°C in an oil bath for 3 hours. After cooling to 60°C, Bu_4NBr (23 mmol, 1.05 eq), CeF (23 mmol, 1.05 eq) and NapBr (23 mmol, 1.05 eq) were added and the mixture was heated to 120°C and stirred for 4 hours after which TLC (Pentane:EtOAc, 3:2) indicated complete conversion of the starting material. The mixture was cooled to room temperature and a 10% aq solution of KF was added. After 30 minutes of vigorous stirring, the layers were separated, the aqueous phase extracted with EtOAc and the combined organic fractions were washed with Brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The desired product **35** was isolated by column chromatography in 82% yield as yellow oil.
TLC analysis: R_f=0.32 (Pentane:EtOAc; 8:2)
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.91-7.79 (4H, H_{arom}, m), 7.60-7.45 (5H, H_{arom}, m), 7.32-7.20 (3H, H_{arom}, m), 5.90 (1H, H_1, $J_{1\text{-}2}$=5.4 Hz, d), 4.92 (1H, CH*H*$_{Nap}$, J=11.5 Hz, d), 4.86 (1H, CH*H*$_{Nap}$, d), 4.29 (1H, H_5, $J_{5\text{-}6}$=6.6 Hz, q), 4.20 (1H, H_2, $J_{2\text{-}3}$=10.2 Hz, dd), 3.90 (1H, H_4, $J_{4\text{-}3}$=3.2 Hz, d), 3.75 (1H, H_3, dd), 1.24 (3H, 3 x H_6, d).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 134.6 (CH_{arom}), 134.5, 133.3 x2 (C_q), 129.2, 128.8 (CH_{arom}), 128.6 (C_q), 128.1, 127.9 x 2, 127.1, 126.5, 126.4, 125.8 (CH_{arom}), 85.3 (C_1), 79.4 (C_3), 72.4 (CH_{2_Nap}), 68.7 (C_5, C_4), 60.4 (C_2), 16.2 (C_6)
HRMS: $C_{23}H_{23}N_3O_3Se$ + H^+ required 469.0905, found 470.0978

Phenyl 2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(*tert*butyldimethyl)silyl-1-seleno-α-L-fucopyranoside (36)

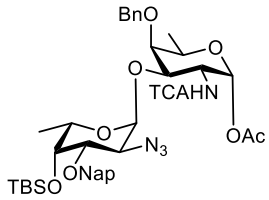


Compound **35** (10 mmol) was dissolved in pyridine (50 mL, 0.2 M). TBSOTf (11 mmol, 1.1 eq) and DMAP (0.05 mmol, 0.5%) were added at 0°C and after 10 minutes stirring the reaction mixture was heated to 70°C. After 16 hours TLC analysis (Pentane:EtOAc, 8:2) showed complete consumption of starting material and the reaction mixture was cooled to room temperature. After quenching with cold MeOH and diluting with EtOAc, the mixture was washed with 10% aq solution of $CuSO_4$ (x2), H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography afforded the title compound **36** in quantitative yield as a light-yellow oil.
TLC analysis: R_f=0.30 (Pentane:EtOAc; 95:5)
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.91-7.79 (4H, H_{arom}, m), 7.63-7.43 (5H, H_{arom}, m), 7.32-7.20 (3H, H_{arom}, m), 5.93 (1H, H_1, $J_{1\text{-}2}$=5.4 Hz, d), 4.94-4.82 (2H, CH_{2_Nap}, m), 4.28 (1H, H_2, $J_{2\text{-}3}$=10.4 Hz, dd), 4.22 (1H, H_5, $J_{5\text{-}6}$=6.5 Hz, q), 3.98-3.89 (1H, H_4, m), 3.66 (1H, H_3, $J_{3\text{-}4}$=2.5 Hz,

dd), 1.16 (3H, 3 x H_6, d), 0.88 (9H, 3 x CH_{3_tBu}, m), 0.06 (3H, CH_{3_Me}, s), 0.05 (3H, CH_{3_Me}, s).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 135.0 (C_q), 134.9 (CH_{arom}), 133.4, 133.2 (C_q), 129.2 (CH_{arom}), 128.8 (C_q), 128.3, 128.1, 127.9 x 2, 126.9, 126.3, 126.1, 126.0 (CH_{arom}), 85.9 (C_1), 80.2 (C_3), 73.1 (CH_{2_Nap}), 71.2 (C_4), 70.2 (C_5), 61.1 (C_2), 26.2 (CH_{3_tBu}), 18.7 (C_q), 17.1 (C_6), -3.8, -4.5 (CH_{3_Me}).

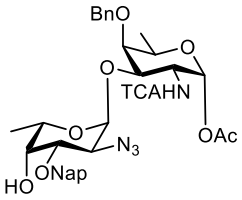
HRMS: $C_{17}H_{20}Cl_3NO_6$ + Na^+ required 606.1662, found 606.1664

2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(*tert*butyldimethyl)silyl-α-L-fucopyranosyl)-4-*O*-benzyl-1-*O*-acetyl-α-D-fucopyranoside (37)



Donor **36** (1.3 mmol, 1.3 eq) and acceptor **34** (1 mmol) were co-evaporated together with toluene. After dissolving in DCM (20 mL, 0.05 M), freshly activated 4 Å MS were added and the reaction mixture was cooled to -78°C. NIS (1.3 mmol, 1.3 eq) ad TBSOTf (0.3 mmol, 0.3 eq) were added and the reaction mixture was left to stir at -55°C. After 1 hour TLC analysis (Pentane:EtOAc 7:3) showed complete consumption of acceptor **34** and the reaction was quenched by addition of TEA (0.3 mmol, 0.3 eq). The reaction mixture was diluted in EtOAc and washed with a saturated aq. solution of $Na_2S_2O_3$, H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (Pentane:EtOAc, 8:2→7:3) afforded **37** in quantitative yield.
TLC analysis: R_f= 0.43 (Pentane:EtOAc; 7:3)
^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.78-7.42 (4H, H_{arom}, m), 7.59-7.41 (4H, H_{arom}, NH_{TCA}, m), 7.40-7.24 (5H, H_{arom}, m), 6.53 (1H, H_{1D}, J_{1-2}=3.6 Hz, d), 5.06 (1H, H_{1L}, J=3.6 Hz, d), 4.94-4.78 (2H, CH_2, m), 4.78-4.63 (2H, CH_2, m), 4.63-4.52 (1H, H_{2D}, m), 4.10 (1H, H_{2L}, J_{2-3}=10.4 Hz, dd), 4.05-3.90 (4H, H_{5L}, H_{5D}, H_{3D}, H_{4L}, m), 3.85 (1H, H_{3L}, J_{3-4}=2.4 Hz, dd), 3.75 (1H, H_{4D}, J_{3-4}=2.8 Hz, d), 2.11 (3H, CH_{3_OAc}, s), 1.19 (3H, 3 x H_6, J_{6-5}=6.5 Hz, d), 1.14 (3H, 3 x H_6, J_{6-5}=6.5, d) 0.88 (9H, 3 x CH_{3_tBu}, m), 0.03 (3H, CH_{3_Me}, s), 0.02 (3H, CH_{3_Me}, s).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.0, 162.3, 138, 134.7, 133.4, 133.1 (C_q), 128.6, 128.5, 128.3, 128.1, 127.9 x 2, 127.6, 126.4, 126.3, 126.1, 125.4 (CH_{arom}), 99.6 (C_{1L}) , 92.7 (C_q), 90.3 (C_{1D}), 78.9 (C_{3L}), 78.3 (C_{3D}), 77.4 (C_{4L}), 75.1 (CH_2), 72.8 (CH_2), 70.8 (C_{4D}), 69.5 (C_5), 69.3 (C_5), 61.2 (C_{2L}), 50.7 (C_{2D}), 26.1 (CH_{3_tBu}), 21.0 (CH_{3_OAc}), 18.7 (C_q), 17.5 (C_6),17.0 (C_6), -3.8, -4.5 (CH_{3_Me}).
HRMS: $C_{40}H_{51}Cl_3N_4O_9Si$ + Na^+ required 887.2383, found 887.2383

2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-α-L-fucopyranosyl)-4-*O*-benzyl-1-*O*-acetyl-α-D-fucopyranoside (38)



Compound **37** (1 mmol) was dissolved in dry THF (10 mL, 0.1 M) and TEA*HF (20 mmol, 20 eq) was added. The reaction mixture was stirred at reflux 3 days. After reaching room temperature, the reaction mixture was diluted with EtOAc and transfer in a beaker containing a stirring cold solution of saturated aq. solution of $NaHCO_3$. Once the ice melted, the organic layer was separated and the aqueous phase was reextracted (x2). The combined EtOAc solutions were washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound **38** was isolated by column chromatography in 92% yield as white solid.

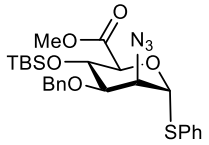
TLC analysis: R_f= 0.35 (Pentane:EtOAc; 1:1)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.91-7.75 (4H, H_{arom}, m), 7.58-7.42 (4H, H_{arom}, NH_{TCA}, m), 7.40-7.24 (5H, H_{arom}, m), 6.52 (1H, H_{1D}, J_{1-2}=3.6 Hz, d), 5.04 (1H, H_{1L}, J=3.4 Hz, d), 4.90-4.67 (4H, 2 x CH_2, m), 4.63-4.55 (1H, H_{2D}, m), 4.10-3.92 (5H, H_{2L}, H_{5L}, H_{5D}, H_{3D}, H_{3L}, m), 3.85 (1H, H_{4D}, J_{3-4}=2.9 Hz, dd), 3.75 (1H, H_{4L}, J_{3-4}=2.8 Hz, d), 2.11 (3H, CH_{3_OAc}, s), 1.22 (3H, 3 x H_6, J_{6-5}=6.5 Hz, d), 1.18 (3H, 3 x H_6, J_{6-5}=6.5, d).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.0, 162.2, 138.1, 134.3, 133.3 (C_q), 128.9, 128.6, 128.1, 127.9 x 2, 127.8, 127.7, 127.1, 126.6, 126.5, 125.7 (CH_{arom}), 99.0 (C_{1L}) , 92.7 (C_q), 90.4 (C_{1D}), 78.3 (C_{3L}, C_{3D}), 76.8 (C_{4L}), 75.0 (CH_2), 72.3 (CH_2), 69.2 (C_5), 68.3 (C_{4D}), 67.5 (C_5), 60.7 (C_{2L}), 50.7 (C_{2D}), 21.0 (CH_{3_OAc}), 17.0 (C_q), 16.4 (C_6).

HRMS: $C_{35}H_{37}Cl_3N_4O_9$ + Na^+ required 773.1518, found 773.1518

Methyl (phenyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-1-thio-α-D-mannopyranosiduronate) (11)



Compound **46** (6.6 mmol) was dissolved in pyridine (33 mL, 0.2 M). TBSOTf (19.8 mmol, 3 eq) and DMAP (0.06 mmol, 1%) were added at 0°C and after 10 minutes stirring the reaction mixture was heated to 70°C. After 5 hours TLC analysis (Pentane:EtOAc, 8:2) showed complete consumption of starting material and the reaction mixture was cooled to room temperature. After quenching with cold MeOH and diluting with EtOAc, the mixture was washed with 10% aq solution of $CuSO_4$ (x2), H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (Pentane:EtOAc, 98:2→8:2) afforded the title compound **11** in quantitative yield as a light-yellow oil.

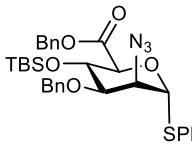
TLC analysis: R_f= 0.55 (Pentane:EtOAc; 9:1)

^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.68-7.58 (2H, H_{arom}, m), 7.39-7.23 (8H, H_{arom}, m), 7.24-7.14 (5H, H_{arom}), 5.61 (1H, H_1, J_{1-2}=8.9 Hz, d), 4.76-4.52 (2H, 2 x $HCHBn$, m) 4.89 (1H, $HCHBn$, d), 4.50 (1H, $HCHBn$, J=11.4 Hz, d), 4.47-4.37 (3H, $HCHBn$, H_4, H_5, m), 3.70 (1H, H_3, J_{3-4}=4.6 Hz, J=2.9 Hz, dd), 3.49 (1H, H_2, dd), 0.81 (9H, 3 x CH_{3_tBu}, s), 0.03, -0.02 (3H, CH_{3_Me}, s).4.48-4.26 (2H, H_5, H_4, m), 3.73 (1H, H_3, J_{3-2}=4.9 Hz, J_{3-4}=2.8 Hz, dd), 3.59-3.48 (4H, CH_{3_Me}, H_2, m), 0.82 (9H, 3 x CH_{3_tBu}, s), 0.06 (3H, CH_{3_Me}, s), 0.01 (3H, CH_{3_Me}, s).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.7, 137.2, 135.2 (C_q), 128.9, 128.8, 128.6 x 2, 128.4, 128.3 x 2, 128.1, 128.0 (CH_{arom}), 92.0 (C_1), 78.6 (C_3), 76.2 (C_4), 73.1 (CH_2), 68.6 (C_5), 60.4 (C_2), 52.4 (CH_3), 25.8 (CH_{3_tBu}), 18.1 (C_q), -4.5 (CH_3), -5.02 (CH_3).

HRMS: $C_{26}H_{35}N_3O_5SSi$ + Na^+ required 552.1959, found 552.1959

Benzyl (phenyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-1-thio-α-D-mannopyranosiduronate) (12)



Compound **47** (4.3 mmol) was dissolved in pyridine (21 mL, 0.2 M). TBSOTf (13 mmol, 3 eq) and DMAP (0.04 mmol, 1%) were added at 0°C and after 10 minutes stirring the reaction mixture was heated to 70°C. After 16 hours TLC analysis (Pentane:EtOAc, 8:2) showed complete consumption of starting material and the reaction mixture was cooled to room temperature. After quenching with cold MeOH and diluting with EtOAc, the mixture was washed with 10% aq. solution of CuSO (x2), H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (Pentane:EtOAc, 98:2→8:2) afforded the title compound in quantitative yield as a light yellow oil.

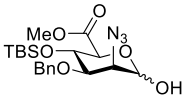
TLC analysis: R_f= 0.57 (Pentane:EtOAc; 9:1)

^{1}H-NMR (400 MHz, $CDCl_3$), δ: δ: 7.63-7.54 (2H, H_{arom}, m), 7.35-7.24 (8H, H_{arom}, m), 7.24-7.14 (5H, H_{arom}), 5.66 (1H, H_1, $J_{1\text{-}2}$=9.4 Hz, d), 5.03 (1H, $HC\mathit{H}HBn$, J=12.1 Hz, d), 4.89 (1H, $HC\mathit{H}HBn$, d), 4.50 (1H, $HC\mathit{H}HBn$, J=11.4 Hz, d), 4.47-4.37 (3H, $HC\mathit{H}HBn$, H_4, H_5, m), 3.70 (1H, H_3, $J_{3\text{-}4}$=4.6 Hz, J=2.9 Hz, dd), 3.49 (1H, H_2, dd), 0.81 (9H, 3 x CH_{3_tBu}, s), 0.03, -0.02 (3H, CH_{3_Me}, s).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.0, 136.8, 135.2 (C_q), 132.8 (CH_{arom}), 131.6 (C_q), 128.9, 128.6 x 2, 128.4, 128.3, 127.9 (CH_{arom}), 80.9 (C_1), 78.0 (C_3), 77.1 (C_4), 73.1 (CH_2), 68.6 (C_5), 68.6 (C_5), 67.2 (CH_2), 57.3 (C_2), 25.7 (CH_{3_tBu}), 17.9 (C_q), -4.3 (CH_3), -5.6 (CH_3).

HRMS: $C_{32}H_{39}N_3O_5SSi$ + Na^+ required 628.2272, found 628.2272

Methyl (2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-α/β-D-mannopyranosiduronate) (48)



To a solution of compound **11** (1 mmol) in acetone:H_2O (10 mL, 0.1M, 4:1), NBS (3 mmol, 3 eq) was added at 0°C. The reaction mixture assumed an orange-brown colour and after 1.5 hour TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of starting material. The reaction mixture was diluted with EtOAc and washed with saturated aq. solution of $Na_2S_2O_3$ (x2), H_2O and brine. After drying over $MgSO_4$ and filtration, the organic phase was concentrated *in vacuo*. The final product was isolated by column chromatography (Pentane:EtOAc, 9:1→7:3) in quantitative yield as a white foam and as a mixture of α/β mixture 1:4.

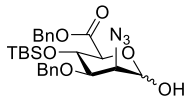
TLC analysis: R_f= 0.33 (Pentane:EtOAc; 8:2)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.43-7.26 (5H, H_{arom}, m), 5.57-5.49 (1H, H_1, m), 4.69-4.54 (2H, CH_{2_Bn}, m), 4.40-4.34 (2H, H_5, H_4, m), 3.82-3.75 (1H, H_3, m), 3.68 (1H, H_2, $J_{2\text{-}1}$=6.5 Hz, $J_{2\text{-}3}$=2.9 Hz, dd), 3.60 (3H, CH_{3_OMe}, s), 3.58-3.50 (1H, OH, b), 0.9 (9H, 3 x CH_{3_tBu}, s), 0.08 (3H, CH_3, s), 0.06 (3H, CH_3, s).

^{13}C-NMR (500 MHz, $CDCl_3$), δ: 169.7, 137.2 (C_q), 128.9, 128.8, 128.6 x 2, 128.4, 128.3 x 2, 128.1, 128.0 (CH_{arom}), 92.0 (C_1), 78.6 (C_3), 76.2 (C_4), 73.1 (CH_2), 68.6 (C_5), 60.4 (C_2), 52.4 (CH_3), 25.8 (CH_{3_tBu}), 18.1 (C_q), -4.5 (CH_3), -5.02 (CH_3).

HRMS: $C_{20}H_{31}N_3O_6Si$ + Na^+ required 460.1874, found 460.1874

Benzyl (2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-α/β-D-mannopyranosiduronate) (49)



To a solution of compound **12** (1 mmol) in acetone:H_2O (10 mL, 0.1 M, 4:1) NBS (3 mmol, 3 eq) was added at 0°C. The reaction mixture assumed an orange-brown colour and after 1.5 hour TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of starting material. The reaction mixture was diluted with EtOAc and washed with a saturated aq. solution of $Na_2S_2O_3$ (x2), H_2O and brine. After drying over $MgSO_4$ and filtration, the organic phase was concentrated *in vacuo*. The final product was isolated by column chromatography (Pentane:EtOAc, 9:1→7:3) in quantitative yield as a white foam and as a mixture of α/β mixture 1:6.7.

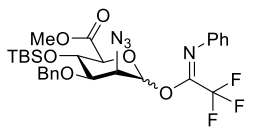
TLC analysis: R_f= 0.35 (Pentane:EtOAc; 8:2)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.40-7.23 (10H, H_{arom}, m), 5.62-5.54 (1H, H_1, m), 5.08 (1H, CH*H*, J=12.2 Hz, d), 4.95 (1H, CH*H*, J=12.2 Hz, d), 4.55 (1H, CH*H*, J=11.6 Hz, d), 4.49 (1H, CH*H*, J=11.6 Hz, d), 4.42-4.36 (2H, H_5, H_4, m), 3.78-3.72 (1H, H_3, m), 3.64 (1H, H_2, $J_{2\text{-}1}$=7.0 Hz, $J_{2\text{-}3}$=2.9 Hz, dd), 3.31 (1H, OH, d), 0.9 (9H, 3 x CH_{3_tBu}, s), 0.06 (3H, CH_3, s), 0.04 (3H, CH_3, s).

^{13}C-NMR (500 MHz, $CDCl_3$), δ: 169.2, 137.1, 135.2 (C_q), 128.8, 128.7 x 2, 128.6, 128.5 x 2, 128.4, 128.1 x 2 (CH_{arom}), 91.7 (C_1), 78.2 (C_3), 76.5 (C_4), 73.0 (CH_2), 68.7 (C_5), 67.2 (CH_2), 60.3 (C_2), 25.8 (CH_{3_tBu}), 18.1 (C_q), -4.7 (CH_3), -5.06 (CH_3).

HRMS: $C_{26}H_{35}N_3O_6Si$ + Na^+ required 536.2187, found 536.2187

Methyl (2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-1-*O*-(*N*-phenyl-2,2,2-trifluoroacetimidoyl)-α/β-D-mannopyranosiduronate) (50)



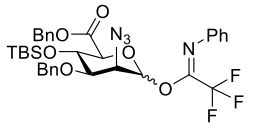
Compound **48** (0.5 mmol) was dissolved in dry acetone (1.5 mL, 0.33 M) and *N*-phenyl-2,2,2-trifluoroimidoyl chloride (0.65 mmol, 1.5 eq) and Cs_2CO_3 (0.6 mmol, 1.2 eq) were added. After1.5 hour, TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of the starting material. The reaction mixture was diluted with acetone, filtered and concentrated *in vacuo*. Compound **50** was isolated by column chromatography (Pentane:EtOAc, 95:5, 1% TEA) in 74% yield as transparent oil.

TLC analysis: R_f= 0.7 (Pentane:EtOAc; 9:1)

^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.47-7.28 (7H, H_{arom}, m), 7.20-7.10 (1H, H_{arom}, m), 6.88 (2H, H_{arom}, J=7.7 Hz, d), 6.42-6.20 (1H, H_1, bs), 4.71 (1H, $HC\mathit{H}HBn$, J=11.6 Hz, d), 4.65 (1h, $HC\mathit{H}HBn$, d), 4.36-4.17 (2H, H_5, H_4, m), 4.12-4.01 (1H, H_3, m), 3.92-3.83 (1H, H_2, m), 3.65 (3H, CH_3, s), 0.87 (9H, 3 x CH_{3_tBu}, s), 0.07 (3H, CH_{3_Me}, s), 0.05 (3H, CH_{3_Me}, s).

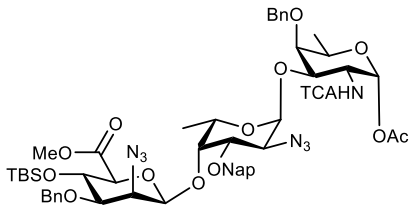
^{13}C-NMR (500 MHz, $CDCl_3$), δ: 169.7, 137.2 (C_q), 128.9, 128.8, 128.6 x 2, 128.4, 128.3 x 2, 128.1, 128.0 (CH_{arom}), 92.0 (C_1), 78.6 (C_3), 76.2 (C_4), 73.1 (CH_2), 68.6 (C_5), 60.4 (C_2), 52.4 (CH_3), 25.8 (CH_{3_tBu}), 18.1 (C_q), -4.5 (CH_3), -5.02 (CH_3).

Benzyl (2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-1-*O*-(*N*-phenyl-2,2,2-trifluoroacetimidoyl)-α/β-D-mannopyranosiduronate) (51)



Compound **49** (0.5 mmol) was dissolved in dry acetone (1.5 mL, 0.33 M) and *N*-phenyl-2,2,2-trifluoroimidoyl chloride (0.65 mmol, 1.2 eq) and Cs_2CO_3 (0.6 mmol, 1.2 eq) were added. After1.5 hour, TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of the starting material. The reaction mixture was diluted with acetone, filtered and concentrated *in vacuo*. Compound **51** was isolated by column chromatography (Pentane:EtOAc, 95:5, 1% TEA) in 77% yield as transparent oil.
TLC analysis: R_f= 0.7 (Pentane:EtOAc; 9:1)
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.51-7.21 (12H, H_{arom}, m), 7.18-7.09 (1H, H_{arom}, m), 6.83-6.75 (2H, H_{arom}, m), 6.50-6.21 (1H, H_1, bs), 5.11 (1H, $HCHBn$, J=12.4 Hz, d), 5.00 (1H, $HCHBn$, d), 4.64 (1H, $HCHBn$, J=11.6 Hz, d), 4.58 (1H, $HCHBn$, d), 4.38-4.28 (2H, $H_{5,\ H}4$, m), 4.06-3.97 (1H, H_3, m), 3.91-3.82 (1H, H_2, m), 0.85 (9H, 3 x CH_{3_tBu}, s), 0.05 (6H, 2 x CH_3, s).
^{13}C-NMR (500 MHz, $CDCl_3$), δ: 169.2, 137.1, 135.2 (C_q), 128.8, 128.7 x 2, 128.6, 128.5 x 2, 128.4, 128.1 x 2 (CH_{arom}), 91.7 (C_1), 78.2 (C_3), 76.5 (C_4), 73.0 (CH_2), 68.7 (C_5), 67.2 (CH_2), 60.3 (C_2), 25.8 (CH_{3_tBu}), 18.1 (C_q), -4.7 (CH_3), -5.06 (CH_3).

2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(methyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-1-*O*-acetyl-α-D-fucopyranoside (52)

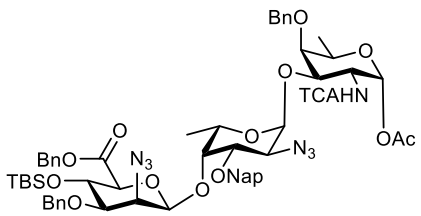


Method A: Donor **11** (0.2 mmol, 2.5 eq) and acceptor **38** (0.08 mmol) were co-evaporated with toluene three times and dissolved in DCM (0.8 mL, 0.1 M). Freshly activated 4 Å MS were added to the reaction mixture, which was then cooled to -60°C. NIS (0.2 mmol, 2.5 eq) and TBSOTf (0.024 mmol, 0.3 eq) were added and the reaction was kept at -40°C for 8 hours, after which TLC analysis (Pentane:EtOAc, 1:1) showed complete consumption of acceptor. The reaction mixture was quenched by addition of TEA (0.024 mmol, 0.3 eq) and let to warm up to room temperature. The reaction was diluted with DCM and washed with saturated aq. solution of $Na_2S_2O_4$, H_2O and brine. Purification by column chromatography (Pentane:EtOAc, 8:2→6:4) afforded the title compound **52** in 67% yield as white solid.

Method B: Donor **50** (0.242 mmol, 2.5 eq) and acceptor **38** (0.097 mmol) were co-evaporated with toluene three times and dissolved in dry DCM (1 mL, 0.1 M). Freshly activated 4 Å MS were added to the reaction mixture, which was then cooled to -60°C. TBSOTf (0.03 mmol, 0.3 eq) was added dropwise and the reaction was kept at -40°C for 10 hours, after which TLC analysis (Pentane:EtOAc, 1:1) showed complete consumption of acceptor. The reaction mixture was quenched by addition of TEA (0.03 mmol, 0.3 eq) and let to warm up to room temperature. The reaction was diluted with DCM and washed with H_2O and brine. Purification by column chromatography (Pentane:EtOAc, 8:2→6:4) afforded the title compound **52** in 70% yield as white solid.
TLC analysis: R_f= 0.35 (DCM:Acetone; 98:2)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.90-7.75 (4H, H_{arom}, m), 7.73 (1H, NH_{TCA}, J_{NH-H2}=5.4 Hz, d), 7.53-7.43 (3H, H_{arom}, m), 7.40-7.24 (10H, H_{arom}, m), 6.53 (1H, H_{1D}, J_{1-2}=3.6 Hz, d), 5.02 (1H, H_{1L}, J_{1-2}=3.6 Hz, d), 4.95 (1H, CH*H*, J=11.1 Hz, d), 4.85 (1H, CH*H*, J=11.6 Hz, d), 4.72-4.42 (6H, 4 x CH*H*, H_{1M}, H_{2D}, m), 4.22 (1H, H_{2L}, J_{2-3}=10.4 Hz, dd), 4.12-3.99 (4H, H_{4M}, H_{5L}, H_{3D}, H_{4L}, m), 3.97-3.91 (1H, H_{5D}, m), 3.89 (1H, H_{3L}, J_{3-4}=2.8 Hz, dd), 3.85 (1H, H_{2M}, J_{2-1}=1.1 Hz, J_{2-3}=3.6 Hz, dd), 3.73 (1H, H_{4D}, J_{3-4}=2.8 Hz, J_{4-5}=1.2 Hz, dd), 3.64 (1H, H_{5M}, J_{5-4}=9.4 Hz, d), 3.33-3.24 (4H, H_{3M}, OMe, m), 2.07 (3H, CH_{3_OAc}, s), 1.15 (3H, 3 x H_{6D}, J_{6-5}=6.4 Hz, d), 1.09 (3H, 3 x H_6, J_{6-5}=6.6, d), 0.78 (9H, *t*Bu, s), 0.02 (3H, Me, s), -0.06 (3H, Me, s).
^{13}C-NMR (500 MHz, $CDCl_3$), δ: 169.0, 167.8, 162.3, 138.1, 137.4, 135.1, 133.4, 133.1 (C_q), 128.7, 128.5, 128.5, 128.3, 128.2, 128.1 x 2, 127.9, 127.8 x 2, 127.7 x 2, 126.4, 126.1 x 2, 126.0, 125.8 (CH_{arom}), 101.0 (C_{1M}), 98.6 (C_{1L}), 92.6 (C_q), 90.0 (C_{1D}), 80.2 (C_{3M}), 78.2 (C_{3D}), 74.5 (H_{5M}), 77.0 (C_{3L}), 75.3 (C_{4L}), 74.9, 72.3, 71.3 (CH_2), 69.1 (C_{5D}), 68.4 (C_{4D}), 68.1 (C_5), 61.4 (C_{2M}), 60.5 (C_{2L}), 52.2 (CH_{3_OMe}), 50.7 (C_{2D}), 25.8 (CH_{3_tBu}), 20.9 (CH_{3_OAc}), 18.0 (C_q), 17.1 (C_{6L}), 16.9 (C_{6D}), -3.8 (CH_3), -5.4 (CH_3).
HRMS: $C_{54}H_{66}Cl_3N_7O_{14}Si$ + Na^+ required 1192.3395, found 1192.3395

2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(benzyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-1-*O*-acetyl-α-D-fucopyranoside (53)



Method A: Donor: Donor **12** (0.125 mmol, 2.5 eq) and acceptor **38** (0.05 mmol) were co-evaporated with toluene three times and dissolved in DCM (0.5 mL, 0.1 M). Freshly activated 4 Å MS were added to the reaction mixture, which was then cooled to -60°C. NIS (0.125 mmol, 2.5 eq) and TBSOTf (0.015 mmol, 0.3 eq) were added and the reaction was kept at -40°C for 7 hours, after which TLC analysis (Pentane:EtOAc, 1:1) showed complete consumption of acceptor. The reaction mixture was quenched by addition of TEA (0.04 mmol, 0.5 eq) and let to warm up to room temperature. The reaction was diluted with DCM and washed with saturated aq. solution of $Na_2S_2O_4$, H_2O and brine. Purification by column chromatography (Pentane:EtOAc, 8:2→6:4) afforded the title compound **53** in 70% yield as white solid.

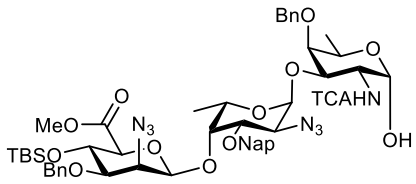
Method B: Donor **51** (0.195 mmol, 2.4 eq) and acceptor **38** (0.08 mmol) were co-evaporated with toluene three times and dissolved in DCM (0.8 mL, 0.1 M). Freshly activated 4 Å MS were added to the reaction mixture, which was then cooled to -60°C. TBSOTf (0.024 mmol, 0.3 eq) was added dropwise and the reaction was kept at -40°C for 10 hours, after which TLC analysis (Pentane:EtOAc, 1:1) showed complete consumption of acceptor. The reaction mixture was quenched by addition of TEA (0.02 mmol, 0.2 eq) and let to warm up to room temperature. The reaction was diluted with DCM and washed with H_2O and brine. Purification by column chromatography (Pentane:EtOAc, 8:2→6:4) afforded the title compound **53** in 69% yield as white solid.
TLC analysis: R_f= 0.36 (DCM:Acetone; 98:2)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: δ: 7.91-7.75 (5H, H_{arom}, NH, m), 7.60-7.00 (4H, H_{arom}, NH_{TCA}, m), 6.57 (1H, H_{1D}, J_{1-2}=3.6 Hz, d), 5.04 (1H, H_{1L}, J=3.6 Hz, d), 4.95-4.81 (4H, 4 x CH*H*, m), 4.74-4.58 (4H, 3 x CH*H*, H_{1M}, m), 4.57-4.59 (1H, H_{2D}, m), 4.38 (1H, CH*H*, J=11.1 Hz, d),4.62-4.14 (2H, H_{2L}, H_{4M}, m), 4.12-4.00 (3H, H_{3D}, H_{5L}, H_{4D}, m), 4.00-3.84 (3H, H_{5D}, H_{2M}, H_{3L}, m), 3.78-3.68 (2H, H_{5M}, H_{4L}, m), 3.35 (1H, H_{3M}, J_{2-3}=3.6, J_{3-4}=8.9 Hz, dd), 2.11 (3H, CH_{3_OAc}, s), 1.18 (3H, 3 x H_6, J_{6-5}=6.4 Hz, d), 1.12 (3H, 3 x H_6, J_{6-5}=6.5, d), 0.81 (9H, *t*Bu, s), 0.05 (3H, CH_3, s), -0.02 (3H, CH_3, s).

^{13}C-NMR (500 MHz, $CDCl_3$), δ: 169.0, 167.4, 162.3, 138.1, 137.4, 135.0, 134.7, 133.3, 133.1 (C_q), 128.7 x 2, 128.5 x 3, 128.4, 128.3, 128.2 x 2, 128.1, 127.9, 127.8, 127.7, 126.8, 126.1, 126.0 (CH_{arom}), 101.1 (C_{1M}), 98.7 (C_{1L}), 92.6 (C_q), 90.0 (C_{1D}), 80.3 (C_{3M}), 78.2 (C_{3D}), 77.3 (H_{5M}), 76.6 (C_{3L}, C_{4L}), 74.9 (C_{4D}), 74.9, 72.1, 71.1 (CH_2), 69.1 (C_{5D}), 68.4 (C_{4M}), 68.0 (C_5), 67.5 (CH_2), 61.1 (C_{2M}), 60.4 (C_{2L}), 50.7 (C_{2D}), 25.9 (CH_{3_tBu}), 21.0 (CH_{3_OAc}), 18.1 (C_q), 17.1 (C_{6L}), 16.9 (C_{6D}), -3.8 (CH_3), -5.3 (CH_3).

HRMS: $C_{60}H_{70}Cl_3N_7O_{14}Si$ + Na^+ required 1268.3708, found 1268.3708

2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(methyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-α-D-fucopyranoside (54)



52 (0.160 mmol) was dissolved in dry MeOH (1.6 mL, 0.1M) and under inert atmosphere Bu_3SnOMe (0.16 mmol, 1 eq) was added. The reaction mixture was stirred 6 hours at 50°C, after which TLC analysis (DCM:Acetone, 98:2) showed complete consumption of starting material. After cooling at room temperature, the reaction mixture was diluted with EtOAc and washed with H_2O. The aqueous phase was extracted with EtOAc and the combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo*. Product **54** was isolated in 81% by column chromatography (DCM:Acetone, 99:1→90:10) as white solid.

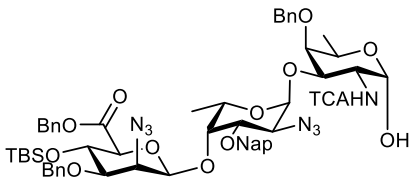
TLC analysis: Rf= 0.33 (DCM:Acetone; 9:1)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.91-7.75 (4H, H_{arom}, m), 7.58-7.42 (4H, H_{arom}, NH_{TCA}, m), 7.40-7.24 (5H, H_{arom}, m), 6.52 (1H, H_{1D}, J_{1-2}=3.6 Hz, d), 5.04 (1H, H_{1L}, J=3.4 Hz, d), 4.90-4.67 (4H, 2 x CH_2, m), 4.63-4.55 (1H, H_{2D}, m), 4.10-3.92 (5H, H_{2L}, H_{5L}, H_{5D}, H_{3D}, H_{3L}, m), 3.85 (1H, H_{4D}, J_{3-4}=2.9 Hz, dd), 3.75 (1H, H_{4L}, J_{3-4}=2.8 Hz, d), 2.11 (3H, CH_{3_OAc}, s), 1.22 (3H, 3 x H_6, J_{6-5}=6.5 Hz, d), 1.18 (3H, 3 x H_6, J_{6-5}=6.5, d).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.0, 162.2, 138.1, 134.3, 133.3 (C_q), 128.9, 128.6, 128.1, 127.9 x 2, 127.8, 127.7, 127.1, 126.6, 126.5, 125.7 (CH_{arom}), 99.0 (C_{1L}), 92.7 (C_q), 90.4 (C_{1D}), 78.3 (C_{3L}, C_{3D}), 76.8 (C_{4L}), 75.0 (CH_2), 72.3 (CH_2), 69.2 (C_5), 68.3 (C_{4D}), 67.5 (C_5), 60.7 (C_{2L}), 50.7 (C_{2D}), 21.0 (CH_{3_OAc}), 17.0 (C_q), 16.4 (C_6).

HRMS: $C_{52}H_{64}Cl_3N_7O_{13}Si$ + Na^+ required 1150.3289, found 1150.3289

2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(benzyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-α-D-fucopyranoside (55)



53 (0.3 mmol) was dissolved in DMF (10 mL, 0.03 M) and under inert atmosphere NH_2NH_2OAc (1.5 mmol, 5 eq) was added. The reaction mixture was stirred for one hour, after which TLC analysis (DCM:Acetone, 98:2) showed complete consumption of starting material. After cooling at room temperature, the reaction mixture was diluted with EtOAc and washed with H_2O. The aqueous phase was extracted with EtOAc and the combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo*. Product **55** was quantitatively isolated by column chromatography (DCM:Acetone, 99:1→90:10) as white solid.

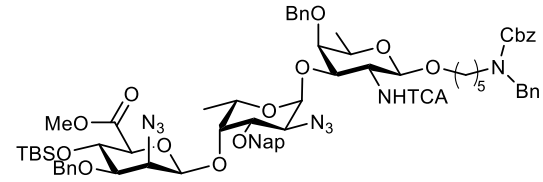
TLC analysis: Rf= 0.33 (DCM:Acetone; 9:1)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.91-7.75 (4H, H_{arom}, m), 7.58-7.42 (4H, H_{arom}, NH_{TCA}, m), 7.40-7.24 (5H, H_{arom}, m), 6.52 (1H, H_{1D}, J_{1-2}=3.6 Hz, d), 5.04 (1H, H_{1L}, J=3.4 Hz, d), 4.90-4.67 (4H, 2 x CH_2, m), 4.63-4.55 (1H, H_{2D}, m), 4.10-3.92 (5H, H_{2L}, H_{5L}, H_{5D}, H_{3D}, H_{3L}, m), 3.85 (1H, H_{4D}, J_{3-4}=2.9 Hz, dd), 3.75 (1H, H_{4L}, J_{3-4}=2.8 Hz, d), 2.11 (3H, CH_{3_OAc}, s), 1.22 (3H, 3 x H_6, J_{6-5}=6.5 Hz, d), 1.18 (3H, 3 x H_6, J_{6-5}=6.5, d).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 169.0, 162.2, 138.1, 134.3, 133.3 (C_q), 128.9, 128.6, 128.1, 127.9 x 2, 127.8, 127.7, 127.1, 126.6, 126.5, 125.7 (CH_{arom}), 99.0 (C_{1L}), 92.7 (C_q), 90.4 (C_{1D}), 78.3 (C_{3L}, C_{3D}), 76.8 (C_{4L}), 75.0 (CH_2), 72.3 (CH_2), 69.2 (C_5), 68.3 (C_{4D}), 67.5 (C_5), 60.7 (C_{2L}), 50.7 (C_{2D}), 21.0 (CH_{3_OAc}), 17.0 (C_q), 16.4 (C_6).

HRMS: $C_{58}H_{68}Cl_3N_7O_{13}Si + Na^+$ required 1226.3602, found 1226.3602

5-(benzyl(benzyloxicarbonyl)amino)pentyl 2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(methyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-β-D-fucopyranoside (5)



54 (0.060 mmol) was dissolved in dry acetone (0.3 mL, 0.2 M) and under inert atmosphere $CsCO_3$ (0.066 mmol, 1.1 eq) was added. The reaction mixture was cooled to 0°C and $CF_3CN(Ph)Cl$ (0.072 mmol, 1.2 eq) was added. After 1 hour, TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of starting material. The reaction was diluted in acetone, filtered over Celite® and concentrated *in vacuo*. Acceptor **58** (0.120 mmol, 2 eq) was added and the two compounds were coevaporated three times with toluene. The resulting mixture was dissolved in dry DCM (0.6 mL, 0.1 M) and 3Å MS were added. After10 minutes the reaction was cooled to -78°C and TBSOTf (0.02 mmol, 0.3 eq) was added. The reaction mixture was allowed to warm up and after 1 hour at -33 °C, TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of donor. The reaction mixture was diluted with DCM, washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound was purified by column chromatography (Pentane:EtOAc, 8:2→65:25) in 68% yield as white foam.

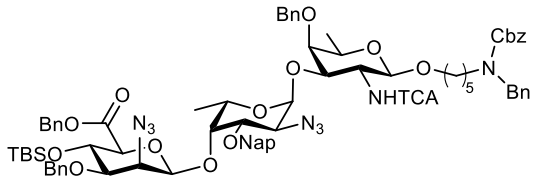
TLC analysis: R_f= 0.38 (Pentane:EtOAc; 7:3)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.91-7.75 (4H, H_{arom}, m), 7.58-7.46 (3H, H_{arom}, m), 7.44-7.13 (23H, H_{arom}, NH_{TCA}, m), 5.25-5.14 (2H, H_{arom}, m), 4.98 (1H, H_{1L}, J=3.7 Hz, d), 4.94-4.82 (2H, $H_{CH}HBn$, H_{1D}, m), 4.79-4.45 (8H, 7 x $H_{CH}HBn$, H_{1M}, m), 4.35-4.25 (1H, H_{3D}, m), 4.15 (1H, H_{4M}, $J_{4\text{-}3}$=$J_{4\text{-}5}$=9.1 Hz, t), 4.01-3.80 (6H, H_{2L}, H_{2L}, H_{2M}, $H_{CH2OLinker}$, H_{4L}, H_{5L}, m), 3.77 (1H, H_{3L}, $J_{3\text{-}2}$=10.6 Hz, $J_{3\text{-}4}$=2.8Hz, dd), 3.70 (1H, H_{5M}, d), 3.64 (1H, 3.67-3.60 (1H, H_{5D}, m), 3.56 (1H, H_{4D}, $J_{4\text{-}3}$=2.8 Hz, d), 3.46-3.30 (5H, $H_{CH2OLinker}$, OMe, H_{3M}, m), 3.30-3.13 (2H, $CH_{2\_NLinker}$, m), 1.65-1.42 (4H, 2 x CH_{2_Linker}, m), 1.39-1.22 (5H, $CH_{2\_NLinker}$, H_{6D}, m), 1.12 (3H, H_{6L}, $J_{6\text{-}5}$=6.5 Hz, d), 0.84 (9H, 3 x CH_{3_tBu}, s), 0.06 (3H, CH_3, s), -0.01 (3H, CH_3, s).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 167.86, 162.0, 138.5, 138.0, 135.4, 133.4, 133.0 (C_q), 128.6 x 2, 128.5, 128.4, 128.2, 128.1 x 2, 128.0, 127.9 x 2, 127.8, 127.7 x 2, 127.5, 127.3, 126.4, 126.1, 125.9 x 2 (CH_{arom}), 100.1 (C_{1M}), 99.3 (C_{1L}, C_{1D}) , 92.6 (C_q), 80.3 (C_{3M}), 79.3 (C_{4D}), 78.1 (C_{3D}), 77.5 (C_{5M}), 75.4 (C_{3M}), 75.3 (CH_{4L}), 72.2, 71.1, 70.6 (CH_2), 70.4 (C_{5D}), 69.7 ($CH_{2OLinker}$), 68.3 (C_{4M}), 67.1 (CH_2), 67.0 (C_{5L}), 61.1 (C_{2D}), 59.3 (C_{2L}), 55.9 (C_{2M}), 52.2 (CH_{3OMe}), 50.3 (CH_2), 47.1, 46.2 ($CH_{2NLinker}$), 28.7 (CH_2), 25.9 (3 x CH_{3tBu}), 17.9 (C_q), 17.1 (C_{6L}, C_{6D}), -3.9, -5.4 (CH_{3Me}).

HRMS: $C_{71}H_{85}Cl_3N_8O_{15}Si$ + Na^+ required 1445.4861, found 1445.4861

5-(benzyl(benzyloxicarbonyl)amino)pentyl 2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(benzyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-β-D-fucopyranoside (6)



55 (0.060 mmol) was dissolved in dry acetone (0.3 mL, 0.2 M) and under inert atmosphere $CsCO_3$ (0.066 mmol, 1.1 eq) was added. The reaction mixture was cooled to 0°C and $CF_3CN(Ph)Cl$ (0.072 mmol, 1.2 eq) was added. After 1 hour, TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of starting material. The reaction was diluted in acetone, filtered over Celite® and concentrated *in vacuo*. Acceptor **58** (0.120 mmol, 2 eq) was added and the two compounds were coevaporated three times with toluene. The resulting mixture was dissolved in dry DCM (0.6 mL, 0.1 M) and 3Å MS were added. After10 minutes the reaction was cooled to -78°C and TBSOTf (0.02 mmol, 0.3 eq) was added. The reaction mixture was allowed to warm up and after 1 hour at -33 °C, TLC analysis (Pentane:EtOAc, 9:1) showed complete consumption of donor. The reaction mixture was diluted with DCM, washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound was purified by column chromatography (Pentane:EtOAc, 8:2→65:25) in 71% yield as white foam.

TLC analysis: R_f= 0.38 (Pentane:EtOAc; 7:3)

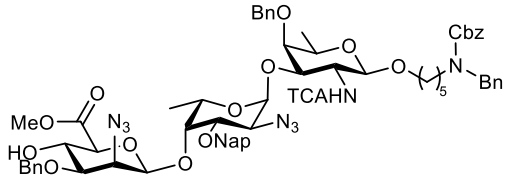
^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.89-7.75 (4H, H_{arom}, m), 7.51-7.05 (29H, H_{arom}, NH, m), 5.22-5.10 (2H, 2 x $H_{CH}H$, m), 4.96-4.88 (3H, H_{1L}, 2 x $H_{CH}H$, m), 4.87-4.73 (2H, H_{1D}, $H_{CH}H$, m), 4.73-4.54 (5H, 4 x $H_{CH}H$, H_{1M}, m), 4.54-4.36 (3H, $H_{CH}H$, m), 4.32-4.22 (1H, H_{3D}, m), 4.19 (1H, H_{4M}, $J_{4\text{-}5}$=$J_{4\text{-}3}$=9.1 Hz, pt), 3.91-3.74 (5H, H_{2L}, H_{2D}, $H_{CH}HOLinker$, H_{5L}, H_{4D}, m) 3.75-3.64 (2H, H_{5M}, H_{3M}, m), 3.60 (1H, H_{5D}, $J_{5\text{-}6}$=6.4 Hz, q), 3.51 (1H, H_{4L}, $J_{4\text{-}3}$=3.51 Hz, d), 3.45-3.29 (2H, $H_{CH}HOLinker$, H_{3M}, m), 3.28-3.09 (2H, $H_{CH}HNLinker$), 1.62-1.39 (4H, 4 x $H_{CH}HLinker$, m), 1.39-1.12 (5H, 2 x

$H_{CHHLinker}$, H_{6D}, m),1.10 (3H, H_{6L}, $J_{6\text{-}5}$=6.4 Hz, d), 0.81 (9H, H_{tBu}, s), 0.03 (3H, CH_3, s), -0.03 (3H, CH_3, s).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 167.4, 162.0, 138.5, 137.9, 137.3 x 2, 134.7, 133.3, 133.0 (C_q), 128.9, 128.8, 128.6, 128.6, 128.6, 128.5, 128.5, 128.4 x 2, 128.3, 128.2, 128.1, 128.0, 127.9, 127.9, 127.8, 127.7, 127.6, 127.5, 127.3, 126.7, 126.4, 126.1, 126.0 x 2, 125.8 (CH_{arom}), 101.0 (C_{1M}), 99.3 (C_{1L}), 99.1 (C_{1D}), 92.6 (C_q), 80.3 (C_{3M}), 79.2 (C_{4D}), 77.9 (C_{4L}), 77.4 (C_{5M},), 75.1 x 2 (CH_2, C_{5D}), 72.0, 70.8 (CH_2), 70.6 (C_{5L}), 69.8 (CH_2), 69.7 ($CH_{2OLinker}$), 68.3 (C_{4M}), 67.4 (CH_2), 67.2 (C_{6L}), 60.8 (C_{2M}), 59.2 (C_{2L}), 56.0 (C_{2D}), 50.5 (CH_2), 47.2, 46.2 (CH_{2NLink}), 29.2, 28.0 ($CH_{2Linker}$), 27.4 (CH_{3tBu}), 25.8 ($CH_{2Linker}$), 17.2 (CH_{3_6D}), 17.0 (CH_{3_6L}), -3.9, -5.3 (CH_{3_Me}).

HRMS: $C_{77}H_{89}Cl_3N_8O_{15}Si$ + H^+ required 1521.5174, found 1521.9714

5-(benzyl(benzyloxicarbonyl)amino)pentyl 2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(methyl 2-azido-2-deoxy-3-*O*-benzyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-β-D-fucopyranoside (59)



5 (0.035 mmol) was dissolved in THF (0.350 mL, 0.1 M) and subsequently TEA*3HF (0.35 mmol, 10 eq) was added. The reaction was stirred for 24 hours at reflux, after which TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of starting material. The reaction was diluted in EtOAc and transfer in a beaker containing a cold saturated aq. solution of $NaHCO_3$. Once the ice melted, the organic layer was separated and the aqueous phase was reextracted (x2). The combined EtOAc solutions were washed with water and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound **59** was isolated by column chromatography in quantitative yield as white solid.

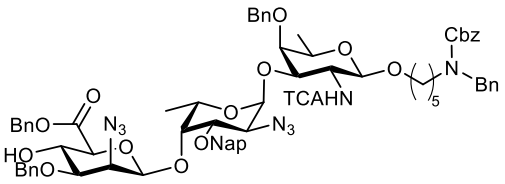
TLC analysis: R_f= 0.31 (DCM:Acetone; 95:5)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.91-7.75 (4H, H_{arom}, m), 7.58-7.46 (3H, H_{arom}, m), 7.44-7.13 (23H, H_{arom}, NH_{TCA}, m), 5.25-5.14 (2H, H_{arom}, m), 4.98 (1H, H_{1L}, J=3.7 Hz, d), 4.94-4.82 (2H, H_{CHHBn}, H_{1D}, m), 4.79-4.45 (8H, 7 x H_{CHHBn}, H_{1M}, m), 4.35-4.25 (1H, H_{3D}, m), 4.15 (1H, H_{4M}, $J_{4\text{-}3}$=$J_{4\text{-}5}$=9.1 Hz, t), 4.01-3.80 (6H, H_{2L}, H_{2L}, H_{2M}, $H_{CH2OLinker}$, H_{4L}, H_{5L}, m), 3.77 (1H, H_{3L}, $J_{3\text{-}2}$=10.6 Hz, $J_{3\text{-}4}$=2.8Hz, dd), 3.70 (1H, H_{5M}, d), 3.64 (1H, 3.67-3.60 (1H, H5D, m), 3.56 (1H, H_{4D}, $J_{4\text{-}3}$=2.8 Hz, d), 3.46-3.30 (5H, $H_{CH2OLinker}$, OMe, H_{3M}, m), 3.30-3.13 (2H, $CH_{2\_NLinker}$, m), 1.65-1.42 (4H, 2 x CH_{2_Linker}, m), 1.39-1.22 (5H, $CH_{2\_NLinker}$, H_{6D}, m), 1.12 (3H, H_{6L}, $J_{6\text{-}5}$=6.5 Hz, d), 0.84 (9H, 3 x CH_{3_tBu}, s), 0.06 (3H, CH_3, s), -0.01 (3H, CH_3, s).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 167.86, 162.0, 138.5, 138.0, 135.4, 133.4, 133.0 (C_q), 128.6 x 2, 128.5, 128.4, 128.2, 128.1 x 2, 128.0, 127.9 x 2, 127.8, 127.7 x 2, 127.5, 127.3, 126.4, 126.1, 125.9 x 2 (CH_{arom}), 100.1 (C_{1M}), 99.3 (C_{1L}, C_{1D}) , 92.6 (C_q), 80.3 (C_{3M}), 79.3 (C_{4D}), 78.1 (C_{3D}), 77.5 (C_{5M}), 75.4 (C_{3M}), 75.3 (CH_{4L}), 72.2, 71.1, 70.6 (CH_2), 70.4 (C_{5D}), 69.7 ($CH_{2OLinker}$), 68.3 (C_{4M}), 67.1 (CH_2), 67.0 (C_{5L}), 61.1 (C_{2D}), 59.3 (C_{2L}), 55.9 (C_{2M}), 52.2 (CH_{3OMe}), 50.3 (CH_2), 47.1, 46.2 ($CH_{2NLinker}$), 28.7 (CH_2), 25.9 (3 x CH_{3tBu}), 17.9 (C_q), 17.1 (C_{6L}, C_{6D}), -3.9, -5.4 (CH_{3Me}).

HRMS: $C_{65}H_{71}Cl_3N_8O_{15}$ + Na^+ required 1331.3997, found 1331.3997

5-(benzyl(benzyloxicarbonyl)amino)pentyl 2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(benzyl 2-azido-2-deoxy-3-*O*-benzyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-β-D-fucopyranoside (60)



6 (0.035 mmol) was dissolved in THF (0.350 mL, 0.1 M) and subsequently TEA*3HF (0.35 mmol, 10 eq) was added. The reaction was stirred for 24 hours at reflux, after which TLC analysis (Pentane:EtOAc, X:X) showed complete consumption of starting material. The reaction was diluted in EtOAc and transfer in a beaker containing a cold saturated aq. solution of $NaHCO_3$. Once the ice melted, the organic layer was separated and the aqueous phase was reextracted (x2). The combined EtOAc solutions were washed with water and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound **60** was isolated by column chromatography in quantitative yield as white solid.

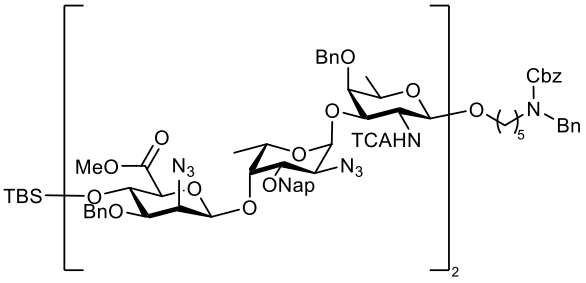
TLC analysis: R_f= 0.31 (DCM:Acetone; 95:5)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.80-7.67 (4H, H_{arom}, m), 7.51-7.11 (27H, H_{arom}, NH, m), 7.11-7.05 (4H, H_{arom}, m), 5.19-5.09 (2H, 2 x H_{CHH}, m), 4.95-4.82 (3H, H_{1L}, 2 x H_{CHH}, m), 4.81 (1H, H_{1D}, $J_{1\text{-}2}$=7.89 Hz, d), 4.77-4.55 (6H, 5 x H_{CHH}, H_{1M}, m), 4.49-4.36 (3H, H_{CHH}, m), 4.30-4.22 (1H, H_{3D}, m), 4.16 (1H, H_{4M}, m), 4.03-3.94 (2H, H_{2M}, H_{4L}, m), 3.91-3.73 (4H, H_{2L}, H_{5L}, H_{2D}, $H_{CHHOLinker}$), 3.71-3.62 (2H, H_{5M}, H_{3L}), 3.59 (1H, H_{5D}, $J_{5\text{-}6}$=6.4 Hz, q), 3.51 (1H, H_{4D}, $J_{4\text{-}3}$=2.88 Hz, d), 3.43-3.29 (2H, $H_{CHHOLinker}$, H_{3M}, m), 3.25-3.08 (2H, $H_{CHHNLinker}$), 1.62-1.39 (4H, 4 x $H_{CHHLinker}$, m), 1.39-1.12 (5H, 2 x $H_{CHHLinker}$, H_{6D}, m),1.10 (3H, H_{6L}, $J_{6\text{-}5}$=6.4 Hz, d).

^{13}C-NMR (400 MHz, $CDCl_3$), δ:

HRMS: $C_{71}H_{75}Cl_3N_8O_{15}$ + Na^+ required 1407.4310, found 1407.4310

Fully protected hexasaccharide (7)



54 (0.030 mmol) was dissolved in dry acetone (0.150 mL, 0.2 M) and under inert atmosphere $CsCO_3$ (0.033 mmol, 1.1 eq) was added. The reaction mixture was cooled to 0°C and $CF_3CN(Ph)Cl$ (0.035 mmol, 1.2 eq) was added. After 1 hour, TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of starting material. The reaction was diluted in acetone, filtered over Celite® and concentrated *in vacuo*. Acceptor **59** (0.040 mmol, 1.3 eq) was added and the two compounds were coevaporated three times with toluene. The resulting mixture was dissolved in dry DCM (0.300 mL, 0.1 M) and 3Å MS were added. After 10 minutes the reaction was cooled to -78°C and a freshly prepared 0.6 M solution of TBSOTf in dry DCM (0.006 mmol, 0.02 eq) was added. The reaction mixture was allowed to warm up and after 1 hour at -50 °C, TLC analysis (Pentane:EtOAc, 7:3) showed

complete consumption of donor. The reaction mixture was diluted with DCM, washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound was purified by column chromatography (Pentane:EtOAc, 9:1→6:4) in 88% yield as white foam.

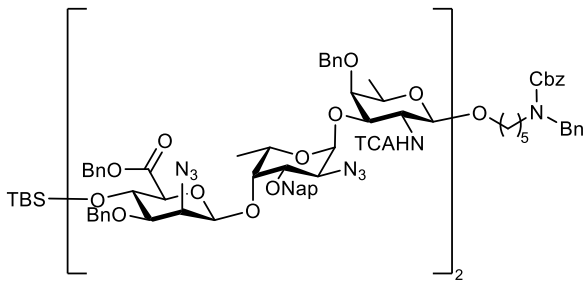
TLC analysis: R_f= 0.31 (Pentane:EtOAc; 7:3)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.90-7.72 (8H, H_{arom}, m), 7.56-7.08 (38H, 46 x H_{arom}, 2 x NH, m), 5.20-5.10 (2H, 2 x H_{CHH}, m), 5.00-4.73 (7H, 2 x H_{1L}, H_{1D}, 2 x H_{CHH}, m), 4.72-4.41 (15H, 2 x H_{1M}, H_{1D}, 6 x CH_2), 4.31-4.16 (3H, H_{3D}, H_{4M}, H_{2D}, m), 4.16-4.05 (2H, H_{4M}, H_{3D}, m), 4.05-3.88 (6H, 2 x H_{2M}, 2 x H_{2L}, H_{2D}, H_{5L}, m), 3.88-3.25 (19H, 2 x H_{3L}, H_{5L}, 2 x H_{4L}, 2 x H_{5D}, 2 x H_{4D}, 2 x $H_{CHHOLinker}$, 2 x H_{5M}, 2 x H_{3M}, 2 x CH_3, m), 3.25-3.09 (2H, 2 x $H_{CHHNLinker,}$ m), 1.58-1.39 (4H, 4 x $H_{CHHLinker}$, m), 1.39-1.00 (5H, 2 x $H_{CHHLinker}$, 2 x H_{6D}, 2 x H_{6L}, m), 0.81 (9H, H_{tBu}, s), 0.03 (3H, CH_3, s), -0.03 (3H, CH_3, s).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 168.8, 167.9, 167.7, 162.1, 161.8, 139.4, 138.7, 138.6, 138.5, 138.4, 138.0, 137.6, 137.4, 137.0, 136.0, 135.5, 133.4, 133.4, 133.3, 133.0, 133.0 (C_q), 128.9, 128.8, 128.7 x 2, 128.6, 128.5 x 2, 128.4 x 2, 128.3 x 2, 128.2 x 3, 128.1, 128.1, 128.0 x 5, 127.9, 127.8 x 2, 127.7, 127.6 x 3, 127.5 x 3, 127.3, 127.2, 126.6, 126.4, 126.3 x 2, 126.2, 126.2, 126.1, 126.0, 126.0 x 2, 125.9, 125.8, 125.7, 125.7, 124.9 (CH_{arom}), 100.9 (C_{1M}), 100.8 (C_{1D}), 100.7 (C_{1M}), 99.2 x 2 (C_{1L}), 99.0 (C_{1D}), 93.1 (C_q), 92.7 (C_q), 80.6 (C_{4D}), 80.3 x 2 (C_{3M}), 79.3 (C_{4D}), 78.6 (C_{4L}), 78.1 (C_{3D}), 77.8 (C_{4L}), 77.7 (C_{5M},), 77.5 (C_{5M}), 75.7, 75.6 (C_{5L}), 75.3 (CH_2), 75.2 (C_{4D}), 75.0 (CH_2), 73.8 (CH_2), 72.6 (CH_2), 72.0 (CH_2), 71.3 (CH_2), 70.6 x 2 (C_{5D}), 70.0 ($CH_{2OLinker}$), 68.7 x 2 (C_{4M}), 67.7 (CH_2), 62.6 (C_{2D}), 61.7 x 2 (C_{2L}, C_{2D}), 59.6 (C_{2L}) 56.3 x 2(C_{2M}), 52.6 x 2 (CH_{3OMe}), 50.7 (CH_2), 47.5, 46.7 (CH_{2NLink}), 29.7, 28.0 ($CH_{2Linker}$), 25.9 (CH_{3tBu}), 23.9 ($CH_{2Linker}$), 17.1 x 4 (CH_{3_6L}, CH_{3_6D}), -3.7, -5.3 (CH_{3_Me}).

HRMS: $C_{118}H_{135}Cl_3N_{15}O_{27}Si + NH_4^+$ required 2449.7891, found 2453.7888.

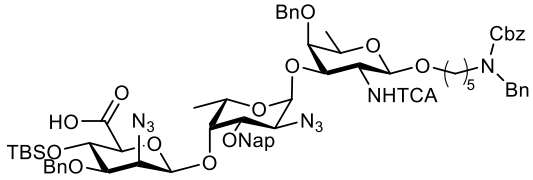
Fully protected hexasaccharide (8)



55 (0.18mmol) was dissolved in dry acetone (0.900 mL, 0.2 M) and under inert atmosphere $CsCO_3$ (0.19 mmol, 1.1 eq) was added. The reaction mixture was cooled to 0°C and $CF_3CN(Ph)Cl$ (0.23 mmol, 1.2 eq) was added. After 1 hour, TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of starting material. The reaction was diluted in acetone, filtered over Celite® and concentrated *in vacuo*. Acceptor **60** (0.23 mmol, 1.3 eq) was added and the two compounds were coevaporated three times with toluene. The resulting mixture was dissolved in dry DCM (1.8 mL, 0.1 M) and 3Å MS were added. After 10 minutes the reaction was cooled to -78°C and a freshly prepared 0.6 M solution of TBSOTf in dry DCM (0.036 mmol, 0.02 eq) was added. The reaction mixture was allowed to warm up and after 45 min at -50 °C, TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of donor. The reaction mixture was diluted with DCM, washed with H_2O and brine, dried

over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound was purified by column chromatography (Pentane:EtOAc, 9:1→6:4) in 96% yield as white foam.
TLC analysis: R_f= 0.33 (Pentane:EtOAc; 65:35)
^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.89-7.75 (8H, H_{arom}, m), 7.51-6.98 (48H, 46 x H_{arom}, 2 x NH, m), 5.21-5.09 (2H, 2 x $HCHH$, m), 4.96-4.51 (19H, 2 x H_{1L}, H_{1D}, 14 x $HCHH$, H_{1M}, m), 4.51-4.10 (11H, H_{1D}, 6 x $HCHH$, 2 x H_{3D}, 2 x H_{4M}, m), 4.01-3.90 (3H, 2 x H_{2M}, H_{4D}, m), 3.90-3.63 (9H, 2 x H_{2L}, 2 x H_{2D}, 2 x H_{5L}, 2 x H_{5M}, $HCHHOLinker$, m), 3.25-3.09 (3H, 2 x $HCHHNLinker$, H_{5D}, m), 1.58-1.39 (4H, 4 x $HCHHLinker$, m), 1.39-1.00 (5H, 2 x $HCHHLinker$, 2 x H_{6D}, 2 x H_{6L}, m), 0.81 (9H, H_{tBu}, s), 0.03 (3H, CH_3, s), -0.03 (3H, CH_3, s).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 168.0, 167.4, 167.2, 162.1, 161.9, 138.7, 138.6, 138.3, 138.0, 137.5, 137.4, 137.0, 135.5, 135.3, 134.9, 134.8, 133.4, 133.1 x 2 (C_q), 129.3, 129.1, 128.9, 128.8, 128.7 x 2, 128.6 x 3, 128.5 x 3, 128.4 x 2, 128.2 x 2, 128.1 x 3, 128.0, 127.9, 127.8 x 2, 127.7 x 2, 127.6 x 4, 127.5 x 2, 127.4, 127.2 x 2, 126.7 x 2, 126.6, 126.2, 126.1 x 3, 126.0 x 2, 125.9 2 (CH_{arom}), 101.0 (C_{1M}), 100.7 (C_{1M}), 99.9 (C_{1D}), 99.3 x 2 (C_{1L}), 99.1 (C_{1D}), 93.2 (C_q), 92.7 (C_q), 80.4 x 2 (C_{3M}), 79.3 (C_{4D}), 78.3 (C_{4L}), 78.2 (C_{3D}), 77.8 (C_{4L}), 77.5 (C_{5M},), 75.4 x 3 (2 x C_{5L}, C_{5M}), 75.2 (CH_2), 75.1 (C_{4D}), 74.9 (CH_2), 73.8 (CH_2), 72.1 (CH_2), 71.2 (CH_2), 71.0 (CH_2), 70.7 x 2 (C_{5D}), 69.8 ($CH_{2OLinker}$), 68.6 x 2 (C_{4M}), 67.4 (CH_2), 67.2 (CH_2), 63.0 (C_{2M}), 61.1 x 2 (C_{2L}), 59.7 (C_{2M}), 59.3 (C_{2D}), 56.2 (C_{2D}), 50.6 (CH_2), 47.4, 46.5 (CH_{2NLink}), 29.7, 28.0 ($CH_{2Linker}$), 25.9 (CH_{3tBu}), 23.9 ($CH_{2Linker}$), 17.1 x 4 (CH_{3_6L}, CH_{3_6D}), -3.7, -5.3 (CH_{3_Me}).
HRMS: $C_{118}H_{135}Cl_3N_{15}O_{27}Si + NH_4^+$ required 2601.8517, found 2605.8520

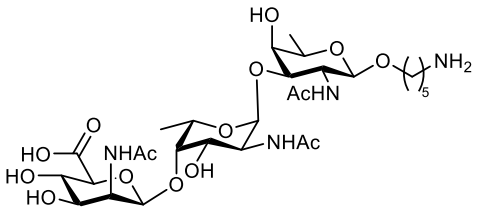
5-(benzyl(benzyloxicarbonyl)amino)pentyl 2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-(2-naphthylmethyl)-4-*O*-(2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronic)-α-L-fucopyranosyl)-4-*O*-benzyl-β-D-fucopyranoside (61)



Compound **5** (0.013 mmol) was dissolved in THF. After cooling to 0°C, 50 µL of a solution containing H_2O_2 (0.195 mmol, 15 eq) and TBAOH (40%w in H_2O, 0.065 mmol, 5 eq) were slowly added. The temperature was raised at 40°C and after 1 hour TLC analysis (Pentane:EtOAc, 7:3) showed complete consumption of starting material. The solution was acidified with 1M HCl (pH ≈ 3), diluted with DCM and washed one with water. The aqueous layer was reextracted 5 times with DCM, the organic phase dried, filtered and concentrated *in vacuo*. The desired product was isolated by column chromatography (DCM:MeOH, 99:1→97:3→95:5) in quantitative yield.
TLC analysis: R_f= 0.34 (DCM:MeOH; 9:1 + 1%AcOH)
^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.94 (1H, H_{1L}, J_{1-2}=3.9 Hz, d), 4.90 (1H, H_{1M}, J_{1-2}=1.6 Hz, d), 4.64 (1H, H_{2M}, J_{2-3}=4.3 Hz, dd), 4.40 (1H, H_{1D}, J_{1-2}=8.4 Hz, d), 4.16 (1H, H_{5D}, J_{5-6}=8.3 Hz, d), 4.38 (1H, H_{2L}, dd), 4.22 (1H, H_{4L}, d), 4.19 (1H, H_{5L}, J_{5-6}=6.6 Hz, q), 4.01-3.96 (1H, H_{2D}, m), 3.91-3.86 (1H, H $CHHOlinker$, m), 3.82-3.75 (4H, H_{4D}, H_{5L}, H_{3D}, H_{3M}, m), 3.65 (1H, H_{4M}, J_{4-3}=J_{4-5}=9.7 Hz, t), 3.61-3.55 (2H, H_{5M}, H $CHHOlinker$, m), 3.03-2.96 (2H, $HCHHNlinker$, m), 2.14 (3H, CH_{3_NHAcM}, s), 2.08 (3H, CH_{3_OAc}, s), 2.01 (3H, CH_{3_NHAcL}, s), 1.99 (3H, CH_{3_NHAcD}, s), 1.71- 1.64

(2H, $H_{CH2Linker}$, m), 1.63-1.55 (2H, $H_{CH2Linker}$, m), 1.43-1.33 (2H, $H_{CH2Linker}$, m), 1.28 (3H, H_{6D}, $J_{6\text{-}5}$=6.5 Hz, d), 1.25 (3H, H_{6L}, d).
^{13}C NMR (214 MHz, $CDCl_3$) δ: 176.5, 176.3, 175.2, 175.0, 174.7 (C_q), 102.4 (C_{1D}), 100.7 (C_{1M}), 99.9 (C_{1L}), 79.4 (C_{5M}), 78.0 (C_{4D}), 76.9 (C_{4L}), 72.5, 71.6, 71.3 (C_{5D}, C_{3D}, C_{3M}), 71.0 ($CH_{2OLinker}$), 70.7 (C_{3L}), 70.4 (C_{4M}), 67.7 (C_{4L}), 53.9 (C_{2M}), 52.2 (C_{2D}), 48.0 (C_{2L}), 40.2 ($CH_{2NLinker}$), 29.1, 27.3 ($CH_{2Linker}$), 23.1 (CH_{3NAcD}), 23.0 ($CH_{2Linker}$), 22.9 (CH_{3NAcL}, CH_{3NAcM}), 21.2 (CH_{3OAc}), 16.3 (CH_{3_6D}), 16.2 (CH_{3_6L}).
HRMS: $C_{118}H_{135}Cl_3N_{15}O_{27}Si + Na^+$ required 1431.4705, found 1431.4705

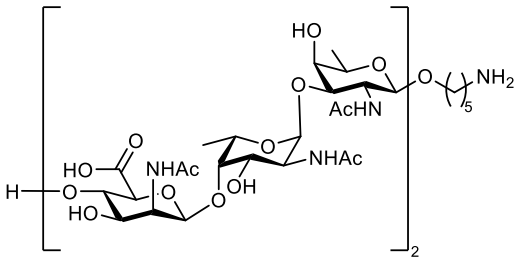
5-amino-pentyl 2-acetylamido-2-deoxy-3-*O*-(2-acetylamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-β-D-mannopyranosiduronyl)-α-L-fucopyranosyl)-β-D-fucopyranoside (1)



Compound **6** (0.025 mmol) was dissolved in THF (0.25 mL, 0.1M) and under inert atmosphere AcOH (1.75 mmol, 70 eq), Ac_2O (2.11 mmol, 84 eq) and Zn dust (1,22 mmol, 50 eq) were added. The reaction mixture was heated at 50°C and left stirring overnight. After TLC analysis (DCM:MeOH, 95:5) showed complete consumption of the starting material, the reaction was diluted with DCM, filtered and concentrated *in vacuo*. The desired product was isolated after column chromatography (DCM:MeOH, 99:1→97:3→95:5→9:1) in 67% yield as white solid. Intermediate **63** was dissolved in dry THF (1mL, 0.01M) and under inert atmosphere TEA*HF (0.25 mmol, 10 eq) was added. The reaction was stirred overnight at 50°C, after which TLC analysis (DCM:MeOH, 9:1) showed complete consumption of starting material. The reaction was diluted in EtOAc and washed with a saturated aq. solution of $NaHCO_3$ (x3), water and brine. The aqueous phases were reextracted and the combined organic phases dried over $MgSO_4$, filtered and concentrated *in vacuo*. The removal of the TBS group was confirmed by ^{1}H-NMR. The resulting crude was dissolved in a mixture of *t*Bu:H_2O (4:1, 0.01M) and one drop of AcOH was added. After purging Ar for 30 min, $Pd(OH)_2$ (20 weight% on carbon, 50% water) was added and the mixture was purged with Ar for 15 min, followed by H_2 (30s). The reaction was stirred under H_2 for three days. After filtration over Celite® and concentration *in vacuo*, the product was purified by size exclusion chromatography (HW40, 10 mM NH_4OAc in water). After repeated lyophilization to remove NH_4OAc, the final trisaccharide was isolated in 96% yield.
^{1}H-NMR (500 MHz, D_2O) δ: 4.94 (1H, H_{1L}, $J_{1\text{-}2}$=3.9 Hz, d), 4.90 (1H, H_{1M}, $J_{1\text{-}2}$=1.6 Hz, d), 4.64 (1H, H_{2M}, $J_{2\text{-}3}$=4.3 Hz, dd), 4.40 (1H, H_{1D}, $J_{1\text{-}2}$=8.4 Hz, d), 4.16 (1H, H_{5D}, $J_{5\text{-}6}$=8.3 Hz, d), 4.38 (1H, H_{2L}, dd), 4.22 (1H, H_{4L}, d), 4.19 (1H, H_{5L}, $J_{5\text{-}6}$=6.6 Hz, q), 4.01-3.96 (1H, H_{2D}, m), 3.91-3.86 (1H, H *CH*OlinkerH, m), 3.82-3.75 (4H, H_{4D}, H_{5L}, H_{3D}, H_{3M}, m), 3.65 (1H, H_{4M}, $J_{4\text{-}3}$=$J_{4\text{-}5}$=9.7 Hz, t), 3.61-3.55 (2H, H_{5M}, H *CH*OlinkerH, m), 3.03-2.96 (2H, H*CH*HNlinkerH, m), 2.14 (3H, CH_{3_NHAcM}, s), 2.08 (3H, CH_{3_OAc}, s), 2.01 (3H, CH_{3_NHAcL}, s), 1.99 (3H, CH_{3_NHAcD}, s), 1.71- 1.64 (2H, $H_{CH2Linker}$, m), 1.63-1.55 (2H, $H_{CH2Linker}$, m), 1.43-1.33 (2H, $H_{CH2Linker}$, m), 1.28 (3H, H_{6D}, $J_{6\text{-}5}$=6.5 Hz, d), 1.25 (3H, H_{6L}, d).

^{13}C NMR (214 MHz, D_2O) δ: 176.5, 176.3, 175.2, 175.0, 174.7 (C_q), 102.4 (C_{1D}), 100.7 (C_{1M}), 99.9 (C_{1L}), 79.4 (C_{5M}), 78.0 (C_{4D}), 76.9 (C_{4L}), 72.5, 71.6, 71.3 (C_{5D}, C_{3D}, C_{3M}), 71.0 ($CH_{2OLinker}$), 70.7 (C_{3L}), 70.4 (C_{4M}), 67.7 (C_{4L}), 53.9 (C_{2M}), 52.2 (C_{2D}), 48.0 (C_{2L}), 40.2 ($CH_{2NLinker}$), 29.1, 27.3 ($CH_{2Linker}$), 23.1 (CH_{3NAcD}), 23.0 ($CH_{2Linker}$), 22.9 (CH_{3NAcL}, CH_{3NAcM}), 21.2 (CH_{3OAc}), 16.3 (CH_{3_6D}), 16.2 (CH_{3_6L}).
HRMS: $C_{28}H_{48}N_4O_{15}$ + Na^+ required 703.3008, found 703.3008

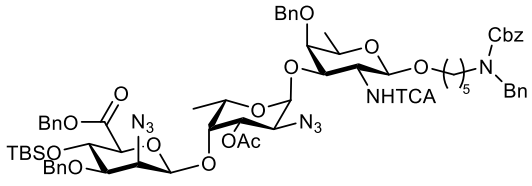
Hexasaccharide without 3-*O*-acetyl (3)



Compound **8** (0.014 mmol) was dissolved in THF (0.60 mL, 0.03M) and under inert atmosphere AcOH (0.98 mmol, 70 eq), Ac_2O (1.18 mmol, 84 eq) and Zn dust (0.70 mmol, 50 eq) were added. The reaction mixture was heated at 50°C and left stirring overnight. After TLC analysis (DCM:MeOH, 95:5) showed complete consumption of the starting material, the reaction was diluted with DCM, filtered and concentrated *in vacuo*. The desired product was isolated after column chromatography (DCM:MeOH, 99:1→97:3→95:5→9:1) in 62% yield as white solid. Intermediate **64** was dissolved in dry THF (0.860 mL, 0.01M) and under inert atmosphere TEA*HF (0.086 mmol, 10 eq) was added. The reaction was stirred overnight at 50°C, after which TLC analysis (DCM:MeOH, 9:1) showed complete consumption of starting material. The reaction was diluted in EtOAc and washed with a saturated aq. solution of $NaHCO_3$ (x3), water and brine. The aqueous phases were reextracted and the combined organic phases dried over $MgSO_4$, filtered and concentrated *in vacuo*. The removal of the TBS group was confirmed by ^{1}H-NMR. The resulting crude was dissolved in a mixture of *t*Bu:H_2O (4:1, 0.01M) and one drop of AcOH was added. After purging Ar for 30 min, $Pd(OH)_2$ (20 weight% on carbon, 50% water) was added and the mixture was purged with Ar for 15 min, followed by H_2 (30s). The reaction was stirred under H_2 for three days. After filtration over Celite® and concentration *in vacuo*, the product was purified by size exclusion chromatography (HW40, 10 mM NH_4OAc in water). After repeated lyophilization to remove NH_4OAc, the final trisaccharide was isolated in 55% yield.
^{1}H-NMR (500 MHz, D_2O) δ: 4.93-4.88 (4H, 2 x H_{1L}, 2 x H_{1M}, m), 4.64 (1H, H_{2MB}, s), 4.59 (1H, H_{2MA}, s), 4.40-4.32 (4H, 2 x H_{1D}, 2x H_{2L}, m), 4.22-4.18 (4H, 2 x H_{4L}, 2 x H_{5L}, d), 4.16-3.96 (4H, H_{5D}, 2 x H_{2D}, m), 3.91-3.86 (1H, H *CH*HOlinker, m), 3.82-3.61 (8H, 2 x H_{4D}, 2 x H_{5L}, 2 x H_{3D}, 2 x H_{3M}, m), 3.65-3.52 (5H, 2 x H_{4M}, 2 x H_{5M}, H *CH*HOlinker, m), 3.03-2.96 (2H, H*CH*HNlinker, m), 2.14-1.96 (18H, 2 x CH_{3_NHAcM}, 2 x CH_{3_OAc}, 2 x CH_{3_NHAcL}, 2 x CH_{3_NHAcD}, m), 1.73- 1.65 (2H, $H_{CH2Linker}$, m), 1.62-1.57 (2H, $H_{CH2Linker}$, m), 1.44-1.33 (2H, $H_{CH2Linker}$, m), 1.29 (3H, H_{6D}, J_{6-5}=6.5 Hz, d), 1.27 (3H, H_{6L}, d).
^{13}C NMR (214 MHz, D_2O) δ: 176.5, 176.2, 175.2, 175.1, 174.9, 173.8, 173.5 (C_q), 102.4 (2 x C_{1D}), 100.5 (2 x C_{1M}), 99.3 (2 x C_{1L}), 79.4 (2 x C_{5M}), 77.9 (2 x C_{4D}), 76.9 (2 x C_{4L}), 72.5, 71.6, 71.3 (2 x C_{5D}, 2 x C_{3D}, 2 x C_{3M}), 71.0 ($CH_{2OLinker}$), 70.5 (2 x C_{3L}), 70.2 (2 x C_{4M}), 67.8 (2 x C_{4L}), 53.9 (2 x C_{2M}), 52.2 (2 x C_{2D}), 48.0 (2 x C_{2L}), 41.1 (2 x $CH_{2NLinker}$), 29.7, 27.2 (2 x $CH_{2Linker}$),

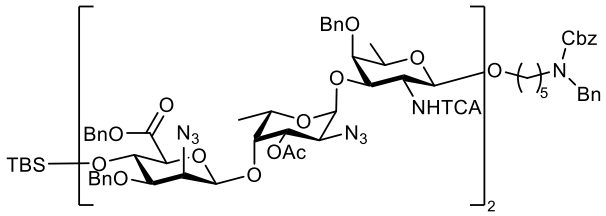
23.1 (2 x CH_{3NAcD}), 23.0 ($CH_{2Linker}$, 2 x CH_{3NAcL}, 2 x CH_{3NAcM}), 21.1 (CH_{3OAc}), 17.1 (CH_{3_6D}), 15.9 (CH_{3_6L}).
HRMS: $C_{53}H_{87}N_7O_{29}$ + 2xH^+ required 643.7847, found 643.7847

5-(benzyl(benzyloxicarbonyl)amino)pentyl 2-trichloroacetylamido-2-deoxy-3-*O*-(2-azido-2-deoxy-3-*O*-acetyl-4-*O*-(benzyl 2-azido-2-deoxy-3-*O*-benzyl-4-*O*-(*tert*butyldimethyl)silyl-β-D-mannopyranosiduronate)-α-L-fucopyranosyl)-4-*O*-benzyl-β-D-fucopyranoside (65)



6 (0.021 mmol) was dissolved in a mixture of DCM/H_2O (8:2, 0.1M) and DDQ (0.053 mmol, 2.5 eq) was added. The reaction mixture was stirred at room temperature for 2 hours, after which TLC analysis (Pent:EtOAc, 7:3) showed complete consumption of starting material. The reaction was diluted with DCM and washed with a 10% aq. solution of $Na_2S_2O_3$, a saturated aq. solution of $NaHCO_3$, H_2O and brine subsequently. The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The crude mixture was then dissolved in a mixture of DCM/Pyridine (1:1, 0.05M) and acetic anhydride (0.196 mmol, 10 eq) was added. The reaction mixture was stirred overnight, after which TLC analysis (DCM:Acetone, 96:4) showed complete consumption of starting material. The reaction mixture was diluted with DCM and washed with HCl 1M solution, saturated $NaHCO_3$ and water. After reextraction of the aqueous layers, the combined organic phases were dried over $MgSO_4$, filtered and concentrated *in vacuo*. After purification by column chromatography (Pent:EtOAc, 8:2→7:3→6:4), compound **65** was obtained in 98% yield as white solid over the two steps.
TLC analysis: R_f= 0.33 (Pentane:EtOAc; 7:3)
^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.41-7.08 (21H, H_{arom}, NH_{TCA}, m), 5.26-5.04 (4H, 4 x H_{CHHBn}, m), 5.00-4.91 (2H, H_{1L}, H_{3L}, m), 5.00-4.92 (2H, H_{1L}, H_{3L}, m), 4.90-4.83 (1H, H_{1D}, m), 4.80 (1H, H_{CHHBn}, J_{1-2}=11.8 Hz, d),4.74 (1H, H_{CHHBn}, d), 4.64-4.54 (2H, 2 x H_{CHHBn}, m), 4.52-4.40 (3H, H_{CHHBn}, H_{1M}, m), 4.37-4.28 (1H, H_3, m), 4.14 (1H, H_{4M}, J_{4M-5M}= J_{4M-3M}=9.1 Hz, pt), 4.03 (1H, H_{4L}, J_{4-3}=3.3 Hz, d), 3.94-3.73 (5H, H_{2L}, H_{2M}, H_{2D}, $H_{CHHOlinker}$, H_{5L}, m), 3.67 (1H, H_{5M}, d), 3.62 (1H, H_{5D}, J=6.5 Hz, q), 3.56 (1H, H_{4D}, J_{4-3}=2.8 Hz, d), 3.45-3.29 (2H, $H_{CHHOlinker}$, H_{3D}, m),3.27-3.10 (2H, $H_{CHHNlinker}$, m), 1.97 (3H, CH_{3_OAc}, s), 1.60-1.40 (4H, $H_{CH2Linker}$, m), 1.33-1.24 (5H, $H_{CH2Linker}$, H_{6D}, m), 1.00 (3H, 3 x H_{6L}, d), 0.78 (9H, H_{tBu}, s), 0.02 (3H, H_{CH3_Me}, s), -0.01 (3H, H_{CH3_Me}).
^{13}C-NMR (400 MHz, $CDCl_3$), δ: 170.7 (C_q), 167.8 (C_q), 162.1 (C_q), 138.3 (C_q), 138.0(C_q), 137.3 (C_q), 134.8 (2 x C_q), 129.2, 128.8, 128.8, 128.7, 128.7, 128.7, 128.6, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.8, 127.7, 127.5, 127.3 (CH_{arom}), 101.2 (C_{1M}), 99.1 (C_{1D}), 99.04 (C_{1D}), 92.6 (C_q), 80.2 (C_{3M}), 78.6 (C_{4D}), 78.1 (C_{5M}), 77.23 (C_{3D}), 75.5 (C_{4L}), 75.1 (CH_{2Bn}), 72.1 (CH_{2Bn}), 70.8 (C_{5D}), 70.3 (C_{3L}), 70.0 ($CH_{2OLinker}$), 68.6 (C_{4M}), 67.8 (CH_{2Bn}), 67.7 (CH_{2Bn}), 66.3 (C_{5L}), 60.5 (C_{2M}), 57.7 (C_{2L}), 57.3 (C_{2D}), 50.6 (CH_{2Bn}), 47.8, 46.7 ($CH_{2NLinker}$), 29.8, 29.5, 29.3 ($CH_{2Linker}$), 25.9 (CH_{3tBu}), 23.5 ($CH_{2Linker}$),20.9 (CH_{3OAc}), 17.7 (CH_{3_6D}), 17.3 (CH_{3_6L}), -3.8, -5.2 (CH_{3_Me}).
HRMS: $C_{68}H_{83}Cl_3N_8O_{16}Si$ + Na^+ required 1425.4625, found 1425.4648

Fully protected (3-*O*-acetyl) hexasaccharide (66)



8 (0.017 mmol) was dissolved in a mixture of DCM/H_2O (8:2, 0.1M) and DDQ (0.088 mmol, 5 eq) was added. The reaction mixture was stirred at room temperature for 2 hours, after which TLC analysis (Pent:EtOAc, 7:3) showed complete consumption of starting material. The reaction was diluted with DCM and washed with a 10% aq. solution of $Na_2S_2O_3$, a saturated aq. solution of $NaHCO_3$, H_2O and brine subsequently. The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The crude mixture was then dissolved in a mixture of DCM/Pyridine (1:1, 0.05M) and acetic anhydride (0.196 mmol, 10 eq) was added. The reaction mixture was stirred overnight, after which TLC analysis (DCM:Acetone, 96:4) showed complete consumption of starting material. The reaction mixture was diluted with DCM and washed with HCl 1M solution, saturated $NaHCO_3$ and water. After reextraction of the aqueous layers, the combined organic phases were dried over $MgSO_4$, filtered and concentrated *in vacuo*. After purification by column chromatography (Pent:EtOAc, 8:2→7:3→6:4), compound **66** was obtained in 88% yield as white solid over the two steps.

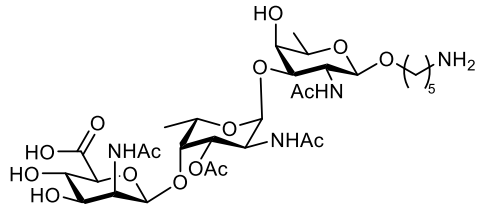
TLC analysis: R_f= 0.33 (Pentane:EtOAc; 7:3)

^{1}H-NMR (500 MHz, $CDCl_3$), δ: 7.46-7.11 (42H, H_{arom}, NH_{TCA}, m), 5.26-5.04 (4H, 4 x H_{CHHBn}, m), 5.00-4.42 (16H, 2 x H_{1L}, 2 x H_{3L}, 2 x H_{1D}, 8 x H_{CHHBn}, 2 x H_{1M}, m), 4.36-4.11 (1H,2 x H_3, 2 x H_{4M}, m), 4.03-3.31 (1H, 2 x H_{4L}, 2 x H_{2L}, 2 x H_{2M}, 2 x H_{2D}, 2 x $H_{CHHOlinker}$, 2 x H_{5L}, 2 x H_{5M}, 2 x H_{5D}, 2 x H_{4D}, 2 x H_{3D}, m), 3.27-3.10 (2H, $H_{CHHNlinker}$, m), 2.01 (3H, CH_{3_OAc}, s), 1.97 (3H, CH_{3_OAc}, s), 1.60-1.40 (4H, $H_{CH2Linker}$, m), 1.33-1.24 (5H, $H_{CH2Linker}$, 2 x CH_{3_6D}, m), 1.00 (3H, 2 x CH_{3_6L}, d), 0.78 (9H, H_{tBu}, s), 0.01 (3H, H_{CH3_Me}, s), -0.02 (3H, H_{CH3_Me}).

^{13}C-NMR (400 MHz, $CDCl_3$), δ: 170.7, 169.6, 167.7, 162.1, 138.3, 138.0, 137.9, 137.3, 134.8 (C_q), 129.2, 129.0, 128.8, 128.7, 128.7, 128.6, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, (CH_{arom}), 101.1 (2 x C_{1M}), 98.9 (2 x C_{1D}), 99.04 (2 x C_{1D}), 79.8 (2 x C_{3M}), 78.6 (2 x C_{4D}), 78.2 (2 x C_{5M}), 77.3 (2 x C_{3D}), 75.5 (2 x C_{4L}), 75.3 (CH_{2Bn}), 72.4 (CH_{2Bn}), 70.4 (2 x C_{5D}), 70.3 (2 x C_{3L}), 69.7 ($CH_{2OLinker}$), 68.7 (C_{4M}), 67.8 (CH_{2Bn}), 67.5 (CH_{2Bn}), 66.3 (2 x C_{5L}), 60.5 (2 x C_{2M}), 58.2 (2 x C_{2L}), 57.4 (2 x C_{2D}), 51.0 (CH_{2Bn}), 47.8, 46.7 ($CH_{2NLinker}$), 29.8, 29.5, 29.3 ($CH_{2Linker}$), 25.9 (CH_{3tBu}), 23.5 ($CH_{2Linker}$), 20.9 (2 x CH_{3OAc}), 17.5 (2 x CH_{3_6D}), 17.2 (2 x CH_{3_6L}), -3.8, -5.2 (CH_{3_Me}).

HRMS: $C_{68}H_{83}Cl_3N_8O_{16}Si + Na^+$ required 2412.7001, found 2412.7094

5-amino-pentyl 2-acetylamido-2-deoxy-3-*O*-(2-acetylamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-β-D-mannopyranosiduronyl)-α-L-fucopyranosyl)-β-D-fucopyranoside (2)



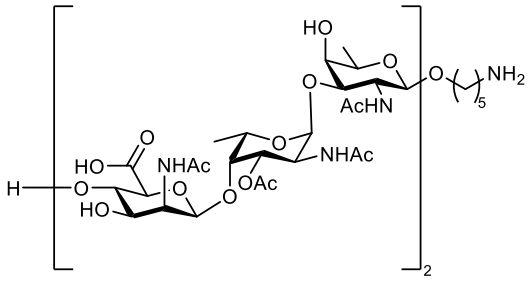
Compound **65** (0.023 mmol) was dissolved in THF (0.23 mL, 0.1M) and under inert atmosphere AcOH (1.6 mmol, 70 eq), Ac_2O (1.93 mmol, 84 eq) and Zn dust (1.15 mmol, 50 eq) were added. The reaction mixture was heated at 50°C and left stirring overnight. After TLC analysis (DCM:MeOH, 95:5) showed complete consumption of the starting material, the reaction was diluted with DCM, filtered and concentrated *in vacuo*. A column chromatography (DCM:MeOH, 99:1→97:3→95:5→9:1) was performed to remove minor impurities. Intermediate **67** was dissolved in dry THF (1mL, 0.01M) and under inert atmosphere TEA*HF (0.25 mmol, 10 eq) was added. The reaction was stirred overnight at 50°C, after which TLC analysis (DCM:MeOH, 9:1) showed complete consumption of starting material. The reaction was diluted in EtOAc and washed with a saturated solution of $NaHCO_3$ (x3), water and brine. The aqueous phases were reextracted and the combined organic phases dried over $MgSO_4$, filtered and concentrated *in vacuo*. The removal of the TBS group was confirmed by ^{1}H-NMR. The resulting crude was dissolved in a mixture of *t*Bu:H_2O (4:1, 0.01M) and one drop of AcOH was added. After purging Ar for 30 min, $Pd(OH)_2$ (20 weight% on carbon, 50% water) was added and the mixture was purged with Ar for 15 min, followed by H_2 (30s). The reaction was stirred under H_2 for three days. After filtration over Celite® and concentration *in vacuo*, the product was purified by size exclusion chromatography (HW40, 10 mM NH_4OAc in water). After repeated lyophilization to remove NH_4OAc, the final trisaccharide was isolated in 88% yield.

^{1}H-NMR (850 MHz, D_2O) δ: 5.03 (1H, H_{3L}, $J_{3\text{-}2}$=11.6 Hz, $J_{3\text{-}4}$=3.0 Hz, dd), 5.01 (1H, H_{1L}, $J_{1\text{-}2}$=4.0 Hz, d), 4.74 (1H, H_{1M}, m), 4.59 (1H, H_{2M}, $J_{2\text{-}3}$=4.3 Hz, d), 4.41 (1H, H_{1D}, $J_{1\text{-}2}$=8.3 Hz, d), 4.38 (1H, H_{2L}, dd), 4.22 (1H, H_{4L}, d), 4.19 (1H, H_{5L}, $J_{5\text{-}6}$=6.6 Hz, q), 4.01-3.96 (1H, H_{2D}, m), 3.91-3.86 (1H, H *CH*HOlinker, m), 3.82-3.75 (4H, H_{4D}, H_{5L}, H_{3D}, H_{3M}, m), 3.65 (1H, H_{4M}, $J_{4\text{-}3}$=$J_{4\text{-}5}$=9.7 Hz, t), 3.61-3.55 (2H, H_{5M}, H *CH*HOlinker, m), 3.03-2.96 (2H, H*CH*HNlinker, m), 2.14 (3H, CH_{3_NHAcM}, s), 2.08 (3H, CH_{3_OAc}, s), 2.01 (3H, CH_{3_NHAcL}, s), 1.99 (3H, CH_{3_NHAcD}, s), 1.71- 1.64 (2H, $H_{CH2Linker}$, m), 1.63-1.55 (2H, $H_{CH2Linker}$, m), 1.43-1.33 (2H, $H_{CH2Linker}$, m), 1.28 (3H, H_{6D}, $J_{6\text{-}5}$=6.5 Hz, d), 1.25 (3H, H_{6L}, d).

^{13}C NMR (214 MHz, D_2O) δ: 176.5, 176.3, 175.2, 175.0, 174.7 (C_q), 102.4 (C_{1D}), 100.7 (C_{1M}), 99.9 (C_{1L}), 79.4 (C_{5M}), 78.0 (C_{4D}), 76.9 (C_{4L}), 72.5, 71.6, 71.3 (C_{5D}, C_{3D}, C_{3M}), 71.0 ($CH_{2OLinker}$), 70.7 (C_{3L}), 70.4 (C_{4M}), 67.7 (C_{4L}), 53.9 (C_{2M}), 52.2 (C_{2D}), 48.0 (C_{2L}), 40.2 ($CH_{2NLinker}$), 29.1, 27.3 ($CH_{2Linker}$), 23.1 (CH_{3NAcD}), 23.0 ($CH_{2Linker}$), 22.9 (CH_{3NAcL}, CH_{3NAcM}), 21.2 (CH_{3OAc}), 16.3 (CH_{3_6D}), 16.2 (CH_{3_6L}).

HRMS: $C_{30}H_{50}N_4O_{16}$ + H^+ required 723.3295, found 723.3295

3-O-acetyl Hexasaccharide (4)



Compound **66** (0.012 mmol) was dissolved in THF (0.12 mL, 0.1M) and under inert atmosphere AcOH (0.84 mmol, 70 eq), Ac_2O (1.00 mmol, 84 eq) and Zn dust (0.6 mmol, 50 eq) were added. The reaction mixture was heated at 50°C and left stirring overnight. After TLC analysis (DCM:MeOH, 95:5) showed complete consumption of the starting material, the reaction was diluted with DCM, filtered and concentrated *in vacuo*. A column chromatography (DCM:MeOH, 99:1→97:3→95:5→9:1) was performed to remove minor impurities. Intermediate **68** was dissolved in dry THF (1mL, 0.01M) and under inert atmosphere TEA*HF (0.12 mmol, 10 eq) was added. The reaction was stirred overnight at 50°C, after which TLC analysis (DCM:MeOH, 9:1) showed complete consumption of starting material. The reaction was diluted in EtOAc and washed with a saturated solution of $NaHCO_3$ (x3), water and brine. The aqueous phases were reextracted and the combined organic phases dried over $MgSO_4$, filtered and concentrated *in vacuo*. The removal of the TBS group was confirmed by ^{1}H-NMR. The resulting crude was dissolved in a mixture of *t*Bu:H_2O (4:1, 0.01M) and one drop of AcOH was added. After purging Ar for 30 min, $Pd(OH)_2$ (20 weight% on carbon, 50% water) was added and the mixture was purged with Ar for 15 min, followed by H_2 (30s). The reaction was stirred under H_2 for three days. After filtration over Celite® and concentration *in vacuo*, the product was purified by size exclusion chromatography (HW40, 10 mM NH_4OAc in water). After repeated lyophilization to remove NH_4OAc, the final trisaccharide was isolated in 62% yield.

^{1}H-NMR (850 MHz, D_2O) δ: 4.94-4.90 (4H, 2 x H_{3L}, 2 x H_{1L}, m), 4.70-4.63 (2H, 2 x H_{1M}, n.d.), 4.57 (1H, H_{2MB}, s), 4.52 (1H, H_{2MA}, s), 4.34-4.23 (4H, 2 x H_{1D}, 2 x H_{2L}, m), 4.15-4.06 (4H, 2 x H_{4L}, 2 x H_{5L}, m), 3.91-3.85 (2H, 2 x H_{2D}, m), 3.83-3.77 (2H, H $_{CH}$Olinker, H_{3MB}, m), 3.77-3.60 (9H, 2 x H_{4D}, 2 x H_{5D}, 2 x H_{3D}, H_{3MA}, 2 x H_{4M}, m), 3.59-3.55 (2H, 2 x H_{5M}, m), 3.51-3.46 (1H, H $_{CH}$Olinker, m), 2.93-2.88 (2H, H_{CH}HNlinker, m), 2.06-1.84 (24H, 2 x CH_{3_NHAcM}, 2 x CH_{3_OAc}, 2 x CH_{3_NHAcL}, 2 x CH_{3_NHAcD}, m), 1.71- 1.64 (2H, $H_{CH2Linker}$, m), 1.63-1.55 (2H, $H_{CH2Linker}$, m), 1.43-1.33 (2H, $H_{CH2Linker}$, m), 1.23-1.10 (12H, 2 x H_{6D}, 2 x H_{6L}, m).

^{13}C NMR (214 MHz, D_2O) δ: 175.6, 175.5, 174.3, 174.2, 174.1, 173.7, 173.6 (C_q), 101.5 (2 x C_{1D}), 99.9 (2 x C_{1M}), 99.0 (2 x C_{1L}), 79.0 (2 x C_{4M}), 77.2 (2 x C_{5M}), 76.1 (2 x C_{4L}), 71.7 (2 x C_{4D}) 70.8 (2 x C_{5D}, 2 x C_{3D}), 70.3 (2 x C_{3M}), 70.1 ($CH_{2OLinker}$), 69.1 (2 x C_{3L}), 66.7 (2 x C_{5L}), 52.9 (2 x C_{2MA}), 51.9 (2 x C_{2MB}), 51.3 (2 x C_{2D}), 47.1 (2 x C_{2L}), 39.5 ($CH_{2NLinker}$), 28.2, 26.6, 22.0 ($CH_{2Linker}$), 21.9 (6 x CH_{3NAc}), 20.3 (CH_{3OAc}), 15.4-15.2 (CH_{3_6D}, CH_{3_6L}).

HRMS: $C_{57}H_{91}N_7O_{31}$ + 2xH^+ required 685.7963, found 685.7953

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Chapter 3

Development of a teichoic acid microarray tool for antibodies profiling

D. van der Es*, F. Berni*, W. F. J. Hogendorf, N. Meeuwenoord, D. Laverde, A. van Diepen, H. S. Overkleeft, D. V. Filippov, C. H. Hokke, J. Huebner, G. A. van der Marel, J. D. C. Codée; *Chem. Eur. J.*, **2018**, *24*, 4014–4018.

INTRODUCTION

Our immune system protects us against possible dangerous infections.[1] The first line of defence is constituted by the innate immune response which is characterized by a non-specific but fast reaction, to recognize and eradicate pathogens.[2] The second arm of the immune system, the adaptive immune system, develops during the lifetime of an individual as the adaptation to infections by pathogens. This immune response develops slower, but lasts longer and serves to protect form reinfection with the establishment of immunological memory.[3]

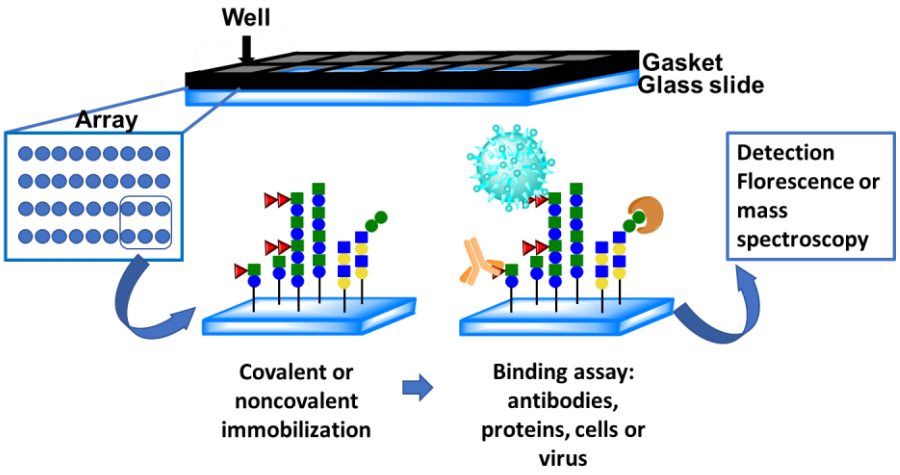
The development of vaccines for the prevention of infectious diseases relies on the activation of the adaptive (humoral and cellular) immune response. The ultimate goal is to confer long-term protection and the nature of the vaccine determines the type of antigen-specific immune effectors and the production of immune memory cells.[4] One of the parameters for the evaluation of vaccine efficacy is the detection of high affinity antibodies. These are released by B-lymphocytes and upon maturation into memory cells the isotype of the immunoglobulins (Ig) switches from low-affinity IgM to high affinity IgG.[1,5] The evaluation of the (specific) recognition of antigens of the pathogen constitutes a critical parameter for vaccine and diagnostic purposes but also for basic and clinical research.[6] In the last decades anti-carbohydrate antibody profiling has significantly advanced thanks to the advent of glycan-microarray technology, which allows the analysis of carbohydrate-mediated interactions in a high throughput manner using minute amounts of material.[7] For example, Gildersleeve and co-workers used this tool to probe the anti-carbohydrate antibody profile of sera of 48 healthy volunteers against 122 different glycans (98 well-defined synthetic fragments and 24 natural glycoproteins). High levels of IgG antibodies were detected against α-L-rhamnose (α-Rha), β-L-rhamnose (β-Rha), the Forssman disaccharide as well as the well-known α-Gal epitope.[8]

Generally, a glycan microarray is created by positioning different carbohydrates (from natural sources or chemically and/or enzymatically synthesized) in a dot matrix array fashion on a glass microscope slide via an automated arraying robot (Figure 1).[9] On a single slide multiple arrays can be printed, as the applied gasket can contain up to 64 wells for the simultaneous screening of different samples. The immobilization of the fragments can occur either via noncovalent interaction or covalent coupling and once printed the slides can be stored for several months in the dark and inert atmosphere. Carbohydrate-microarrays have been employed for the analysis of glycan binding specificity of not only antibodies but also proteins (such as lectins from the immune system), viruses and cells, through fluorescent measurement or mass spectroscopy detection.[10]

As described in Chapter 1, teichoic acids (TA) are immunogenic cell-wall components present in many Gram-positive bacteria species. Since the isolation from native sources leads to heterogenous mixtures, synthetic strategies have been developed both in solution and using automated solid phase techniques, to deliver well-defined TA-fragments.[11] Libraries have been generated comprising both glycerol phosphate (GroP) and ribitol phosphate (RboP)-based TAs. [12,13,14] In order to evaluate these compounds in a high throughput fashion, herein the development of a TA-microarray is described as a qualitative tool to access the binding specificity of monoclonal antibodies as well as

polyclonal sera. The availability of this technique enables the rapid screening of binding interactions to establish structure-immunogenicity relationship studies, aid in monoclonal antibody development and profile sera for both basic and clinical research.

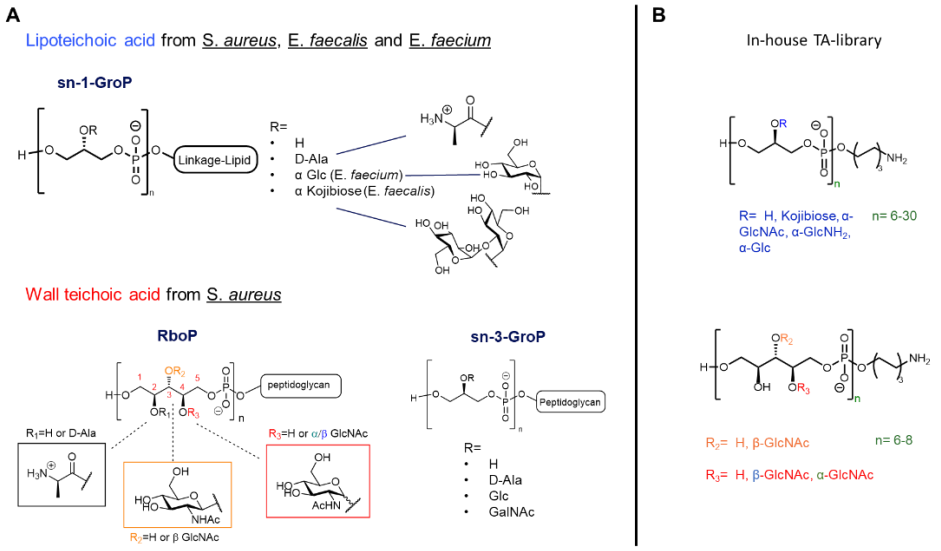
Figure 1: Schematic representation of a glycan-microarray assay.



RESULTS AND DISCUSSION

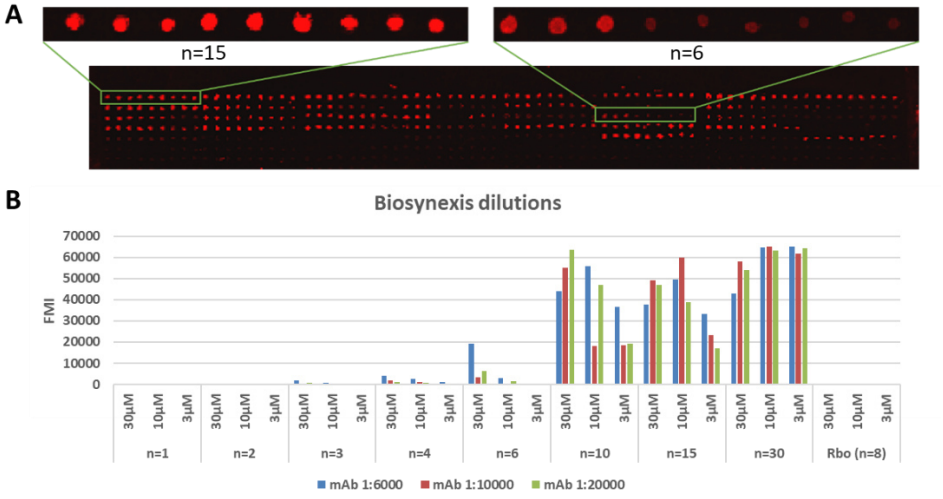
In Figure 2, the structures of the TAs found in *Staphylococcus aureus* and *Enterococcus faecalis* and *faecium* are shown (A), with a schematic overview of the TA-library that has been previously been generated. The well-defined GroP and RboP based fragments differ in the number of repeating units, the type of glycosyl substituent, the position of the carbohydrates along the chain and the degree of substitution. All of the fragments are equipped with the same aminohexanol linker for ligation purposes. The primary amine was used for the immobilization on reactive surfaces and for this purpose epoxide functionalized glass slides were chosen for the development of the TA-microarray.[15, 9]

***Figure 2**: A) TA-structures found in Enterococcos faecalis and faecium and Staphylococcus aureus. B) General overview of the in-house generated TA-library.*



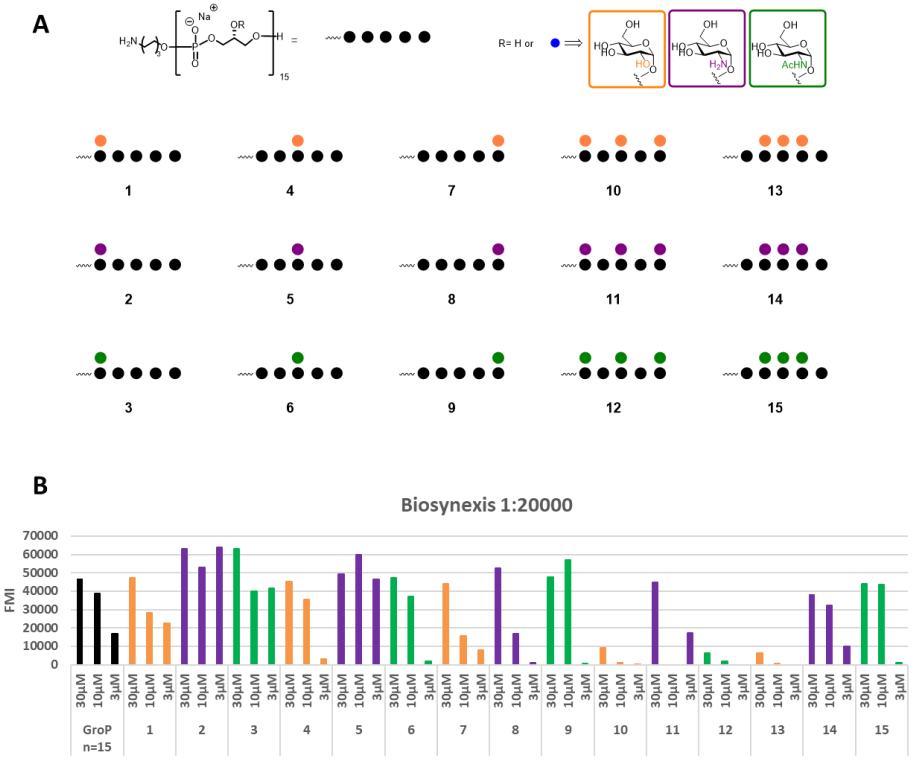
To assess the TA microarrays, first the binding specificity of a commercially available monoclonal antibody (mAb, Biosynexis)[16] was evaluated. This mAb is a mouse monoclonal antibody that has been raised against native S.*epidermidis* LTA, the structure of which is characterized by a GroP repeating unit having D-alanine moieties as the major substituent at the C-2 position.[17] Different dilutions of the antibody were employed (1:6000, 1:10000, 1:20000) and binding was detected by fluorescent scanning of binding of a secondary antibody for which a rabbit anti-mouse IgG labelled with DyLight 650 was used. In Figure 3A an example of an illustrative scan is provided, with a focus on two unsubstituted GroP fragments, a pentadecamer and a hexamer, showing good morphology and homogenicity among the different spots.[18] The binding of the mAb to non-substituted fragments differing in the number (n) of repeating units are shown in Figure 3B. In this experiment a non-substituted RboP octamer was used as negative control. A clear length-dependence was observed for the binding of the mAb to the oligo-GroP chains. The array also revealed better binding at higher concentration of the TA fragments. Little binding was detected for fragments with three, four or six repeating unit, while the interaction significantly increased for the 10-, 15- and 30-mer. Of note, the measured fluorescence is not always linearly proportional to the different dilutions of the mAb or the concentration of the printed TAs. The intensity of the detected signal may be affected by minor differences in density, orientation and conformation of each compound on the array, and the constructed tool is therefore only used as a qualitative assay.[19]

***Figure 3**: Example of scan image and microarray results of Biosynexis mAb on unsubstituted GroP fragments. A) Scan image of Biosynexis at 1:10000 dilution and focus on spots related to pentadecamer GroP (left top) and hexamer GroP (right top). B) Array results of Biosynexis at different dilutions: 1:6000 (blue), 1:10000 (red) and 1:20000 (light green). Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); Rbo (n=8), ribitol phosphate octamer from WTA of S. aureus.*



As shown above, TAs from the opportunistic pathogens *E. faecalis*, *E. faecium* and *S. aureus* contain α-kojibiose (D-glucose-(α-1,2)-D-glucose), α-glucose or *N*-acetyl α-glucosamine residues, respectively.[20] The array used above was expanded with a set of fragments based on a pentadecamer glycerolphosphate backbone carrying one or three carbohydrate (glucose, glucosamine or *N*-acetyl-glucosamine) appendages (Figure 4A). The compounds also differ in the position of the substituents along the chain. In the case of the monosubstituted fragments the glycosyl moiety has been introduced at the beginning (**1**, **2**, **3**) or middle (**4**, **5**, **6**) or at the end of the chain (**7**, **8**, **9**), relative to the position of the linker. The three glycosyl residues were introduced at the end, middle and beginning of the chain (**10**, **11**, **12**) or on neighbouring residues in the middle of the chains (**13**, **14**, **15**). Binding to the array was evaluated using Biosynexis at a dilution of 1:20000. Figure 4B shows diminished binding to the fragments carrying a terminal substituent (**7**-**12**). This reveals the preference of the mAb to bind to non-substituted GroP-chains. It also indicates that binding of the epitopes is best when the epitopes are most exposed (not hindered by a terminal carbohydrate appendage).

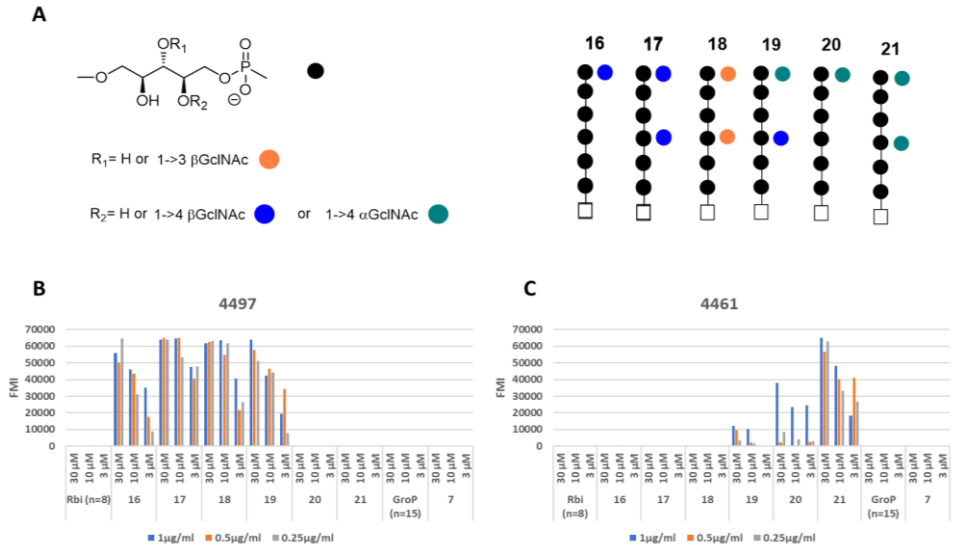
***Figure 4**: Glycosylated GroP pentadecamers. A) Schematic overview of the expanded library of pentadecamers (**1**-**15**). B) Array results of Biosynexis at 1:20000 dilution. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); different colors depict the type of glycosyl substituent (Glc=orange, GlcNH$_2$=purple, GlcNAc=green).*



Lapardus and co-workers recently generated monoclonal antibodies from B-cells of patients infected by *S. aureus* and they have mapped the binding specificity of the antibodies using biosynthesis knock-out strains.[21] The antibodies were found to be directed towards WTA from *S. aureus* specifically recognizing either the β-GlcNAc or the α-GlcNAc glycosylated RboP. More recently, binding studies were performed at the molecular level using fully synthetic RboP fragments or enzymatically[22] glycosylated RboP-oligomers. To establish binding preferences of the monoclonal antibodies 4497 and 4461, raised against β-GlcNAc or α-GlcNAc substituted WTA, a TA-microarray was generated presenting a set of well-defined WTA RboP fragments. The library used is shown in Figure 5A and it includes, alongside the unsubstituted RboP octamer,[22] several hexamers differing in the anomeric configuration of the carbohydrate appendages, as well as the position and number of *N*-acetyl-glucosamine substituents.[21] Both antibodies were used in three different concentrations (1 µg/ml, 0.5 µg/ml and 0.25 µg/ml) and detection was performed using a goat anti-human IgG secondary antibody with an Alexa Fluor® 488 fluorophore. The non-substituted GroP pentadecamer and compound **7** were

used as negative controls. As previously reported, the monoclonal antibody 4497 specifically recognizes both the 1,3- and 1,4-β-GlcNAc glycosylated RboP hexamers (**17**, **18**) and the presence of a single glycosyl substituent is sufficient for recognition (**16**, **19**). The monoclonal antibody 4461 shows clear specificity for the RboP hexamers decorated with α-1,4-GlcNAc substituents (**19**-**21**) with a seemingly lower affinity than antibody 4497. Interestingly, GroP based TA **7** is not recognized, suggesting that the RboP backbone (or unit) plays a crucial role in antibody binding.

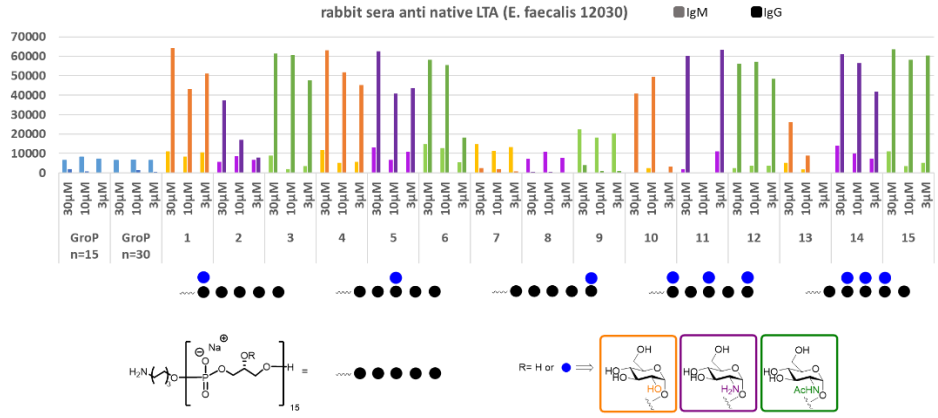
***Figure 5**: A) Schematic overview of the RibP-based library (**16**-**21**). Array results of 4497 (B) and 4461 (C) at different dilutions: 1 µg/ml=blue, 0.5 µg/ml=orange and 0.25 µg/ml=grey. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit.*



After the assessment of the arrays using monoclonal antibodies for both GroP and RboP fragments, the newly generated TA-microarrays were employed for the analysis of more complex biological samples. At first the binding of IgM and IgG antibodies in rabbit sera, obtained after immunization with native LTA of *E. faecalis* strain 12303, was evaluated using the GroP TA microarray.[19] Binding of IgM and IgG antibodies was detected using anti-rabbit IgM- and IgG secondary antibodies, labelled with DyLight 650 and DyLight 550 reporter groups, respectively. In Figure 6 the results are shown, where the IgM signal is depicted in a lighter shade of colour than the IgG signal. Binding to the non-substituted fragments is depicted in blue, while the colour pattern of Figure 4B is used to show binding of the substituted TAs, to differentiate the different glycosyl substituent. As it can be seen in Figure 6, relatively low binding is detected for the IgM antibodies as a result of a low titer or affinity in comparison to the IgG antibodies. The recognition seems to be non-specific. In contrast, the IgG antibodies are directed towards fragments with

glycosyl substituents (**1**-**6**, **10**-**15**), but interestingly no binding was detected for pentadecamers bearing the glycosyl appendage at the end of the chain (**7**-**9**).

***Figure 6**: TA-microarray analysis on rabbit sera after immunization with native LTA from E. faecalis 12303 (1:1000 dilution). Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); different colors depict the type of glycosyl substituent (Glc=orange, GlcNH$_2$=purple, GlcNAc=green).*



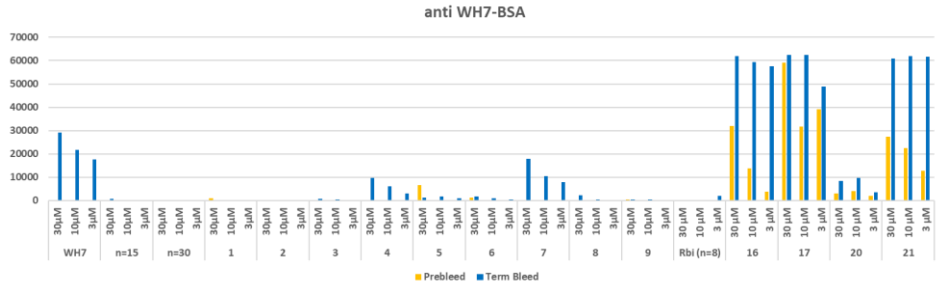
Previously a fully synthetic GroP hexamer bearing a glucosyl substituent at the terminal residue (**WH7**) was used to generate a model vaccine against *E. faecalis*.[23] It was conjugated to BSA as carrier protein, and the conjugate vaccine was able to induce opsonic and protective antibodies against the targeted Gram-positive bacterium. Although the rabbit sera generated after immunization was evaluated for bacterial killing ability, no structural analysis of the antibody repertoire was reported. Therefore, the generated TA-microarray was employed to unravel the preferential binding of the IgG antibodies using the protocol as described above. In Figure 7A the results are reported for the anti-WH7-BSA serum at 1:500 dilution, revealing high specificity of the IgG antibodies towards fragments bearing a glucosyl substituent at the terminal part of the chain or in the middle. The other glycosyl substituted TA fragments and the non-substituted GroP chains are not recognized.

Subsequently, two other **WH7** conjugates were developed, where the synthetic GroP TA was conjugated to either detoxified tetanus toxoid (TT)[24] or the zinc ABC transporter substrate-binding lipoprotein (AdcA) from *E. faecium*.[25] Binding of antibodies in the sera, raised using these conjugates is reported in Figure 7B and 7C, from which it becomes apparent that in these two conjugates elicited a higher antibody titer and the antibodies showed a broader recognition profile, with some recognition of other types of glycan appendages as well as binding to the non-substituted 30-mer.

***Figure 7**: Microarray analysis of rabbit sera raised against different WH7 conjugates. (A) anti WH7-BSA, (B) anti WH7-TT, (C) WH7-AdcA. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); different colors depict the type of glycosyl substituent (Glc=orange, $GlcNH_2$=purple, GlcNAc=green). All three sera were used at 1:500 dilution.*

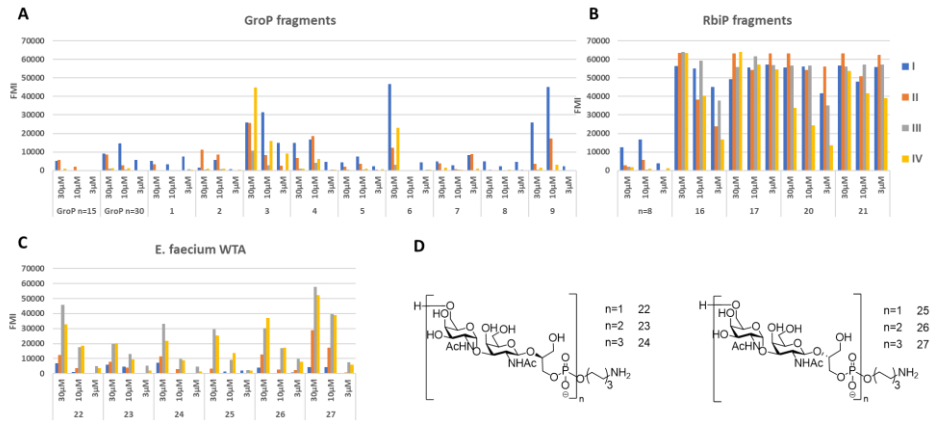
The opsonic activity of the anti-**WH7**-BSA rabbit sera has previously also been evaluated against an *E. faecium* clinical isolate and a community acquired *S. aureus* strain, to explore the potential of model vaccine to combat other multidrug resistance Gram-positive bacteria.[23b] It was observed that the serum generated against **WH7**-BSA was cross-reactive towards these tested strains. When the serum was analysed on the TA-microarray, presenting *S. aureus* WTA fragments, IgG binding to RboP based TA was observed (Figure 8, blue). To exclude the possibility that these antibodies were generated upon immunization with the synthetic GroP conjugate, the pre-bleed serum (taken prior to the immunization) was also tested. Figure 8 reveals the presence of antibodies recognizing the GlcNAc-functionalized RboP fragments in the pre-bleed serum (yellow). This may indicate that the observed opsonic activity towards S. *aureus* could have come from these pre-existing antibodies. Antibodies against the GroP based fragments are present only in the final bleed after immunization with **WH7**-BSA, corroborating the previous results.

Figure 8: *comparison of sera before and after immunization. Prebleed (yellow) and terminal bleed (blue) upon immunization with WH7-BSA conjugate were analyzed on the TA-microarray, both at 1:500 dilution. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations.*



The detection of pre-existing anti-TA antibodies indicates that the TA-microarray could be used as part of the evaluation of an immunization protocol using a TA-based vaccine. Indeed, enterococci and staphylococci are commensal bacteria and IgG antibodies can be present also in the sera of healthy donors.[26] The IgG repertoire against TA-based structures may vary depending on pathogen exposure during the lifetime of an individual. In Figure 9, the anti-TA antibody repertoire of 4 healthy donors was evaluated using the TA-microarray. In panel A binding to the GroP based fragments is shown, where binding to RboP and E. *faecium* WTA are shown in panel B and C respectively. The structures of E. *faecium* WTA fragments are depicted in Figure 9D.[27] As it can be seen, there is a substantial difference among the four subjects in panel A and C. Serum **I** (blue) has a broader content of IgG antibodies against the GroP fragments, while sera **III** (grey) and **IV** (yellow) have relatively higher titer of IgGs directing towards compounds **22**-**27**. In panel B, high signals were instead detected towards **16**, **17**, **20** and **21** across all 4 subjects. It has been previously observed that high level of IgG antibodies can be present in serum from both healthy individuals and infected patients.[28] In particular, GlcNAc substituted RboP oligomers have been identified as one of the immunodominant antigen in *S. aureus* species.[29,20b]

***Figure 9**: Microarray analysis on human sera from healthy volunteers. Four human sera from healthy volunteers were used at 1:200 dilution: **I** (blue), **II** (orange), **III** (grey) and **IV** (yellow). IgG antibodies were detected using goat anti-human IgG secondary antibody, Alexa Fluor® 488 conjugate. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations.*



CONCLUSION

Glycan microarrays have become important tools for the evaluation of glycan binding protein at the molecular level. The high throughput nature of this technology allows the rapid screening of large libraries of glycans, the binding of which can then be further evaluated using other techniques, such as surface plasmon resonance, ITC, X-ray crystallography or STD NMR.[10] In the context of TA-vaccine development, a large library of GroP and RboP based fragments has been generated. This chapter has described the construction of a TA-microarray to probe the binding of monoclonal and polyclonal antibodies from different sources. Epoxide functionalized micro array glass slides were used to immobilize the compounds, that had been equipped with an aminohexanol for this purpose. The feasibility of the technology was at first assessed using monoclonal antibodies generated against either GroP or RboP fragments. Specific binding could be detected although it also became apparent only qualitative binding data could be generated. Next, the arrays were used to probe more complex biological samples such as rabbit sera, obtained in immunization experiments using different TA sources and model vaccine candidates. The arrays have revealed that a very specific immune response can be generated when synthetic TA-conjugate vaccines are used. Preliminary screening of serum from healthy individuals indicates that *S. aureus* WTA featuring GlcNAc-RboP elements is a commonly recognized antigen. The arrays developed here can be expanded when new structures become available. The inclusion of TA fragments carrying D-Ala substituents will be of particular importance as this modification is known to play an important role in TA-biology. The arrays can be used to probe binding to many other biomolecules such as biosynthesis enzymes and lectins.

EXPERIMENTAL SECTION

TA-microarray construction

Synthetic well-defined GroP and RobP based teichoic acids were dissolved in MiliQ in order to obtain three different concentrations (75 µM, 25 µM and 7.5 µM) and from each solution 8 µL were added in a 384-wells V-bottom shape (Genetix, New Milton, UK). Subsequently 12 µL of a solution containing 16% of DMSO in spotting buffer (Nexterion Spot, Schott Nexterio) were added in each well, obtaining a final concentration of respectively 30 µM, 15 µM and 3 µM. Alongside the well-defined TA fragments, wells containing only MilliQ, DMSO and spotting buffer in the same proportion were also included as negative and background control. The plate can be stored at -20°C and used multiple times depending of course on the size of the preceding printing (how many arrays and how many slides performed).[9] Each compound was printed 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. Each array was printed five or eight times on each glass slide. Printed slides were incubated overnight at room temperature at sufficient humidity to prevent drying of the spots and to allow covalent binding to the epoxysilane via reaction with primary amines. The slides were stored in the dark until used, while the plate at -20°C.

General binding assay

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 the appropriate sample (monoclonals or sera) diluted with PBS containing 1% of Tween® 20 and 10 mg/ml of BSA for 60 minutes. The slides were flushed with PBS and PBS containing 5% of Tween® 20 subsequently and finally rinsed with PBS containing 1% of Tween® 20 subsequently. After removal of the PBS containing 1% of Tween® 20, the arrays were shaken with 0.25 ml of fluorescent secondary antibody conjugates, differing upon the type of sample used, diluted with PBS 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 antibody adhesion by subtracting the fluorescence at blank spots, where only spotting buffer was printed without GTA fragment. The average of the triplicate spots was calculated and visualized in bar graphs using Microsoft Excel.

Binding assay using Biosynexis monoclonal antibody

Commercially available (IBT Bioservices) mouse anti-S. epidermidis monoclonal antibody Biosynexis (0.92 mg/ml) was diluted at 1:6000, 1:10000 and 1:20000 and used as described in the general binding assay. Goat anti-mouse IgM heavy chain secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, A21426) and goat anti-mouse IgG (H+L) secondary antibody Alexa Fluor® 555 conjugate (Invitrogen, A21422) were used for detection at 0.5 μg/ml concentration as described in the general binding assay.

Binding assay using 4497 and 4461 monoclonal antibodies

4497 and 4461 monoclonal antibodies were obtained as previously described[22] and used in three different concentrations (1 μg/ml, 0.5 μg/ml and 0.25 μg/ml). as described in the general binding assay. Goat anti-human IgG secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, A-11013) was used at 0.5 μg/ml concentration as described in the general binding assay.

Binding assay using rabbit sera

Rabbit sera raised against the putative vaccine candidate were obtained as previously described (native LTA from *E. faecalis*[23], **WH7**-BSA[23], **WH7**-TT[24] and **WH7**-AdcA[25]). The sera raised against native LTA from *E. faecalis* was diluted at 1:1000, while **WH7** conjugates and the pre-bleed of **WH7**-BSA immunization at 1:500 as described in general binding assay. Detection of IgM and IgG antibodies was performed using specific goat anti rabbit secondary antibodies, labelled with DyLight® 650 (AB_96982) and DyLight® 550 (AB_10942173) reporter groups respectively at 0.5 μg/ml as described in general binding assay.

Binding assay using human sera

Blood was drawn from four healthy volunteers and allowed to clot for 15 minutes at room temperature. After centrifugation for 10 min at 3,220 xg at 4°C, serum was collected and stored at -80°C. Each serum was used at 1:200 dilution as described in general binding assay and IgG detection was performed using goat anti-human IgG secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, A-11013) at 0.5 μg/ml concentration as described in the general binding assay.

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Chapter 4

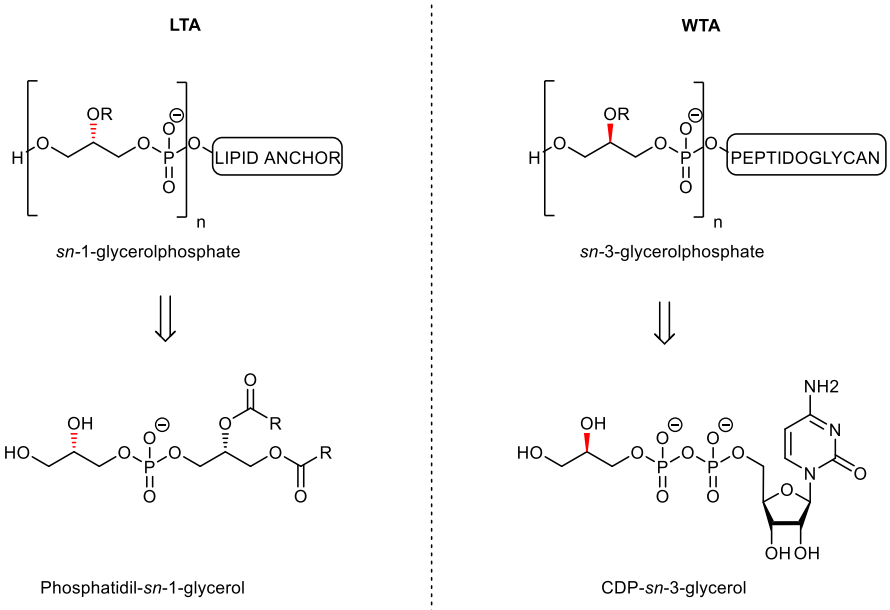
Synthesis of glucosyl sn-1-glycerolphosphate teichoic acids: glycerol stereochemistry affects synthesis and antibody interaction

F. Berni, L. Wang, E. Kalfopoulou, L. D. Nguyen, D. van der Es, J. Huebner, H. S. Overkleeft, C. H. Hokke, G. A. van der Marel, A. van Diepen, J. D. C. Codée; *RSC Chem. Biol*, **2021**, 2: 187-191.

INTRODUCTION

Teichoic acids (TA) are anionic polymeric structures, found in the cell-wall of Gram-positive bacterial species.[1] Among the several biological functions, it has been observed that TA are highly immunogenic and therefore, considered as good antigen candidate for vaccine development against different opportunistic pathogens.[2] As described in Chapter 1, they are divided in lipoteichoic acids (LTA) and wall teichoic acids (WTA) upon the way and location of interconnection with the cell wall. The chemical structure is widely diverse among the different species, but generally most Enterococci and Staphylococci bear a Type I LTA, which is composed by an *sn*-glycerol-1-phosphate (GroP) backbone with either D-Alanine or glycosylic substituents at the C-2 position (Figure 1).[3, 4] The stereospecificity of the GroP chain has been previously carried out based on the differences in the biosynthetic precursors and degradation products compared to GroP based wall teichoic acids (WTA). Indeed, Type I glycerolphosphate based LTA are not only biologically distinct from WTA, but they are structurally enantiomeric polymers composed respectively by *sn*-Gro1P or *sn*-Gro3P.[5] Biochemical evidence of the stereochemical difference between GroP based WTA and LTA has been carried out recently using a stereospecific exo acting sn-glycerol-3-phosphodiesterase (GlpQ) from B. *subtilis* strain 168.[6] It was observed that GlpQ was able to cleave off *sn*-glycerol-3-phosphate repeating unit from the exposed end of WTA but such hydrolysis activity was not occurring with LTA substrate having opposite stereochemistry.

Figure 1*: General structure of sn-Gro-1-P LTA and sn-Gro-3-P WTA and their biosynthesis precursors phosphatidyl-sn-1-glycerol and CDP-sn-3-glycerol*



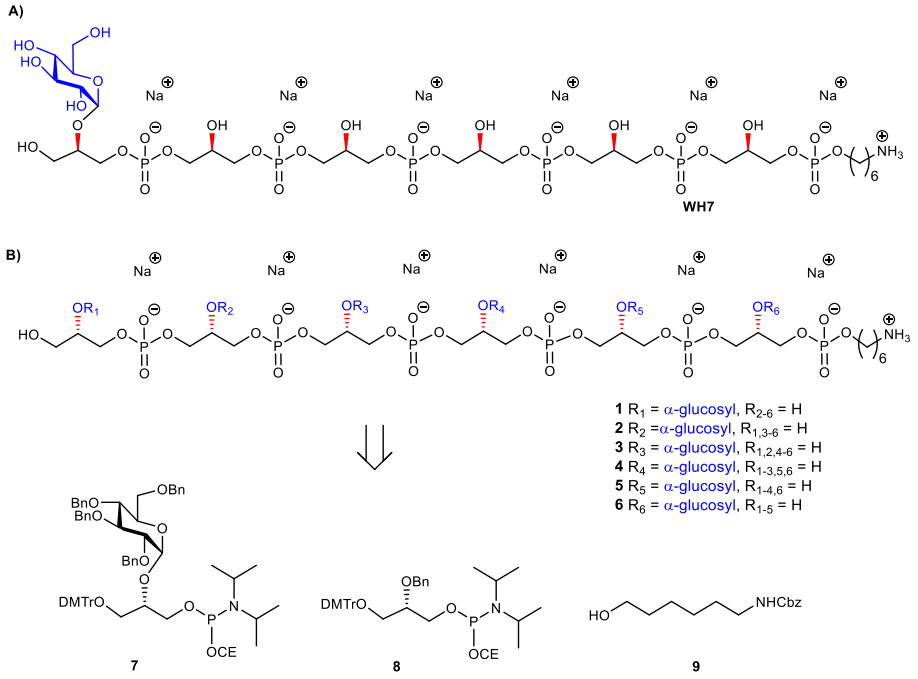
Because of the microheterogenicity of TAs, resulting from the different glycosylation and D-alanylation patterns, it has been difficult to determine the precise antigenic elements

at the molecular level using isolated TAs.[7] Synthetic chemistry can provide instead well-defined structures to establish structure-immunogenicity relationship,[8] and several groups have reported on strategies to assemble both LTA and WTA fragments (See Chapter1).[9] Here attention was focused on glucosyl substituted fragments showing how antibody-TA interaction can be influenced not only for the position of the carbohydrate appendage but also by the stereochemistry of the glycerol unit. The synthetic route towards the pivotal glucosyl-glycerol building block has been improved by employing a recent methodology developed by Wang *et al.* for the construction of 1,2 cis glycosidic linkage.[10] Interestingly the glycosylation outcome was different upon the nature of protecting groups and stereogenic center of the glycerol acceptor.

RESULTS AND DISCUSSION

Different approaches have been described to assemble LTA fragments with well-defined glycosylation patterns. These fragments were equipped with a linker to attach them to either carrier proteins, fluorescent labels or affinity tags as well as microarray surfaces (See Chapter 3).[11] The linker previously was attached to the side of the oligomers, formally generating *sn*-Gro-3-P LTAs. From the pool of synthetic LTA oligomers, a glucosylated fragment was selected as a lead antigen, and this structure, **WH7** (See Figure 2A), was attached to a carrier protein (bovine serum albumin, BSA) to provide a model TA-conjugate vaccine.[11a, 12] Realizing that the chirality of the GroP chains may play a role in the interaction with antibodies, the generation of a set of glucosylated *sn*-Gro-1-P LTA-hexamers **1**-**6** is here described, differing in the position of the α-glucose substituent (Figure 2B).

Figure 2: *A) Lead compound **WH7**; B) The new set of TA hexamers and the building blocks used for their synthesis.*

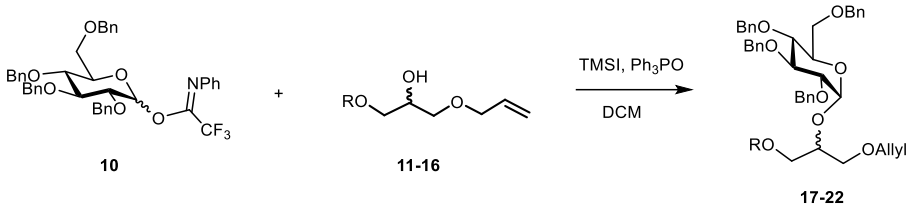


The required LTA-hexamers were assembled using phosphoramidite building blocks **7** and **8** and linker **9** (Figure 2B). The glycerol phosphoramidite building blocks **7** and **8** carry a base labile cyanoethyl protecting group (OCE) and a temporary dimethoxytrityl (DMTr) protecting group to enable the assemble of the target TA hexamers using well-established and highly efficient nucleic acid chemistry.[13-16] The remaining hydroxyl are all substituted with benzyl type protecting group to facilitate final deprotection via hydrogenolysis. While synthesis of compounds **8** and **9** were already optimized previously in our group, attention was focused on the synthesis of building block **7**. The crucial step in the synthesis is the introduction of the 1,2-cis glycosidic linkage. To deliver the desired α-glucosyl glycerol intermediate with good stereoselectivity, previously a glucosyl imidate donor building block carrying a bulky fluorenylmethoxycarbonyl protecting group at the C-6 position was employed.[12] The use of a glucosyl donor, carrying solely benzyl ether protecting groups, would reduce instead the number of required protecting group manipulations.

Among the several strategies for the formation of the 1,2-cis glycosidic linkages, the use of an additive-mediated glycosylation was explored to assemble compound **7**. Recently it has been described that a combination of trimethylsilyl iodide (TMSI) and an excess of triphenylphosphine oxide (Ph_3PO) can be used to glycosylate nucleophilic alcohols with a perbenzylated glucosyl imidate donor in a highly stereoselective manner.[10] This strategy was applied here in the coupling of donor **10** and glycerol acceptor **11**, providing compound **17** in 72% yield. Unfortunately, the stereoselectivity was relatively poor (see

Table 1, entry 1, α/β = 1.3/1). We therefore explored the use of acceptor **12** having the same protecting groups but opposite chirality. As shown in entry 2, the stereoselectivity significantly improved, indicating double stereodifferentiation[17] to play an important role in the union of donor **10** and acceptor **11/12**. This finding is quite unexpected as the acceptor used is relatively flexible and small (as compared to other carbohydrate acceptors, for which this phenomenon has been observed). Upon scale up of the reaction, the yield of the glycosylation dropped to 45%, because of loss of the silyl group, and therefore different protecting groups at this position were probed. Since the stereochemistry of the glycerol acceptor had a strong impact on the stereoselectivity of the glycosylation reactions, we examined both enantiomers of the glycerol acceptor bearing either a para-methoxybenzyl (PMB) ether or a benzoyl (Bz) ester (**13-16**). The results of the glycosylations are summarized in Table 1, showing that the stereoselectivity is actually affected by both chirality and type of substituent. In the case of PMB protecting groups (entry 3 and 4) no double differentiation was observed and the β-by product was detected by ^{1}H-NMR as minor impurity. When the protecting group was replaced with a benzoate ester (entry 5 and 6), the stereoselectivity was good with acceptor **16** (6:1), while no traces of the β anomer were detected in the case of acceptor **15**. The desired α-product (**21**) could be isolated in 68% yield and by extending the reaction time (36h) the yield was further improved to 86%, which was also reproducible on a large scale (up to 15 mmol, Table 1, entry 7).

Table 1: *Glycosylations of donor **10** and glycerol acceptors **11-16**.*[a]



Entry	R	OH	Acc.	Prod.	Yield	α : β
1	TBDPS	OH	**11**	**17**	72%	1.3:1
2	TBDPS	OH	**12**	**18**	68%	>10:1
3	PMB	OH	**13**	**19**	65%	>10:1
4	PMB	OH	**14**	**20**	66%	>10:1
5	Bz	OH	**15**	**21**	68%	>10:1
6	Bz	OH	**16**	**22**[18]	70%	6:1
7[b]	Bz	OH	**15**	**21**	86%[b]	>10:1

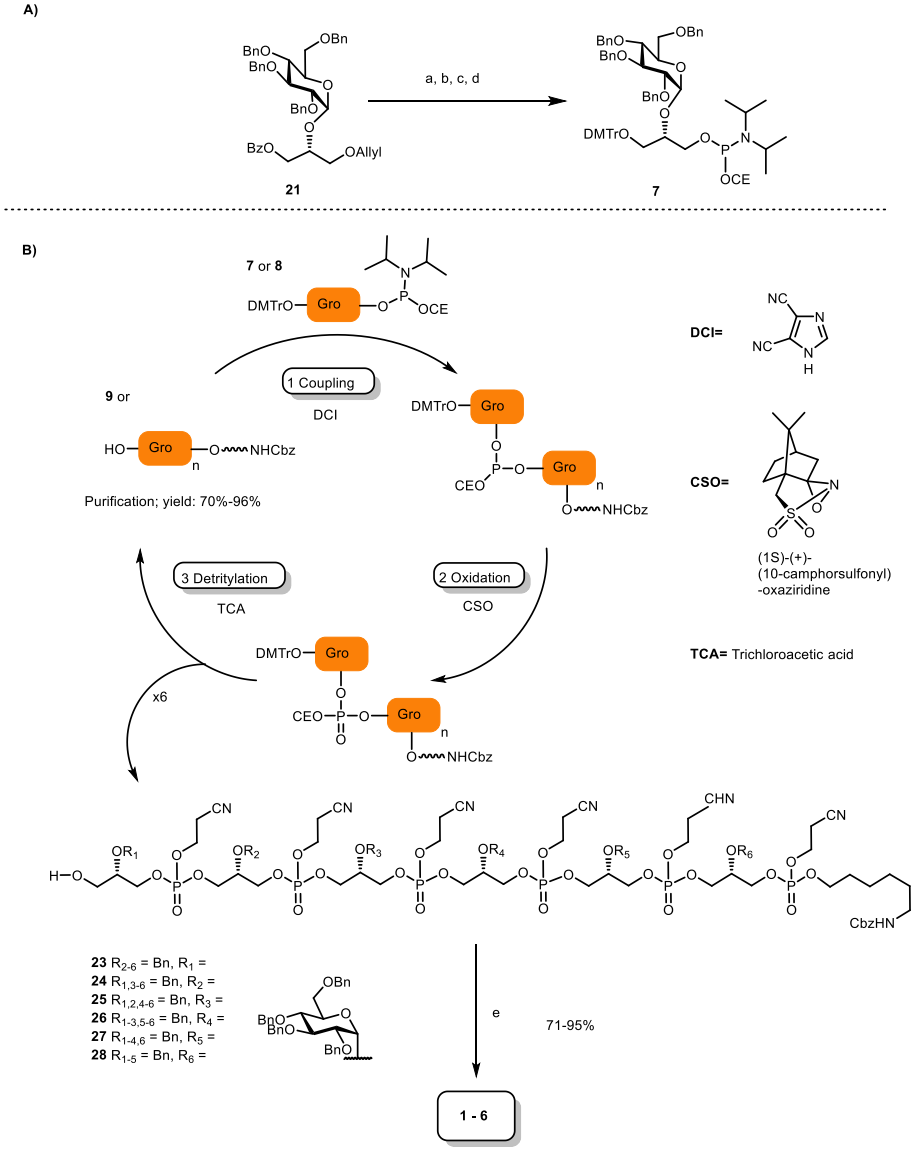
[a] Donor (1 eq), acceptor (0.7 eq), TMSI (1 eq), Ph_3PO (6 eq), DCM (0.1 M), r.t., 24 h.

[b] Reaction time 36h (15 mmol scale)

Next compound **21** was transformed into the required building block phosphoramidite **7** as shown in Scheme 1A. Briefly, the benzoate ester in **21** was exchanged for the required DMTr-ether, after which the allyl ether was removed and the cyanoethyl-protected phosphoramidite installed. With building block **7**, **8** and **9** in hand, the assembly of the

GroP hexamers was performed using repetitive coupling cycles in solution (Scheme 1B). The alcohols, *i.e.* alcohol spacer **9** or the oligomer intermediates, were coupled with phosphoramidite building block **7** or **8** using DCI (4,5-Dicyanoimidazole) as activating agent, followed by CSO [(1S)-(+)-(10-camphorsulfonyl)-oxaziridine] mediated oxidation of the so-formed phosphite triester. After aqueous work up, the DMTr was removed under mild acidic conditions (0.18 M trichloroacetic acid in DCM). The generated alcohol was then purified and used for the subsequent coupling. All coupling-deprotection cycles proceeded uneventfully delivering the elongated structures in 60-96% yield. After construction of the fully protected hexamers **23**-**28**, they were deprotected by first removing the cyanoethyl protecting groups under basic conditions, followed by Pd black catalyzed hydrogenolysis of all benzyl groups and the Cbz carbamate.

Scheme 1: *A) Synthesis of building block **7**. B) Assembly of hexamers **1**-**6***

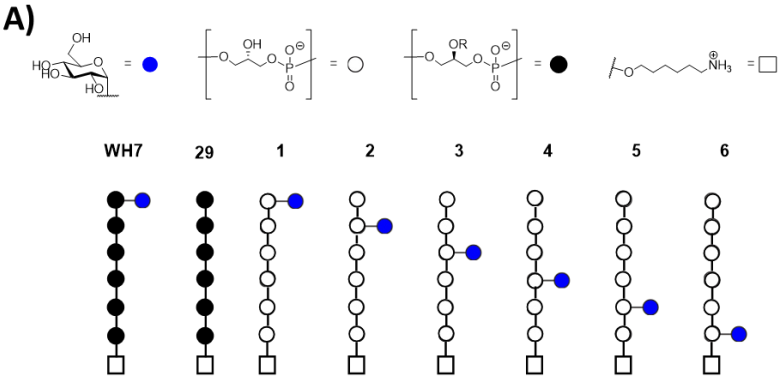


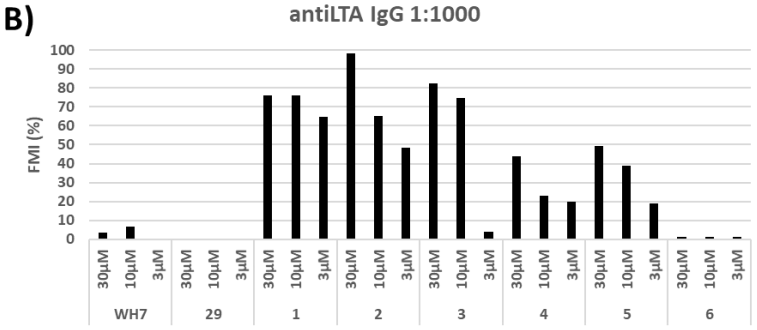
Reagents and conditions: *a) $Na_{(s)}$, MeOH, quant.; b) DMTrCl, TEA, DCM, 88%; c) (i) $Ir(COD)(PPh_2Me)_2PF_6$, H_2, THF; (ii) $NaHCO_{3(aq)}$, I_2, THF, 92%; d) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, TEA, DCM, 70%; e) (i) NH_4OH, H_2O, Dioxane; (ii) Pd black, H_2, H_2O, AcOH.*

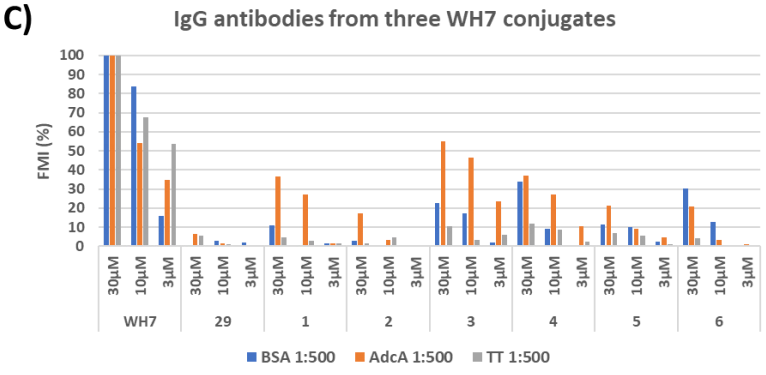
After the generation of the target hexamers, anti-LTA antibodies binding was evaluated using the newly generated library of sn-1-GroP. Chapter 3 dealt with the development of a TA-microarray, which allowed the screening of a library of synthetic TA-fragments for binding with mono- or polyclonal antibodies raised

against isolated LTA from *E. faecalis* **12030** or **WH7** based glycoconjugates vaccine.[11a, 18, 19] It was shown that sera obtained by immunization with native LTA from *E. faecalis* **12030**[20] showed preferential binding to glycosylated TA-fragments. The serum raised against the WH7-BSA glycoconjugate specifically recognized TA-fragments encompassing the **WH7** structure.[11b]
Thus, the six glucosyl hexamers **1**-**6**, the lead antigen **WH7** and an unsubstituted hexamer (**29**, Figure 3A), previously generated,[16] were immobilized on epoxy-silane functionalized glass-slides at three different concentrations (30 μM, 10 μM and 3 μM). The microarrays were then used to probe binding of the serum raised against the native LTA from *E. faecalis* **12030** (Figure 3B) and different **WH7**-conjugate forms as shown in Figure 3C: BSA (blue), AdcA (orange) and TT (grey). IgG binding was visualized using a fluorescently labelled (DyLight550) goat anti-Rabbit IgG antibody. In order to compare the relative signal towards the synthetic fragments among the polyclonal sera, the average of three datapoints from the fluorescence read-out is normalized to the highest peak value (Compound **2** at 30 μM for anti-LTA and **WH7** at 30 μM for sera against the synthetic conjugates). It becomes immediately apparent that IgG binding is influenced not only by the presence of the glucose substituent and its position, but also by the stereochemistry of the Gro-P backbone. The anti-LTA serum did not recognize the bare *sn*-Gro-3-P-backbone nor the **WH7** antigen. In contrast, it bound well to the *sn*-Gro-1-P-hexamers bearing an α-glucosyl moiety. The antibodies seem to show a slightly better binding to fragments that display the glucosyl moiety further away from the linker. Perhaps the display of the glycosylated antigen close to the microarray surface prohibits binding of the antibody. The IgG antibodies present in the sera raised against WH7-conjugates strongly recognized the sn-Gro-3-P-antigen **WH7**, while the signal is significantly attenuated for its sn-Gro-1-P-counterpart **1**, as well as for the other sn-Gro-1-P-hexamers. These results clearly reveal that the stereochemistry of the LTA GroP-backbone is a crucial determinant for antibody binding. From the array results it can be concluded that glycosylated GroP-fragments represent important natural epitopes and anti-LTA antibodies can discriminate between glycosylated *sn*-1 and *sn*-3-glycerol fragments. This exquisite recognition implies that the position of the linker in the synthetic antigens is an important element in the design and construction of synthetic LTA-conjugate vaccines. Also, the position of the glucose appendage plays a major role in recognition by the antibodies, which need sufficient space for binding. The results highlight that a very specific antibody response can be elicited using conjugate vaccines carrying single well-defined synthetic LTA-fragment epitopes.

Figure 3: *A) Overview of the TA-fragments tested; B) IgG binding in rabbit serum raised against native LTA from E. faecalis* ***12030*** *(1:1000 dilution); C) IgG binding in rabbit serum raised against WH7-BSA (blue), WH7-AdcA (orange), WH7-TT (grey). FMI (%): median fluorescent intensity normalized to the highest peak.*







CONCLUSION

In conclusion, the synthesis of a new set of glucosylated GroP-LTA-fragments is here reported, featuring a *sn*-Gro-1-P backbone with an α-glucosyl substituent at different positions along the chain. The synthesis of the pivotal building block **7** was achieved by employing an additive-mediated glycosylation strategy. The stereochemistry of the glycerol acceptor proved to be important for the stereochemistry of the glycosylation reaction linking the glucose moiety to the glycerol alcohol. Evaluation of the set of glucosylated *sn*-Gro-1-P hexamers alongside an unsubstituted *sn*-Gro-3-P LTA hexamer and a glucosylated *sn*-Gro-3-P hexamer (**WH7**) for interactions with anti-LTA antibodies showed that the stereochemistry of the Gro-P backbone plays a decisive role. The position of the α-glucosyl substituent also influenced binding of the antibodies. In the design of conjugate vaccines or diagnostic tools using synthetic TA-fragments, it is therefore important to position the linker connecting the TA fragments to its carrier at the site of the fragment that mimics the natural linkage to the bacterial cell wall.

EXPERIMENTAL SECTION

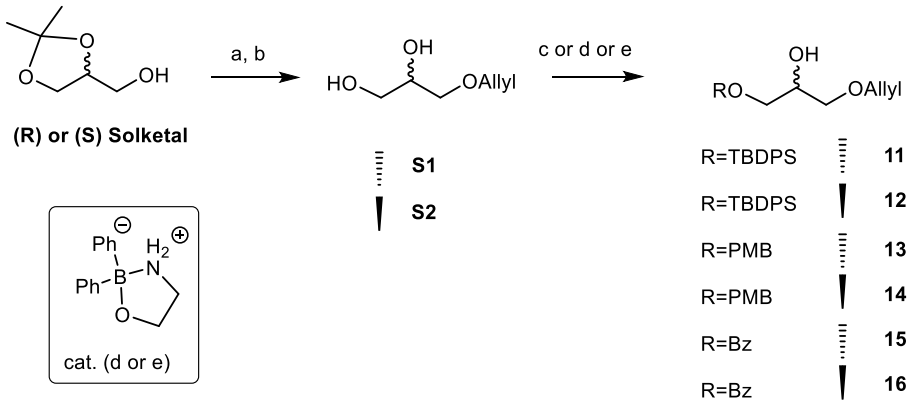
General

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 respectively). ^{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 acceptors 11-16

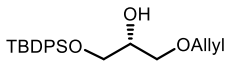
Scheme 2: *Synthetic strategy for synthesis of acceptors* ***11-16****.*



Reagents and conditions: a) AllylBr, NaH, DMF 96%; b) AcOH, H_2O, 50 °C, 300mbar, quant; c) TBDPSCl, Imidazole, DMF, 82% (**11**), 80% (**12**); d) cat., PMBCl, KI, K_2CO_3, ACN, 60 °C, quant (**13**), 96% (**14**); e) cat., BzCl, DIPEA, CAN, 98% (**15**), quant. (**16**).

Note: for experimental procedure and data analysis of steps (a) and (b) see J. Shin, D. H. Thompson, *JOC*, **2003**, 68, 17, 6760-6766

(S)-1-*O*-allyl-3-*O*-(*tert*-butyldiphenylsilyl)-*sn*-glycerol (11)

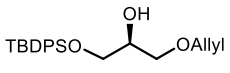


Diol **S1** (0.86 mmol) was diluted in DMF (8.6 ml, 0.1 M) and Imidazole (1 mmol, 1.15 eq) and TBDPSCl (0.86 mmol, 1 eq) were added. After two hours stirring at room temperature, TLC analysis (DCM:MeOH, 95:5) showed complete conversion of the starting material. The reaction mixture was diluted with Et_2O (10 mL) and washed with H_2O (10mL x 3). The aqueous phase was reextracted with Et_2O and the combined organic layers were washed once with brine, dried over $Na_2S_2O_4$, filtered and concentrated *in vacuo*. Compound **11** was isolated by column chromatography (Pentane:EtOAc, 9:1; R_f: 0.31) as transparent oil in 82% yield (0.71 mmol).

^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.70-7.62 (4H, H_{arom}, m), 7.47-7.35 (6H, H_{arom}, m), 5.95-5.82 (1H, H_{allyl}, m), 5.29-5.15 (2H, H_{2_allyl}, m), 4.03-3.97 (2H, CH_{2_allyl}, m), 3.95-3.86 (1H, $CH_{glycerol}$,

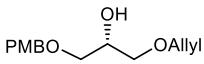
m), 3.71 (2H, $CH_{2\_glycerol}$, J_{CH2-CH}=5.4 Hz, d), 3.58-3.44 (2H, $CH_{2\_glycerol,}$ m), 2.49 (1H, OH, J_{OH-CH}=5.1Hz, d), 1.06 (9H, tBu, s).
^{13}C-NMR (101 MHz, $CDCl_3$), δ: 135.7 (CH_{arom}), 134.7 (CH_{allyl}), 133.1 (C_q), 129.9 (CH_{arom}), 127.9 (CH_{arom}), 117.3 (CH_{allyl}), 72.5 (CH_{2_allyl}), 71.0 ($CH_{2\_glycerol}$), 70.9 ($CH_{glycerol}$), 64.9 ($CH_{2\_glycerol}$), 27.0 (CH_{3_tBu}), 18.9 (C_{q_tBu}).
$([\alpha]_D^{20}$($CHCl_3$): -4.1
HRMS: $C_{22}H_{30}O_3Si$ + Na^+ required 359.1856, found 359.1901.

(R)-1-*O*-(*tert*-butyldiphenylsilyl)-3-*O*-allyl-*sn*-glycerol (12)



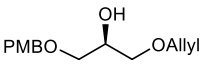
Starting with diol **S2** (1.00 mmol), compound **12** was obtained following the procedure described for **11** in 80% yield (0.80 mmol).
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.70-7.62 (4H, H_{arom}, m), 7.47-7.35 (6H, H_{arom}, m), 5.95-5.82 (1H, H_{allyl}, m), 5.29-5.15 (2H, 2 x H_{allyl}, m), 4.03-3.97 (2H, CH_{2_allyl}, m), 3.95-3.86 (1H, $CH_{glycerol}$, m), 3.71 (2H, $CH_{2\_glycerol}$, J_{CH2-CH}=5.4 Hz, d), 3.58-3.44 (2H, $CH_{2glycerol,}$ m), 2.49 (1H, OH, J_{OH-CH}=5.1Hz, d), 1.06 (9H, tBu, s).
^{13}C-NMR (101 MHz, $CDCl_3$), δ: 135.7 (CH_{arom}), 134.7 (CH_{allyl}), 133.1 (C_q), 129.9 (CH_{arom}), 127.9 (CH_{arom}), 117.3 (CH_{allyl}), 72.5 (CH_{2_allyl}), 71.0 ($CH_{2\_glycerol}$), 70.9 ($CH_{glycerol}$), 64.9 ($CH_{2\_glycerol}$), 27.0 (CH_{3_tBu}), 18.9 (C_{q_tBu})
$[\alpha]_D^{20}$($CHCl_3$): +3.5
HRMS: $C_{22}H_{30}O_3Si$ + Na^+ required 359.1856, found 359.1901.

(R)-1-*O*-allyl-3-*O*-(4-methoxybenzyl)-*sn*-glycerol (13)



Diol **S1** (1.00 mmol) was coevaporated three times with toluene and dissolved under inert atmosphere in dry ACN (2.5 mL, 0.4 M) and the flask was wrapped in aluminium foil. After 10 minutes stirring, PMBCl (1.10 mmol, 1.1 eq) was added followed by K_2CO_3 (1.10 mmol, 1.1 eq) and KI (1 mmol, 1 eq). The reaction was heated to 60 °C and after stirring overnight TLC analysis (DCM:MeOH; 95:5) showed complete consumption of starting material. The reaction mixture was cooled to r.t., diluted with EtOAc and washed with H_2O. The water layer was extract with EtOAc and the combined organic layers were washed with Brine, dried over MgSO4 and concentrated *in vacuo*. The resulting crude was purified by column chromatography (8:2→7:3 Pentane:EtOAc) yielding **13** as a colorless oil in quantitative yield (1.00 mmol).
TLC analysis: R_f= 0.35 (Pentane:EtOAc; 7:3)
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.28-7.23 (2H, H_{arom}, m), 6.91-6.86 (2H, H_{arom}, m), 5.96-5.84 (1H, H_{allyl}, m), 5.31-5.15 (2H, H_{2_allyl}, m), 4.49 (2H, CH_{2_PMB}, s), 4.04-3.95 (3H, CH_{2_allyl}, $CH_{glycerol}$, m), 3.80 (2H, CH_{3_OMe}, s), 3.57-3.43 (4H, $CH_{2\_glycerol}$, m), 2.46 (1H, OH, J_{OH-CH} =4.2 Hz, d).
^{13}C-NMR(101 MHz, $CDCl_3$), δ: 134.5 (CH_{allyl}), 130.1 (C_q), 129.4 (CH_{arom}), 117.3 (CH_{allyl}), 113.9 (CH_{arom}), 73.1 (CH_{2_PMB}), 72.3 (CH_{2_allyl}), 71.3 ($CH_{2\_glycerol}$), 71.04 ($CH_{2\_glycerol}$), 69.6 ($CH_{2\_glycerol}$), 55.3 (CH_{3_OMe}).
$[\alpha]_D^{20}$($CHCl_3$): -7.1
HRMS: $C_{14}H_{20}O_4$ + Na^+ required 275.1254, found 275.1259

(S)-1-*O*-(4-methoxybenzyl)-3-*O*-allyl-*sn*-glycerol (14)



Starting from diol **S2** (1.00), compound **14** was obtained as colorless oil in 96% yield (0.96 mmol) following the procedure described for compound **13**.

TLC analysis: R_f= 0.35 (Pentane:EtOAc; 7:3)

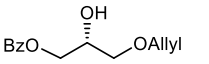
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.28-7.23 (2H, H_{arom}, m), 6.91-6.86 (2H, H_{arom}, m), 5.96-5.84 (1H, H_{allyl}, m), 5.31-5.15 (2H, 2 x H_{allyl}, m), 4.49 (2H, CH_{2_PMB}, s), 4.04-3.95 (3H, CH_{2_allyl}, $CH_{glycerol}$, m), 3.80 (2H, CH_{3_OMe}, s), 3.57-3.43 (4H, $CH_{2\_glycerol}$, m), 2.46 (1H, OH, J=4.2 Hz, d).

^{13}C-NMR(101 MHz, $CDCl_3$), δ: 134.5 (CH_{allyl}), 130.1 (C_q), 129.4 (CH_{arom}), 117.3 (CH_{allyl}), 113.9 (CH_{arom}), 73.1 (CH_{2_PMB}), 72.3 (CH_{2_allyl}), 71.3 ($CH_{2\_glycerol}$), 71.04 ($CH_{2\_glycerol}$), 69.6 ($CH_{2\_glycerol}$), 55.3 (CH_{3_OMe}).

$[\alpha]_D^{20}$($CHCl_3$): +7.5

HRMS: $C_{14}H_{20}O_4$ + Na^+ required 275.1254, found 275.1259

(R)-1-*O*-allyl-3-*O*-benzoyl-*sn*-glycerol (15)



Diol **S1** (10 mmol) was coevaporated with toluene three times and dissolved under inert atmosphere in dry ACN (25 mL, 0.4M). The flask was wrapped in aluminium foil and after ten minutes stirring, BzCl (11 mmol, 1.1 eq), DiPEA (12 mmol, 1.2 eq) and 2-Aminoethyl diphenylborinate (0.1 mmol, 0.01 eq) were subsequently added. The reaction was left to stir at room temperature and after 2h TLC analysis (DCM:MeOH; 95:5) showed complete consumption of starting material. The reaction mixture was diluted with EtOAc and washed with H_2O. The water layer was reextracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The resulting crude was purified by column chromatography (85:15→7:3, pentane:EtOAc) yielding **15** in 98% yield (9.8 mmol).

TLC analysis: R_f= 0.35 (Pentane:EtOAc; 75:25)

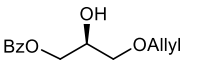
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 8.09-8.03 (2H, H_{arom}, m), 7.61-7.55 (1H, H_{arom}, m), 7.49-7.41 (2H, H_{arom}, m), 5.97-5.85 (1H, H_{allyl}, m), 5.33-5.18 (2H, H_{2_allyl}, m), 4.48-4.36 (2H, $CH_{2\_glycerol}$, m), 4.22-4.11 (1H, $CH_{glycerol}$, m), 4.08-4.03 (2H, CH_{2_allyl}, m), 3.65-3.53 (2H, $CH_{2\_glycerol}$, m), 2.64-2.56 (1H, OH, bs).

^{13}C-NMR(101 MHz, $CDCl_3$), δ: 166.7 (C_q), 134.2 (CH_{allyl}), 133.2 (CH_{arom}), 129.9 (C_q), 129.7 (CH_{arom}), 128.4 (CH_{arom}), 117.6 (CH_{allyl}), 72.5 (CH_{2_allyl}), 710.9 ($CH_{2\_glycerol}$), 69.0 ($CH_{glycerol}$), 66.0 ($CH_{2\_glycerol}$).

$[\alpha]_D^{20}$ ($CHCl_3$) : -5.6

HRMS:$C_{13}H_{16}O_4$ + Na^+ required 259.0941, found 259.1002

(S)-1-*O*-benzoyl-3-*O*-allyl-*sn*-glycerol (16)



Starting from diol **S2** (1 mmol), compound **16** was obtained as colourless oil in quantitative yield (1.00 mmol), following the procedure described for compound **15**.

TLC analysis: R_f= 0.35 (Pentane:EtOAc; 75:25)

^{1}H-NMR (400 MHz, $CDCl_3$), δ: 8.09-8.03 (2H, H_{arom}, m), 7.61-7.55 (1H, H_{arom}, m), 7.49-7.41 (2H, H_{arom}, m), 5.97-5.85 (1H, H_{allyl}, m), 5.33-5.18 (2H, 2 x H_{allyl}, m), 4.48-4.36 (2H,

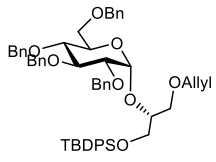
$CH_{2glycerol,}$ m), 4.22-4.11 (1H, $CH_{glycerol}$, m), 4.08-4.03 (2H, CH_{2_allyl}, m), 3.65-3.53 (2H, $CH_{2\_glycerol}$, m), 2.64-2.56 (1H, OH, bs).
^{13}C-NMR(101 MHz, $CDCl_3$), δ: 166.7 (C_q), 134.2 (CH_{allyl}), 133.2 (CH_{arom}), 129.9 (C_{q_arom}), 129.7 (CH_{arom}), 128.4 (CH_{arom}), 117.6 (CH_{allyl}), 72.5 (CH_{2_allyl}), 710.9 ($CH_{2\_glycerol}$), 69.0 ($CH_{glycerol}$), 66.0 ($CH_{2\_glycerol}$).
$[\alpha]_D^{20}$($CHCl_3$): +4.7
HRMS:$C_{13}H_{16}O_4$ + Na^+ required 259.0941, found 259.0993

Glycosylation using TMSI/Ph_3PO.

General procedure

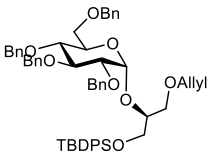
Donor (1 eq) and acceptor (0.75 eq) were co-evaporated three times with toluene. Under argon atmosphere, they were dissolved in dry DCM (0.1M) and after 10 minutes stirring Ph_3PO (6 eq) was added, followed by slow addition of TMSI (1 eq). The reaction mixture was allowed to stir at r.t. overnight. The reaction mixture was diluted with DCM, washed with $Na_2S_2O_3$, H_2O and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude was subjected to size exclusion gel chromatography (DCM:MeOH=1:1, for purification of the final product obtained as mixture of anomers (unless otherwise stated). The ratio α/β was calculated by ^{1}H-NMR.

(S)-1-*O*-allyl-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*-(*ter*t-butyldiphenylsilyl-*sn*-glycerol (17)



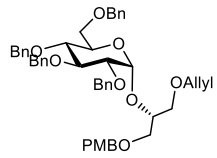
On a scale of 0.10 mmol of donor **10**, following the general procedure, compound **17** was obtained in 72% yield (0.072 mmol) as colourless syrup in a α/β mixture (1.5:1).
TLC analysis: R_f= 0.48 (Pentane:EtOAc; 9:1)
^{1}H-NMR (400 MHz, $CDCl_3$), δ(α): 7.68-7.64 (4H, H_{arom}, m), 7.47-7.07 (26H, H_{arom}, m), 5.94-5.81 (1H, H_{allyl}, m), 5.33-5.07 (3H, 2 x H_{allyl}, H_1 m), 5.02-4.90 (1H, CHH_{Bn}, m), 4.84-4.62 (2H, 3 x CHH_{Bn}, m), 4.68-4.43 (4H, 4 x CHH_{Bn}, m), 4.12-4.05 (1H, H_5, m), 4.01-3.86 (4H, H_3, $CH_{glycerol}$, CH_{2_allyl}, m), 3.82-3.50 (8H, 2 x $CH_{2\_glycerol}$, 2 x H_6, H_4, H_2, m), 1.08 (9H, tBu, s).
^{13}C-NMR (101 MHz, $CDCl_3$), δ(α): 139.1, 138.6, 138.2 (C_q), 135.7, 135.7 (CH_{arom}), 134.9 (CH_{allyl}), 133.1 (C_q), 129.9, 128.5, 128.1, 127.9, 127.8, 127.7 (CH_{arom}), 116.7 (CH_{2_allyl}), 96.2 (C_1), 82.1 (C_3), 79.9 (C_2), 77.8 (C_4), 77.3 ($CH_{glycerol}$), 75.8, 75.1, 73.6, 73.0 (CH_{2_Bn}), 72.2 (CH_{2_Allyl}), 70.4 (C_6), 70.2 (C_5), 68.8, 63.2 ($CH_{2\_glycerol}$), 27.0 (CH_{3_tBu}), 19.4 (C_q).
$[\alpha]_D^{20}$($CHCl_3$): +26.3
HRMS: $C_{56}H_{64}O_8Si$ + Na^+ required 915.4263, found 915.4265

(R)-1-*O*-(*tert*-butyldiphenylsilyl)-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*-allyl-*sn*-glycerol (18)



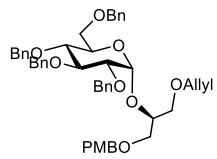
On a scale of 0.1 mmol of donor **10**, following the general procedure described above, compound **18** was isolated as colourless oil in a mixture of α/β anomers (>10:1) in 68% yield (0.068 mmol).
Analytical data in accordance with the one reported in: W. F. J. Hogendorf, L. J. van den Bos, H. S. Overkleeft, J. D. C. Codee, G. A. van der Marel, *Bioorg, Med. Chem.,* **2010**, 18, 3668-3678.

(R)-1-*O*-allyl-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*-(4-methoxybenzyl)-*sn*-glycerol (19)



On a scale of 0.1 mmol of donor **10**, following the general procedure, compound **19** was obtained in 65% yield (0.065 mmol) as colourless syrup in a α/β mixture (>10:1).
TLC analysis: R_f= 0.34 (Pentane:EtOAc; 8:2)
^{1}H-NMR (101 MHz, $CDCl_3$), δ(α): 7.42-7.22 (20H, H_{arom}, m), 7.203-7.12 (2H, H_{arom}, m), 6.90-6.84 (2H, H_{arom}, m), 5.94-5.81 (1H, H_{allyl}, m), 5.30-5.12 (3H, 2 x H_{allyl}, H_1, m), 5.01 (1H, CH*H*$_{Bn}$, J=10.9 Hz, d), 4.88-4.78 (2H, 2 x CH*H*$_{Bn}$, m), 4.71-4.61 (3H, 2 x CH*H*$_{Bn}$, m) 4.54-4.45 (4H, 4 x CH*H*$_{Bn}$, m), 4.17-4.08 (1H, $CH_{glycerol}$, m), 4.07-3.93 (4H, H_5, H_3, CH_{2_allyl}, m), 3.81 (3H, CH_{3_OMe}, s), 3.76 (1H, CH*H*$_{glycerol}$, $J_{CHH\text{-}CHH}$=10.7 Hz, $J_{CHH\text{-}CH}$=3.4 Hz, dd), 3.72-3.51 (7H, CH*H*$_{glycerol}$, $CH_{2\_glycerol}$, 2 x H_6, H_4, H_2, m).
^{13}C-NMR(400 MHz, $CDCl_3$), δ(α): 139.0, 138.5, 138.2, 138.0 (C_q), 134.7 (CH_{allyl}), 130.3 (C_q), 129.2, 128.3, 128.0 x 2, 127.9 x 2, 127.7, 127.6 x 2, 127.5 (CH_{arom}), 117.0 (CH_{2_allyl}), 96.2 (C_1), 81.9 (C_3), 79.5 (C_2), 77.7 (C_4), 75.7, 75.0 (CH_{2_Bn}), 74.7 ($CH_{glycerol}$), 73.5, 73.0, 72.3 (CH_{2_Bn}), 72.2 x 2 (CH_{2_Allyl}, CH_{2_Bn}), 70.4 ($CH_{2\_glycerol}$), 70.2 (C_5), 69.7 (C_6), 55.3 (CH_{3_OMe})
$[\alpha]_D^{20}$($CHCl_3$): +31.2
HRMS: $C_{48}H_{54}O_9$ + Na^+ required 9797.3660, found 797.3667

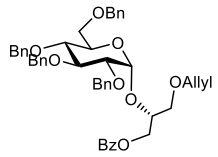
(S)-1-*O*-(4-methoxybenzyl)-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*-allyl-*sn*-glycerol (20)



On a scale of 0.1 mmol of donor **10**, following the general procedure, compound **20** was obtained in 66% (0.066 mmol) yield as colourless syrup in a α/β mixture (9:1).
TLC analysis: R_f= 0.34 (Pentane:EtOAc; 8:2)
^{1}H-NMR (400 MHz, $CDCl_3$), δ(α): 7.39-7.16 (20H, H_{arom}, m), 7.15-7.08 (2H, H_{arom}, m), 6.80-6.73 (2H, H_{arom}, m), 5.93-5.81 (1H, H_{allyl}, m), 5.29-5.11 (3H, 2 x H_{allyl}, H_1, m), 4.98 (1H, CH*H*$_{Bn}$, J=10.8 Hz, d), 4.84-4.77 (2H, 2 x CH*H*$_{Bn}$, m), 4.74 (1H, CH*H*$_{Bn}$, J=12.0 Hz, d), 4.69 (1H, CH*H*$_{Bn}$, J=12.0 Hz, d), 4.57 (1H, CH*H*$_{Bn}$, J=12.1 Hz, d), 4.48-4.34 (4H, 4 x CH*H*$_{Bn}$, m), 4.12-3.94 (5H, $CH_{glycerol}$, H_5, H_3, CH_{2_allyl}, m), 3.79-3.71 (4H, CH*H*$_{glycerol}$, CH_{3_OMe}, s), 3.67-3.51 (6H, 2 x CH*H*$_{glycerol}$, 2 x H_6, H_4, H_2, m), 3,45 (1H, CH*H*$_{glycerol}$, $J_{CHH\text{-}CHH}$=10.6 Hz, $J_{CHH\text{-}CH}$=2,1 Hz, dd),
^{13}C-NMR(101 MHz, $CDCl_3$), δ(α): 139.1, 138.7, 138.5, 138.2 (C_q), 134.8 (CH_{allyl}), 130.4 (C_q), 129.6, 128.5 x 3, 128.2, 128.1, 128.0, 127.8 x 2, 127.7, 127.6 (CH_{arom}), 117.1 (CH_{2_allyl}), 113.8 (CH_{arom}), 96.3 (C_1), 82.1 (C_3), 79.8 (C_2), 77.8 (C_4), 75.8, 75.1 (CH_{2_Bn}), 74.8

($CH_{glycerol}$), 73.6, 73.1, 72.6 (CH_{2_Bn}), 72.5 (CH_{2_Allyl}), 70.8, 70.3 ($CH_{2\_glycerol}$), 70.2 (C_5), 69.6 (C_6), 55.4 (CH_{3_OMe})
$[\alpha]_D^{20}$($CHCl_3$): +19.4
HRMS: $C_{48}H_{54}O_9$ + Na^+ required 797.3660, found 797.3664

(R)-1-*O*-allyl-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*-benzoyl-*sn*-glycerol (21)

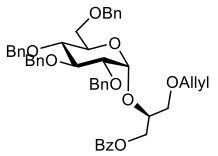


On a scale of 15 mmol of donor **10**, following the general procedure and leaving the reaction stirring for 3 days, compound **21** was obtained in 86% yield (12.9 mmol) as colourless syrup (no presence of β anomer was detected).

TLC analysis: R_f= 0.31 (Pentane:EtOAc; 8:2)

^{1}H-NMR (400 MHz, $CDCl_3$), δ(α): 8.05-8.00 (2H, H_{arom}, m), 7.57-7.52 (1H, H_{arom}, m), 7.43-7.23 (15H, H_{arom}, m), 7.18 (5H, H_{arom}, s), 7.15-7.10 (2H, H_{arom}, m), 5.90-5.79 (1H, H_{allyl}, m), 5.28-5.12 (3H, 2 x H_{allyl}, H_1, m), 4.95 (1H, CH*H*$_{Bn}$, J=10.8 Hz, d), 4.86-4.76 (2H, 2 x CH*H*$_{Bn}$, m), 4.65-4.58 (3H, 3 x CH*H*$_{Bn}$, m), 4.54 (1H, CH*H*$_{glycerol}$, $J_{CH\textit{H}\text{-}\textit{CH}H}$=10.8 Hz, $J_{CH\textit{H}\text{-}CH}$=4.03 Hz, dd), 4.50-4.40 (3H, 2 x CH*H*$_{Bn}$, CH*H*$_{glycerol}$, m*),* 4.28-4.19 (1H, $CH_{glycerol}$, m), 4.04-3.94 (4H, H_5, H_3, CH_{2_allyl}, m), 3.78-3.55 (6H, $CH_{2\_glycerol}$, 2 x H_6, H_4, H_2, m).
^{13}C-NMR(101 MHz, $CDCl_3$), δ(α): 166.5 (C_q), 139.0, 138.5, 138.1, 138.0 (C_q), 134.5 (CH_{allyl}), 133.2 (CH_{arom}), 130.0 (C_q), 129.8, 128.7, 128.6, 128.5, 128.4, 128.0 x 2, 127.9, 127.8 x 2, 127.7, 127.1 (CH_{arom}, 117.4 (CH_{2_allyl}), 96.4 (C_1), 82.0 (C_3), 79.8 (C_2), 77.7 (C_4), 75.7, 75.2 (CH_{2_Bn}), 73.9 ($CH_{glycerol}$), 73.7, 72.9 (CH_{2_Bn}), 72.4 (CH_{2_Allyl}), 70.6 (C_5), 70.0 (C_6), 68.6, 64.7 ($CH_{2\_glycerol}$).
$[\alpha]_D^{20}$($CHCl_3$): +22.4
HRMS: $C_{47}H_{50}O_9$ + Na^+ required 781.3347, found 781.3354

(S)-1-*O*-benzoyl-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*-allyl-*sn*-glycerol (22)



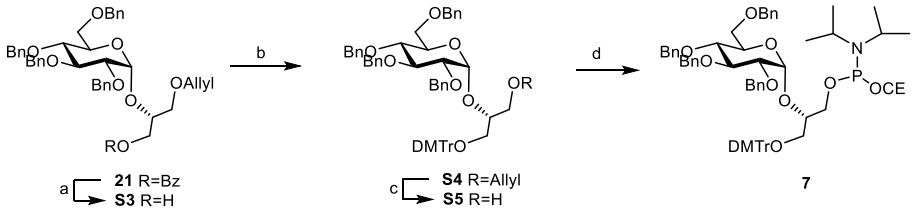
On a scale of 0.1 mmol of donor **10**, following the general procedure, compound **22** was obtained in 70% yield (0.070 mmol) as colourless syrup in a α/β mixture (6:1).

TLC analysis: R_f= 0.31 (Pentane:EtOAc; 8:2)

^{1}H-NMR (400 MHz, $CDCl_3$), δ(α): 8.02-7.97 (2H, H_{arom}, m), 7.53-7.47 (1H, H_{arom}, m), 7.39-7.17 (20H, H_{arom}, m), 7.11-7.03 (2H, H_{arom}, m), 5.94-5.81 (1H, H_{allyl}, m), 5.31-5.15 (3H, 2 x H_{allyl}, H_1, m), 4.97 (1H, CH*H*$_{Bn}$, J=10.8 Hz, d), 4.84-4.76 (2H, 2 x CH*H*$_{Bn}$, m), 4.73 (2H, CH_{2_Bn}, J=2.9 Hz, d) 4.58-4.49 (2H, CH*H*$_{Bn}$, CH*H*$_{glycerol}$, m), 4.44-4.34 (2H, CH*H*$_{Bn}$, CH*H*$_{glycerol}$, m), 4.28 (1H, CH*H*$_{Bn}$, J=12.1 Hz, d), 4.26-4.20 (1H, $CH_{glycerol}$, m), 4.05-3.94 (4H, H_5, H_3, CH_{2_allyl}, m), 3.71-3.53 (4H, 2 x H_6, H_4, H_2, m). 3.49 (1H, *CH*H$_{glycerol}$, $J_{CH\textit{H}\text{-}\textit{CH}H}$ =10.6 Hz, $J_{CH\textit{H}\text{-}CH}$=3.1 Hz, dd), 3.34 (1H, *CH*H$_{glycerol}$, $J_{CH\textit{H}\text{-}CH}$=2.1 Hz, dd).
^{13}C-NMR(101 MHz, $CDCl_3$), δ(α): 166.4 (C_q), 139.0, 138.5, 138.4, 137.9 (C_q), 134.5 (CH_{allyl}), 133.1 ()CH_{arom}), 130.0 (C_q), 129.8, 128.6 x 2, 128.5 x 2, 128.4, 128.2, 128.1, 128.0 x 2, 127.8, 127.7 x 2, (CH_{arom}), 117.4 (CH_{2_allyl}), 96.3 (C_1), 82.0 (C_3), 79.7 (C_2), 77.6 (C_4), 75.8, 75.1 (CH_{2_Bn}), 73.8 ($CH_{glycerol}$), 73.6, 72.8 (CH_{2_Bn}), 72.5 (CH_{2_Allyl}), 70.5 (C_5), 69.8 (C_6), 68.2, 65.2 ($CH_{2\_glycerol}$).
$[\alpha]_D^{20}$($CHCl_3$): +35.6

HRMS: $C_{47}H_{50}O_9$ + Na^+ requires 781.3347, found 781.3357

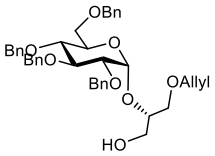
Synthesis of building block 7 from 21



*Scheme S1: Synthetic strategy towards compound **7**.* a) $Na_{(s)}$, MeOH, quant.; b) DMTrCl, TEA, DCM, 88%; c) (i) $Ir(COD)(PPh_2Me)_2PF_6$, H_2, THF; (ii) $NaHCO_3$(aq), I_2, THF, 92%; d) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, TEA, DCM, 70%.

(R)-1-*O*-allyl-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-*sn*-glycerol (S3)

Compound **21** (12 mmol) was dissolved in dry MeOH (60 mL, 0,2 M) and a piece of $Na_{(s)}$ was added. The reaction was stirred for 1 hour, until TLC analysis (Pentane:EtOAc, 8:2) showed complete consumption of the starting material. The reaction mixture was neutralized by addition of Amberlite IR-120 (H^+ form), filtered and concentrated *in vacuo*. The product was obtained quantitatively (12 mmol) and used directly in the subsequent step without further purification.



TLC analysis: R_f= 0.32 (Pentane:EtOAc; 7:3)

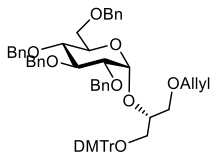
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.41-7.20 (18H, H_{arom}, m), 7.19-7.07 (2H, H_{arom}, m), 5.90-5.75 (1H, H_{allyl}, m), 5.22 (1H, CHH_{allyl}, J = 17.3, 1.7 Hz, dd), 5.14 (1H, CHH_{allyl}, J = 10.4, 1.8 Hz, dd), 4.98-4.89 (2H, H_1, CHH_{Bn}, m), 4.89-4.75 (3H, 3 x CHH_{Bn}, m), 4.67 (1H, CHH_{Bn}, J=11.6 Hz, d), 4.60 (1H, CHH_{Bn}, J=12.1 Hz, d), 4.52-4.41 (2H,2 x CHH_{Bn}, m), 4.05-3.87 (4H, H_3, H_5, $CH_{2\text{-Allyl}}$, m), 3.87-3.78 (1H, $CH_{glycerol}$, m), 3.77-3.38 (8H, 2 x H_6, H_4, H_2, 2 x $CH_{2\_glycerol}$ m), 3.17-3.04 (1H, OH, bs).

^{13}C-NMR(101 MHz, $CDCl_3$), δ: 138.8, 138.3, 138.0, 137.7 (C_q), 134.6 (CH_{allyl}), 128.7, 128.5 x 3, 128.4 x 2, 128.3, 128.2, 128.1 x 2, 128.0 x 2, 127.9, 127.8 x 2, 127.7 (CH_{arom}), 117.3 (CH_{2_allyl}), 98.8 (C_1), 82.4 (C_3), 79.9 (C_2, $CH_{glycerol}$), 77.9 (C_4), 75.7, 75.2, 74.2, 73.6 (CH_{2_Bn}), 72.4 (CH_{2_Allyl}), 70.8 (C_5), 70.3 (C_6), 68.5, 63.0 ($CH_{2\_glycerol}$).

$[\alpha]_D^{20}$($CHCl_3$): +26.7

HRMS: $C_{40}H_{46}O_8$ + H^+ required 655.3265, found 655.3271

(R)-1-*O*-allyl-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*(4,4'-dimethoxytrityl)-*sn*-glycerol (S4)



Compound **S3** (12 mmol) was dissolved in dry DCM (60mL, 0.2M) and under inert atmosphere Et_3N (18 mmol, 1.5eq) and DMTrCl (13.8 mmol, 1.15 eq) were added. The reaction mixture stirred for 3 hours until TLC analysis (Pentane:EtOAc:Et_3N, 7:3:0.1) showed complete consumption of starting material. The reaction was quenched by addition of MeOH (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 **S4** was isolated in 88% yield (10.6 mmol) after column chromatography (Pentane:EtOAc:Et_3N, 97:2:1→80:19:1).

TLC analysis: R_f= 0.31 (Pentane:EtOAc; 8:2)

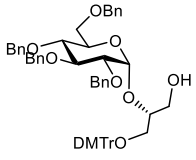
^{1}H-NMR (400 MHz, $CDCl_3$), δ: 7.47-7.38 (2H, H_{arom}, m), 7.37-7.04 (27H, H_{arom}, m), 6.84-6.73 (4H, H_{arom}, m), 5.90-5.73 (1H, H_{allyl}, m), 5.36-5.06 (3H, 2 x H_{allyl}, H_1, m), 4.95 (1H, CH*H*$_{Bn}$, J=10.7 Hz, d), 4.86-4.73 (2H, 2 x CH*H*$_{Bn}$, m), 4.64 (1H, CH*H*, J=12.0 Hz, d) 4.58-4.53 (2H, CH*H*$_{Bn}$, m), 4.51-4.41 (2H, 2 x CH*H*$_{Bn}$, m), 4.19-4.01 (2H, $CH_{glycerol}$, H_5, m), 4.01-3.86 (3H, H_3, CH_{2_allyl}, m), 3.81-3.43 (12H, 2 x CH_{3_OMe}, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$, m). 3.25 (2H, $CH_{2\_glycerol}$, $J_{CH\textit{H}-CH}$=5.7 Hz, dd).

^{13}C-NMR(101 MHz, CD_3CN), δ: 159.6, 146.2, 140.1, 139.8, 139.6, 139.5, 137.0 x 2 (C_q), 136.1 (CH_{allyl}), 131.0, 130.0, 129.3 x 2, 129.2 x 3, 129.0, 128.9 x 2, 128.8 x 4, 128.4 x 2, 127.8 (CH_{arom}), 118.3 (CH_{2_allyl}), 114.0 (CH_{arom}), 97.0 (C_1), 82.5 (C_3), 81.0 (C_2), 78.9 (C_4), 76.8 ($CH_{glycerol}$), 76.09, 75.5, 73.9, 72.9 (CH_{2_Bn}), 72.6 (CH_{2_Allyl}), 71.4 (C_5), 71.2 (C_6), 70.1, 64.5 ($CH_{2\_glycerol}$), 55.9 (2 x CH_{3_OMe}).

$[\alpha]_D^{20}$($CHCl_3$): +21.8

HRMS: $C_{61}H_{60}O_{10}$ + Na^+ required 979.4392, found 979.4401

(R)-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*(4,4'-dimethoxytrityl)-*sn*-glycerol (S5)



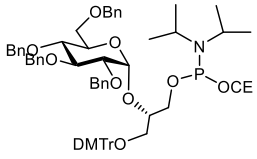
Compound **S4** (10.2 mmol) was dissolved in freshly distilled dry THF (68 mL, 0.15 M). After bubbling $Ar_{(g)}$ for 20 minutes, $Ir(COD)(PPh_2Me)PF_6$ (0,1 mmol, 0.01 eq) was added to the reaction mixture. $Ar_{(g)}$was bubbled for 10 minutes, followed by $H_{2(g)}$ purge for not more than 10 seconds, after which a change in the catalyst colour was observed from red to yellow. After 1 hour TLC analysis (Pentane:Toluene:EtOAc, 85:5:10) showed complete conversion of the starting material to the isomerized intermediate. The reaction mixture was diluted with THF (20 mL) and a sat. aq. solution of $NaHCO_3$ (20 mL) was added together with I_2 (15.9 mmol, 1.6 eq). TLC analysis showed complete consumption of the isomerized intermediate after 18 hours of stirring and the reaction mixture was diluted with EtOAc and washed with $NaS_2O_{3(sat)(aq)}$, $NaHCO_{3(sat.)(aq)}$, H_2O and brine. The organic layer was dried over $Na_2S_2O_4$, filtered and concentrated *in vacuo*. The desired product **S5** was isolated after purification with column chromatography (Pentane:EtOAc:Et_3N, 70:25:5) in 92% yield (9.4 mmol) as colourless syrup.

TLC analysis: R_f= 0.31 (Pentane:EtOAc; 8:2)

^{1}H-NMR (400 MHz, CD_3CN), δ: 7.50-7.44 (2H, H_{arom}, m), 7.38-7.13 (27H, H_{arom}, m), 6.84-6.74 (4H, H_{arom}, m), 5.17 (1H, H_1, J=3.6 Hz, d), 4.92 (1H, CH*H*$_{Bn}$, J=11.0 Hz, d), 4.83-4.74

(2H, 2 x CHH_{Bn}, m), 4.64-4.47 (5H, CHH_{Bn}, m), 4.05-3.98 (1H, H_5, m), 3.94-3.82 (2H, H_3, $CH_{glycerol}$, m), 3.75-3.63 (9H, 2 x CH_{3_OMe}, 2 x H_6, CH$H_{glycerol}$, m). 3.62-3.53 (1H, CH$H_{glycerol}$, m), 3.53-3.44 (2H, H_4, H_2, m), 3.25-3.13 (2H, $CH_{2\_glycerol}$, m), 3.04-2.97 (1H, OH, m).
^{13}C-NMR(101 MHz, CD_3CN), δ: 158.6, 145.3, 139.2, 138.7, 138.5 x 2, 136.1, 136.0 (C_q), 130.1 x 2, 128.4, 128.3 x 2, 128.1, 128.0, 127.9 x 2, 127.8, 127.7, 127.6 x 2, 127.5, 126.8 (CH_{arom}), 96.3 (C_1), 81.6 (C_3), 80.1 (C_2), 78.9 ($CH_{glycerol}$), 78.1 (C_4), 75.1, 74.7, 72.9, 72.0 (CH_{2_Bn}), 70.6 (C_5), 69.1 (C_6), 63.6, 62.5 ($CH_{2\_glycerol}$), 54.9 (2 x CH_3).
$[\alpha]_D^{20}$($CHCl_3$): +27.3
HRMS: $C_{58}H_{60}O_{10}$ + Na^+ required 939.4079, found 939.4090

(S)-1-*O*-([*N,N*-diisopropyl]-2-cyanoethyl-phosphoramidite)-2-*O*-(2,3,4,6-*O*-benzyl-α-D-glucopyranosyl)-3-*O*(4,4'-dimethoxytrityl)-*sn*-glycerol (7)



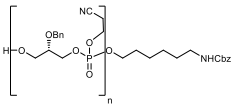
Compound **S5** (8.5 mmol) was dissolved in dry DCM (85 mL, 0.1 M) and Et_3N (12.75 mmol, 1.5 eq) was added. At 0 °C 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (10.2 mmol, 1.2 eq) was added and the reaction was left for 2 hours after which TLC analysis (Pentane:EtOAc:Et_3N, 7:3:0,1) showed complete 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 **7** in 70% (5.95 mmol) as a colourless oil.
Analytical data in accordance with the one reported in W. F. J. Hogendorf, L. J. van den Bos, H. S. Overkleeft, J. D. C. Codee, G. A. van der Marel, *Bioorg, Med. Chem.,* **2010**, 18, 3668-3678.

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 **7** or **8** (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.

List of intermediates from phoshoramidite couling



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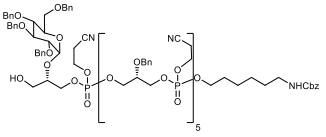
n=2, **S7**
n=3, **S8**
n=4, **S9**
n=5, **S10**



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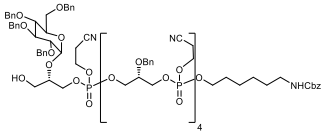
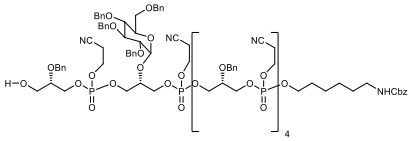


S11



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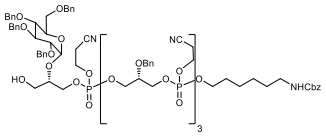
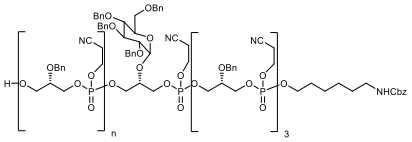


S12



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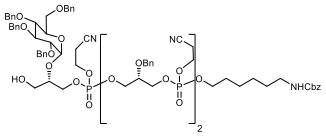
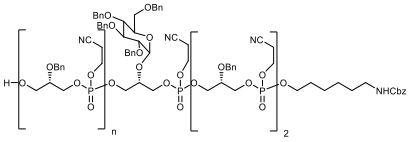
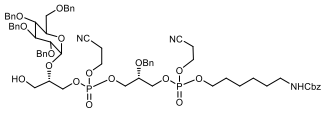
n=2, **25**



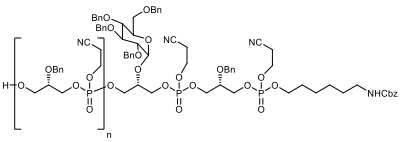
S14



n=1, **S15**
n=2, **S16**
n=3, **26**



S17



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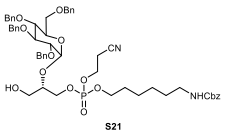
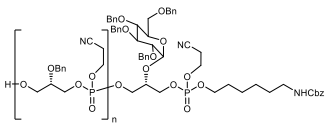
n=2, **S19**
n=3, **S20**
n=4, **27**

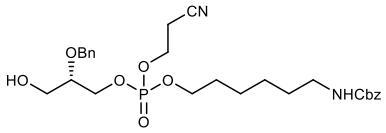


S21



n=1, **S22**
n=2, **S23**
n=3, **S24**
n=4, **S25**
n=5, **28**

(Protected) (GroP)-Spacer or Monomer S6



Alcohol spacer **9** (1.1 mmol) was coupled with phosphoramidite **8** (1.67 mmol, 1.5 eq) following the general procedure. Compound **S6** was obtained after column chromatography (DCM:Acetone, 7.5:2.5) in 90% yield (0.99 mmol).

TLC analysis, R_f:0.48 (DCM:Acetone, 7:3)

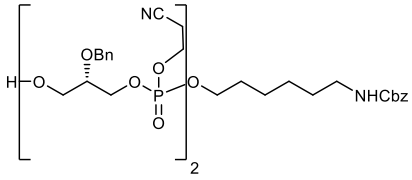
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.42-7.23 (10H, H_{arom}, m), 5.68-5.54 (1H, NH, b), 5.03 (2H, CH_{2_Cbz}, s), 4.63 (2H, CH_{2_Bn}, s), 4.25-3.97 (6H, CH_{2_OCE}, $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.71-3.54 (3H, $CH_{glycerol}$, $CH_{2\_glycerol}$, m), 3.07 (2H, $CH_{2\_Nspacer}$, J=6.6 Hz, q), 3.02-2.92 (1H, OH, b), 2.78-2.68 (2H, CH_{2_OCE}, m), 1.68-1.57 (2H, CH_{2_spacer}, m), 1.51-1.23 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.4, 139.6, 138.4 (C_q), 129.4, 129.2, 128.8, 128.7, 128.6, 128.5 (CH_{arom}), 118.6 (C_q), 79.2-79.1 ($CH_{glycerol}$), 72.4 (CH_{2_Bn}), 69.0 ($CH_{2\_Ospacer}$), 67.6-67.5 ($CH_{2\_glycerol}$), 66.7 (CH_{2_Cbz}), 63.2-63.1 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7, 30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.32, -1.29

HRMS: $C_{27}H_{37}N_2O_8P$ + H^+ required 549.2360, found 549.2361

(Protected) (GroP)$_2$-Spacer or Dimer S7



Alcohol **S6** (0.75 mmol) was coupled with phosphoramidite **8** (1.1 mmol, 1.5 eq) following the general procedure. Compound **S7** was obtained after column chromatography (DCM:Acetone, 6.5:3.5) in quantitative yield (0.75 mmol).

TLC analysis, R_f: 0.43 (DCM:Acetone, 6:4)

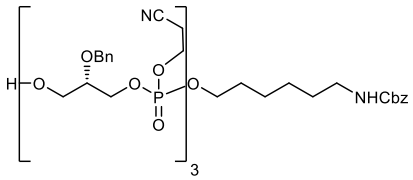
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.47-7.23 (15H, H_{arom}, m), 5.95-5.86 (1H, NH, b), 5.06 (2H, CH_{2_Cbz}, s), 4.71-4.58 (4H, CH_{2_Bn}, m), 4.34-3.98 (12H, 2 x CH_{2_OCE}, 3 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.93-3.81 (1H, $CH_{glycerol}$, m), 3.72-3.56 (3H, $CH_{glycerol}$, $CH_{2\_glycerol}$, m), 3.50-3.35 (1H, OH, b), 3.09 (2H, $CH_{2\_Nspacer}$, J=6.6 Hz, q), 2.77-2.67 (2H, 2 x CH_{2_OCE}, m), 1.71-1.55 (2H, CH_{2_spacer}, m), 1.52-1.22 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.6, 139.1 (C_q), 129.4, 129.3, 128.9, 128.8, 128.7, 128.5 (CH_{arom}), 118.6 (C_q), 79.1-79.0 ($CH_{glycerol}$), 76.8-76.7 ($CH_{glycerol}$), 72.7, 72.4 (CH_{2_Bn}), 69.1-69.0 ($CH_{2\_Ospacer}$), 67.0-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7, 30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.34, -0.33, -0.31, -0.13, -0.10.

HRMS $C_{40}H_{53}N_3O_{13}P_2$ + H^+ required 846.3126, found 846.3119

(Protected) (GroP)$_3$-Spacer or Trimer S8



Alcohol **S7** (0.83 mmol) was coupled with phosphoramidite **8** (1.4 mmol, 1.7 eq) following the general procedure. Compound **S8** was obtained after column chromatography (DCM:Acetone, 6:4) in 97% yield (0.80 mmol).

TLC analysis, R_f: 0.39 (DCM:Acetone, 6.5:3.5)

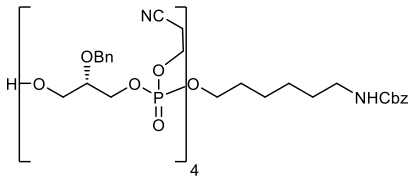
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.47-7.23 (20H, H_{arom}, m), 5.95-5.86 (1H, NH, b), 5.06 (2H, CH_{2_Cbz}, s), 4.71-4.58 (6H, CH_{2_Bn}, m), 4.34-3.98 (18H, 3 x CH_{2_OCE}, 5 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.93-3.81 (2H, 2 x $CH_{glycerol}$, m), 3.72-3.56 (3H, $CH_{glycerol}$, $CH_{2\_glycerol}$, m), 3.09 (2H, $CH_{2\_Nspacer}$, J=6.6 Hz, q), 3.10-2.94 (1H, OH, b), 2.77-2.67 (6H, 3 x CH_{2_OCE}, m), 1.71-1.55 (2H, CH_{2_spacer}, m), 1.52-1.22 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.6, 139.1 (C_q), 129.4, 129.3, 128.9, 128.8, 128.7, 128.5 (CH_{arom}), 118.6 (C_q), 79.1-79.0 ($CH_{glycerol}$), 76.8-76.7 ($CH_{glycerol}$), 72.7, 72.4 (CH_{2_Bn}), 69.1-69.0 ($CH_{2\_Ospacer}$), 67.0-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7, 30.4, 26.8, 25.7 (CH_{2_spacer}), 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: $C_{53}H_{69}N_4O_{18}P_3$ + H^+ required 1143.3893, found 1143.3900

(Protected) (GroP)$_4$-Spacer or Tetramer S9



Alcohol **S8** (0.12 mmol) was coupled with phosphoramidite **8** (0.24 mmol, 2 eq) following the general procedure. Compound **S9** was obtained after column chromatography (DCM:Acetone, 1:1) in 83% yield (0.1 mmol).

TLC analysis, R_f:0.32 (DCM:Acetone, 1:1)

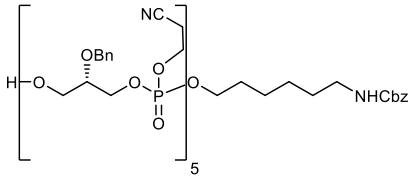
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.47-7.23 (25H, H_{arom}, m), 5.82-5.69 (1H, NH, b), 5.03 (2H, CH_{2_Cbz}, s), 4.67-4.54 (8H, CH_{2_Bn}, m), 4.34-3.98 (24H, 4 x CH_{2_OCE}, 7 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.93-3.81 (3H, 3 x $CH_{glycerol}$, m), 3.72-3.56 (3H, $CH_{glycerol}$, $CH_{2\_glycerol}$, m), 3.23-3.13 (1H, OH, b), 3.07 (2H, $CH_{2\_Nspacer}$, J=6.6 Hz, q), 2.77-2.67 (8H, 4 x CH_{2_OCE}, m), 1.71-1.55 (2H, CH_{2_spacer}, m), 1.52-1.22 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.6, 139.1 (C_q), 129.4 x 2, 129.3, 128.9 x 2, 128.8 x 2, 128.7, 128.5 (CH_{arom}), 118.6 (C_q), 79.1-79.0 ($CH_{glycerol}$), 76.8-76.7 ($CH_{glycerol}$), 72.7, 72.4 (CH_{2_Bn}), 69.1-69.0 ($CH_{2\_Ospacer}$), 67.0-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7, 30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.66, -1.64, -1.62, -1.60, -1.58, -1.40, -1.37.

HRMS: $C_{66}H_{85}N_5O_{23}P_4$ + H^+ required 1440.4659, found 1440.4656

(Protected) (GroP)$_5$-Spacer or Pentamer S10



Alcohol **S9** (35 µmol) was coupled with phosphoramidite **8** (86 µmol, 2.5 eq) following the general procedure. Compound **S10** was obtained after column chromatography (DCM:Acetone, 1:1) in 65% yield (23 µmol).

TLC analysis, R_f: 0.27 (DCM:Acetone, 6:4)

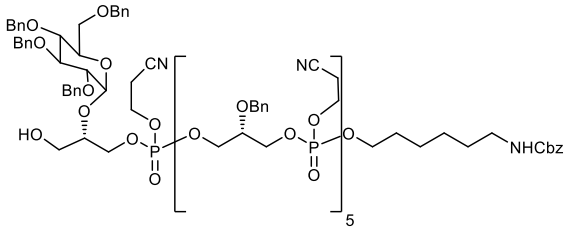
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.47-7.23 (30H, H_{arom}, m), 5.82-5.69 (1H, NH, b), 5.03 (2H, CH_{2_Cbz}, s), 4.67-4.54 (10H, CH_{2_Bn}, m), 4.34-3.98 (30H, 5 x CH_{2_OCE}, 9 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.93-3.81 (4H, 4 x $CH_{glycerol}$, m), 3.72-3.56 (3H, $CH_{glycerol}$, $CH_{2\_glycerol}$, m), 3.20-3.02 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.67 (10H, 5 x CH_{2_OCE}, m), 1.71-1.55 (2H, CH_{2_spacer}, m), 1.52-1.22 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.6, 139.1 (C_q), 129.4 x 2, 129.3, 128.9 x 2, 128.8 x 2, 128.7, 128.5 (CH_{arom}), 118.6 (C_q), 79.1-79.0 ($CH_{glycerol}$), 76.8-76.7 ($CH_{glycerol}$), 72.7, 72.4 (CH_{2_Bn}), 69.1-69.0 ($CH_{2\_Ospacer}$), 67.0-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7, 30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.67, -1.64, -1.63, -1.61, -1.58, -1.44, -1.40, -1.37.

HRMS: $C_{79}H_{101}N_6O_{28}P_5$ + H^+ required 1737.5425, found 1737.5428

(Protected) (GlcGroP)(GroP)$_5$-Spacer or Hexamer 23



Alcohol **S10** (22 µmol) was coupled with phosphoramidite **7** (32 µmol, 1.5 eq) following the general procedure. Compound **23** was obtained after column chromatography (DCM:Acetone, 1:1) in 65% yield (14 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 1:1)

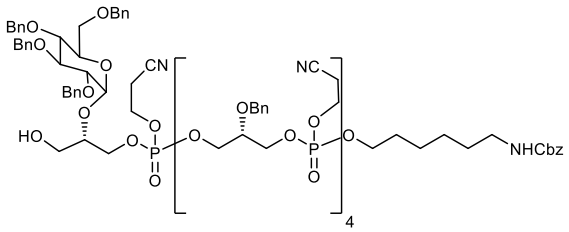
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.45-7.16 (50H, H_{arom}, m), 5.79-5.69 (1H, NH, b), 5.21-5.14 (1H, H_1, m), 5.06 (2H, CH_{2_Cbz}), 4.91-4.45 (18H, CH_{2_Bn}, m), 4.31-3.99 (36H, 6 x CH_{2_OCE}, 11 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.99-3.78 (8H, 6 x $CH_{\_glycerol}$, H_5, H_3, m), 3.78-3.48 (6H, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.28-3.27 (1H, OH, b), 3.09 (2H, $CH_{2\_Nspacer}$, J=6.6 Hz, q), 2.78-2.58 (12H, 6 x CH_{2_OCE}, m), 1.71-1.55 (2H, CH_{2_spacer}, m), 1.52-1.22 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.8, 139.6, 139.1 (C_q), 129.4 x 2, 129.3 X 2, 129.1 x 2, 129.0, 128.9, 128.8 x 2, 128.7, 128.6, 128.4 (CH_{arom}), 118.6 (C_q), 98.4 (C_1), 82.5 (C_3), 81.0 (C_2), 78.7 (C_4), 77.9 ($CH_{glycerol}$), 77.9 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5, 72.7 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 66.8-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.6-63.5 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.9-30.8, 30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.66, -1.63, -1.62, -1.60, -1.58, -1.44, -1.38.

HRMS: $C_{119}H_{145}N_7O_{38}P_6$ + H^+ required 2466.8128, found 2466.8129

(Protected) (GlcGroP)(GroP)$_4$-Spacer or Pentamer S11



Alcohol **S9** (35 µmol) was coupled with phosphoramidite **7** (53 µmol, 1.5 eq) following the general procedure. Compound **S11** was obtained after column chromatography (DCM:Acetone, 1:1) in 67% yield (24 µmol).

TLC analysis, R_f: 0.33 (DCM:Acetone, 6:4)

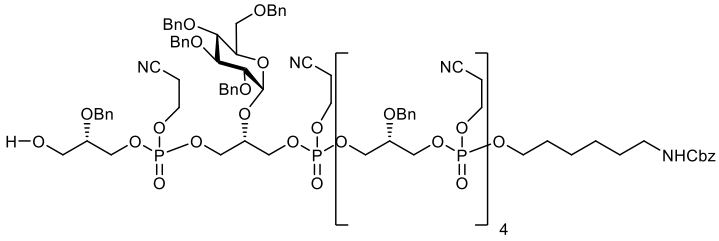
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.45-7.11 (45H, H_{arom}, m), 5.71-5.58 (1H, NH, b), 5.16 (1H, H_1, J=3.6 Hz, d), 5.01 (2H, CH_{2_Cbz}, s), 4.89-4.81 (1H, CH_{2_Bn}, m), 4.80-4.66 (3H, CH_{2_Bn}, m), 4.66-4.43 (12H, CH_{2_Bn}, m), 4.31-3.94 (30H, 5 x CH_{2_OCE}, 9 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.94-3.74 (6H, 4 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.13-2.94 (3H, OH, $CH_{2\_Nspacer}$, m), 2.74-2.54 (10H, 5 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.2, 140.0, 139.6, 139.4, 139.0 (C_q), 129.4 x 2, 129.3 X 2, 129.2 x 2, 129.1, 129.0 x 2, 128.9 x 2, 128.8 x 3, 128.7 x 2, 128.6, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.0 (C_1), 82.3 (C_3), 80.7 (C_2), 79.0 (C_4), 78.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5, 73.0, 72.7, 72.4 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.0 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.41, -0.36, -0.34, -0.32, -0.30, -0.29, -0.14, -0.11

HRMS: $C_{106}H_{129}N_6O_{33}P_5$ + H^+ required 2169.7361, found 2169.7368

(Protected) (GroP)(GlcGroP)(GroP)$_4$-Spacer or Hexamer 24



Alcohol **S11** (20 µmol) was coupled with phosphoramidite **8** (50 µmol, 2.5 eq) following the general procedure. Compound **24** was obtained after column chromatography (DCM:Acetone, 1:1) in 77% yield (15 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 1:1)

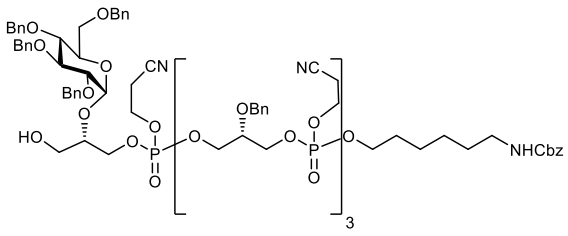
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.47-7.16 (50H, H_{arom}, m), 5.71-5.58 (1H, NH, b), 5.16 (1H, H_1, J=3.6 Hz, d), 5.01 (2H, CH_{2_Cbz}, s), 4.89-4.66 (4H, CH_{2_Bn}, m), 4.66-4.43 (14H, CH_{2_Bn}, m), 4.30-3.94 (36H, 6 x CH_{2_OCE}, 11 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.94-3.74 (7H, 5 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.13-2.94 (3H, OH, $CH_{2\_Nspacer}$, m), 2.74-2.54 (12H, 6 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.2, 140.1, 139.6 x 2, 139.5, 139.0 (C_q), 129.4 x 2, 129.3 X 2, 129.2 x 2, 129.1, 129.0 x 4, 128.9 x 2, 128.8 x 3, 128.7 x 2, 128.6, 128.5 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.0 (C_1), 82.3 (C_3), 80.7 (C_2), 79.1-79.0 ($CH_{glycerol}$), 78.6 (C_4), 76.7 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.0, 72.7, 72.4 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.6-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 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: $C_{119}H_{145}N_7O_{38}P_6$ + H^+ required 2466.8128, found 2466.8133

(Protected) (GlcGroP)(GroP)$_3$-Spacer or Tetramer S12



Alcohol **S8** (59 µmol) was coupled with phosphoramidite **7** (88 µmol, 1.5 eq) following the general procedure. Compound **S12** was obtained after column chromatography (DCM:Acetone, 5.5:4.5) in 86% yield (51 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 1:1)

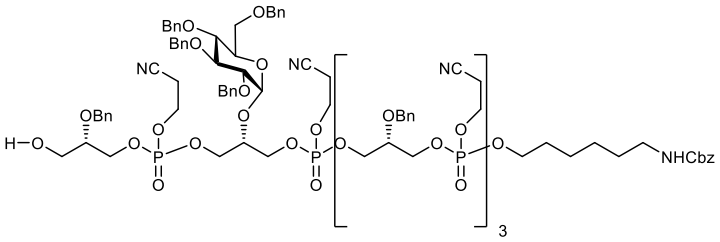
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.50-7.08 (40H, H_{arom}, m), 5.77-5.64 (1H, NH, b), 5.18-5.12 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.92-4.39 (14H, CH_{2_Bn}, m), 4.31-3.97 (34H, 4 x CH_{2_OCE}, 7 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.94-3.75 (6H, 4 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.45 (6H, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.26-3.16 (1H, OH, b), 3.06 (2H, $CH_{2\_Nspacer}$, J=6.6 Hz, q), 2.78-2.50 (8H, 4 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.0, 139.6, 139.5, 139.4, 139.1 (C_q), 129.4 x 2, 129.3 X 2, 129.2, 129.1 x 2, 129.0, 128.9 x 3, 128.8 x 3, 128.7 x 2, 128.6, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.4 (C_1), 82.5 (C_3), 80.9 (C_2), 78.9 (C_4), 78.0-77.8 ($CH_{glycerol}$), 76.8-76.7 ($CH_{glycerol}$), 75.9, 75.6, 73.8, 73.5, 72.7 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.1 ($CH_{2\_Ospacer}$), 68.8-66.06($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.8-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.3-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.39, -0.37, -0.35, -0.33, -0.31, -0.17, -0.10.

HRMS: $C_{93}H_{113}N_5O_{28}P_4$ + H^+ required 1872.6595, found 1872.6603

(Protected) (GroP)(GlcGroP)(GroP)$_3$-Spacer or Pentamer S13



Alcohol **S12** (40 µmol) was coupled with phosphoramidite **8** (100 µmol, 2.5 eq) following the general procedure. Compound **S13** was obtained after column chromatography (DCM:Acetone, 1:1) in 76% yield (30 µmol).

TLC analysis, R_f: 0.38 (DCM:Acetone, 4:6)

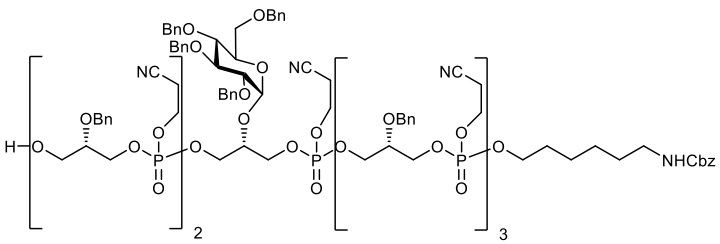
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.47-7.16 (45H, H_{arom}, m), 5.74-5.61 (1H, NH, b), 5.16 (1H, H_1, J=3.6 Hz, d), 5.01 (2H, CH_{2_Cbz}, s), 4.89-4.41 (18H, CH_{2_Bn}, m), 4.30-3.93 (30H, 5 x CH_{2_OCE}, 9 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.94-3.74 (5H, 3 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.22-3.10 (1H, OH, m), 3.08-2.94 (2H, $CH_{2\_Nspacer}$, m), 2.74-2.53 (10H, 5 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.2, 140.0, 139.6, 139.4, 139.0 (C_q), 129.4 x 2, 129.3 X 2, 129.2 x 2, 129.1, 129.0 x 2, 128.9 x 2, 128.8 x 3, 128.7 x 2, 128.6, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.0 (C_1), 82.3 (C_3), 80.7 (C_2), 79.0 (C_4), 78.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5, 73.0, 72.7, 72.4 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.0 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.41, -0.36, -0.34, -0.32, -0.30, -0.29, -0.14, -0.11

HRMS: $C_{106}H_{129}N_6O_{33}P_5 + H^+$ required 2169.7361, found 2169.7355

(Protected) $(GroP)_2(GlcGroP)(GroP)_3$-Spacer or Hexamer 25



Alcohol **S13** (10 µmol) was coupled with phosphoramidite **8** (25 µmol, 2.5 eq) following the general procedure. Compound **25** was obtained after column chromatography (DCM:Acetone, 1:1) in 72% yield (7.2 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 1:1)

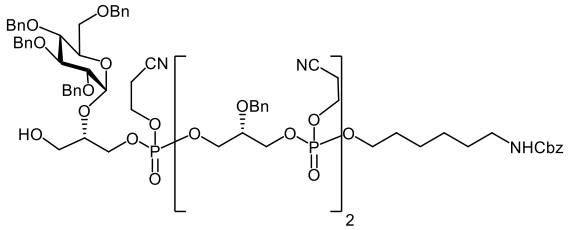
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.50-7.16 (50H, H_{arom}, m), 5.76-5.65 (1H, NH, b), 5.20-5.12 (1H, H_1, m), 5.02 (2H, CH_{2_Cbz}, s), 4.89-4.39 (18H, CH_{2_Bn}, m), 4.30-3.94 (36H, 6 x CH_{2_OCE}, 11 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.94-3.74 (7H, 5 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.17-2.98 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.48 (12H, 6 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.2, 140.0, 139.6, 139.5, 139.1 (C_q), 129.4 x 3, 129.3, 129.2 x 2, 129.1, 129.0, 128.9, 128.8 x 2,128.7, 128.6, 128.4 (CH_{arom}), 118.6 (C_q), 98.1 (C_1), 82.3 (C_3), 80.7 (C_2), 79.1 ($CH_{glycerol}$), 78.6 (C_4), 76.8 ($CH_{glycerol}$), 76.0, 75.7, 73.8, 73.1, 72.7, 72.4 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.1 ($CH_{2\_Ospacer}$), 67.8-66.9 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.77, -1.71, -1.68, -1.63, -1.59, -1.56, -1.43, -1.13, -1.11, -1.03, -1.01.

HRMS: $C_{119}H_{145}N_7O_{38}P_6$ + H^+ required 2466.8128, found 2466.8137

(Protected) (GlcGroP)(GroP)$_2$-Spacer or Trimer S14



Alcohol **S7** (98 µmol) was coupled with phosphoramidite **7** (147 µmol, 1.5 eq) following the general procedure. Compound **S14** was obtained after column chromatography (DCM:Acetone, 5.5:4.5) in 86% yield (84 µmol).

TLC analysis, R_f: 0.35 (DCM:Acetone, 6:4)

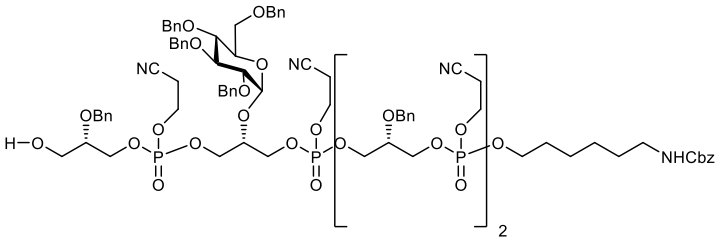
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.44-7.11 (35H, H_{arom}, m), 5.77-5.62 (1H, NH, b), 5.17-5.11 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.90-4.41 (12H, 6 x CH_{2_Bn}, m), 4.29-3.96 (18H, 3 x CH_{2_OCE}, 5 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.96-3.75 (5H, 3 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (6H, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.26-3.14 (1H, OH, b), 3.05 (2H, $CH_{2\_Nspacer}$, J=6.6 Hz, q), 2.77-2.53 (6H, 3 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.4, 140.1, 139.6, 139.5, 139.4, 139.1, 139.0, 138.6 (C_q), 129.4 x 3, 129.3 x 2, 129.2, 129.1, 129.0, 128.9, 128.8 x 2,128.7, 128.6, 128.4 (CH_{arom}), 118.6 (C_q), 97.4 (C_1), 82.5 (C_3), 81.0 (C_2), 78.7 (C_4), 78.0, 76.0 ($CH_{glycerol}$), 76.0, 75.6 x 2, 73.8, 73.6, 72.7, 72.4 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.1 ($CH_{2\_Ospacer}$), 68.3, 67.8-66.9 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.67, -1.65, -1.64, -1.62, -1.61, -1.58, -1.45, -1.42, -1.39, -1.38

HRMS: $C_{80}H_{97}N_4O_{23}P_3$ + H^+ required 1575.5829, found 1575.5833

(Protected) (GroP)(GlcGroP)(GroP)$_2$-Spacer or Tetramer S15



Alcohol **S14** (11 µmol) was coupled with phosphoramidite **8** (28 µmol, 2.5 eq) following the general procedure. Compound **S15** was obtained after column chromatography (DCM:Acetone, 1:1) in 83% yield (9.1 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 1:1)

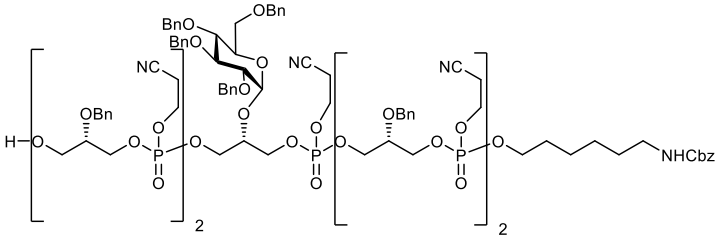
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.11 (40H, H_{arom}, m), 5.73-5.62 (1H, NH, b), 5.19-5.13 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.89-4.42 (14H, 7 x CH_{2_Bn}, m), 4.30-3.96 (24H, 4 x CH_{2_OCE}, 7 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.96-3.75 (5H, 3 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.15-3.00 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (8H, 4 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.7, 139.6, 139.5, 139.1 (C_q), 129.4 x 3, 129.3 x 2, 129.2, 129.1 x 2, 129.0 x 4, 128.9 x 2, 128.8 x 3,128.7 x 2, 128.6 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.1 (C_1), 82.3 (C_3), 80.8 (C_2), 79.2-79.1 ($CH_{gltcerol}$), 78.6 (C_4), 76.9-76.8 ($CH_{glycerol}$), 76.0, 75.6 x 2, 73.9, 73.1, 72.4 (CH_{2_Bn}), 71.8 (C_5), 69.7 (C_6), 69.1 ($CH_{2\_Ospacer}$), 67.9, 67.8, 67.0, 66.7, 66.6, 66.5 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.6-63.3 (CH_{2_OCE}), 61.2 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.71, -1.69, -1.65, -1.63, -1.61, -1.59, -1.55, -1.53, -1.43, -1.42, -1.39, -1.36

HRMS: $C_{93}H_{113}N_5O_{28}P_4$ + H^+ required 1872.6595, found 1872.6594

(Protected) (GroP)$_2$(GlcGroP)(GroP)$_2$-Spacer or Pentamer S16

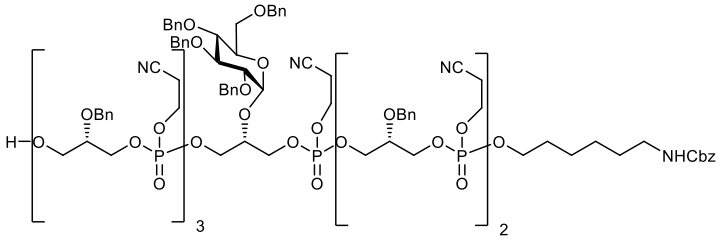


Alcohol **S15** (24 µmol) was coupled with phosphoramidite **8** (60 µmol, 2.5 eq) following the general procedure. Compound **S16** was obtained after column chromatography (DCM:Acetone, 1:1) in 82% yield (19 µmol).

TLC analysis, R_f: 0.38 (DCM:Acetone, 4:6)

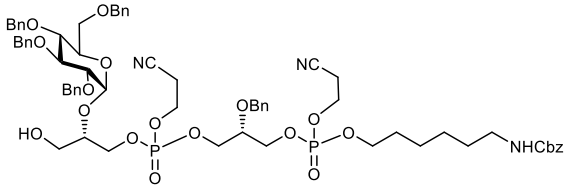
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.12 (45H, H_{arom}, m), 5.77-5.64 (1H, NH, b), 5.20-5.13 (1H, H_1, m), 5.02 (2H, CH_{2_Cbz}, s), 4.87-4.40 (16H, 7 x CH_{2_Bn}, m), 4.30-3.94 (30H, 5 x CH_{2_OCE}, 9 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.94-3.74 (5H, 3 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.19-2.99 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (10H, 5 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).
^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.6 x 2, 139.5, 139.0 (C_q), 129.4 x 2, 129.3 x 2, 129.2 x 2, 129.1, 129.0 x 4, 128.9 x 2, 128.8 x 3,128.7 x 3, 128.6, 128.5 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.0 (C_1), 82.3 (C_3), 80.7 (C_2), 79.1-79.0 ($CH_{gltcerol}$), 78.6 (C_4), 76.7 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.1, 72.7, 72.4 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.6-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).
^{31}P-NMR(162 MHz, CD_3CN), δ: -1.68, -1.65, -1.62, -1.60, -1.42, -1.39.
HRMS: $C_{106}H_{129}N_6O_{33}P_5$ + H^+ required 2169.7361, found 2169.7365

(Protected) $(GroP)_3(GlcGroP)(GroP)_2$-Spacer or Hexamer 26



Alcohol **S16** (35 µmol) was coupled with phosphoramidite **8** (86 µmol, 2.5 eq) following the general procedure. Compound **26** was obtained after column chromatography (DCM:Acetone, 1:1) in 65% yield (23 µmol).
TLC analysis, R_f: 0.41 (DCM:Acetone, 7:3)
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.43-7.12 (50H, H_{arom}, m), 5.75-5.64 (1H, NH, b), 5.20-5.13 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.88-4.41 (18H, 7 x CH_{2_Bn}, m), 4.30-3.94 (36H, 6 x CH_{2_OCE}, 11 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.94-3.74 (6H, 4 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (8H, 2 x $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.17-2.99 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (12H, 6 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).
^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.6 x 2, 139.5, 139.0 (C_q), 129.4 x 2, 129.3 x 3, 129.2, 129.1, 129.0 x 2, 128.9 x 2, 128.8 x 3,128.7 x 2, 128.6, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.0 (C_1), 82.3 (C_3), 80.7 (C_2), 79.1-79.0 ($CH_{gltcerol}$), 78.6 (C_4), 76.7 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.1, 72.7, 72.4 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.6-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).
^{31}P-NMR(162 MHz, CD_3CN), δ: -1.69, -1.67, -1.64, -1.63, -1.61, -1.58, -1.44, -1.40, 1.37.
HRMS: $C_{119}H_{145}N_7O_{38}P_6$ + H^+ required 2466.8128, found 2466.8125

(Protected) (GlcGroP)(GroP)-Spacer or Dimer S17



Alcohol **S6** (160 µmol) was coupled with phospharamidite **7** (200 µmol, 1.3 eq) following the general procedure. Compound **S17** was obtained after column chromatography (DCM:Acetone, 7:3) in 64% yield (102 µmol).

TLC analysis, R_f: 0.38 (DCM:Acetone, 7:3)

^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.11 (30H, H_{arom}, m), 5.73-5.62 (1H, NH, b), 5.19-5.13 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.89-4.42 (10H, 5 x CH_{2_Bn}, m), 4.30-3.96 (12H, 2 x CH_{2_OCE}, 3 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.96-3.75 (4H, 2 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (6H, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.15-3.00 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (4H, 2 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.7, 139.6, 139.5, 139.4 (C_q), 129.4 x 2, 129.3 x 2, 129.2, 129.1 x 2, 129.0, 128.9 x 2, 128.8 x 2,128.7, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.4 (C_1), 82.5 (C_3), 81.0 (C_2), 79.2 ($CH_{gltcerol}$), 78.7 (C_4), 78.0-77.8 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5, 72.7, 72.4 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.0 ($CH_{2\_Ospacer}$), 68.3-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.4-63.1 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

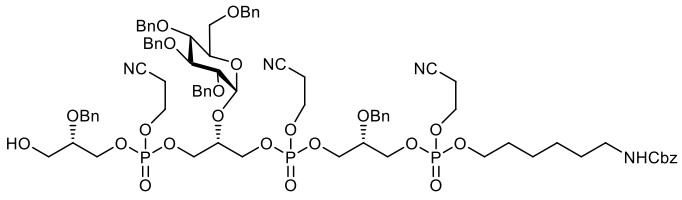
^{31}P-NMR(162 MHz, CD_3CN), δ: -1.64, -1.61, -1.60, -1.48, -1.46, -1.44, -1.43, -1.39, -1.32, -1.28.

HRMS: $C_{67}H_{81}N_3O_{18}P_2 + H^+$ required 1278.5063, found 1278.5064

(Protected) (GroP)(GlcGroP)(GroP) -Spacer or Trimer S18

Alcohol **S17** (86 µmol) was coupled with phosphoramidite **8** (215 µmol, 2.5 eq) following the general procedure. Compound **S18** was obtained after column chromatography (DCM:Acetone, 6:4) in 68% yield (58 µmol).

TLC analysis, R_f: 0.35 (DCM:Acetone, 6:4)



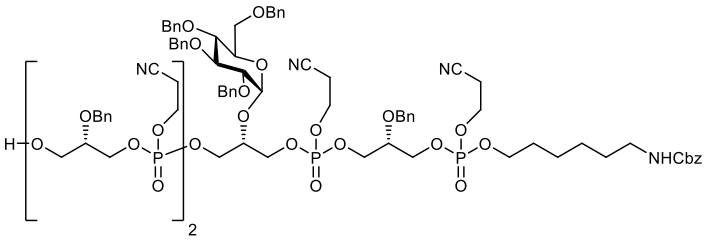
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.11 (35H, H_{arom}, m), 5.73-5.62 (1H, NH, b), 5.19-5.13 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.89-4.42 (12H, 6 x CH_{2_Bn}, m), 4.30-3.96 (18H, 3 x CH_{2_OCE}, 5 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.96-3.75 (4H, 2 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.15-3.00 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (6H, 3 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.0, 139.6 x 2, 139.5, 139.1 (C_q), 129.4 x 2, 129.3 x 3, 129.2, 129.1 x 2, 129.0 x 3, 128.9 x 3, 128.8 x 3,128.7 x 3, 128.6 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.1 (C_1), 82.3 (C_3), 80.7 (C_2), 79.1-79.0 ($CH_{gltcerol}$), 78.6 (C_4), 76.8 ($CH_{glycerol}$), 76.0, 75.6 x 2, 73.9, 73.1, 72.4 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.1 ($CH_{2\_Ospacer}$), 67.9-66.4 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.6-63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.43, -0.42, -0.40, -0.39, -0.37, -0.36, -0.35, -0.12, -0.09, -0.07

HRMS: $C_{80}H_{97}N_4O_{23}P_3$ + H^+ required 1575.5829, found 1575.5832

(Protected) $(GroP)_2(GlcGroP)(GroP)$ -Spacer or Tetramer S19



Alcohol **S18** (41 µmol) was coupled with phosphoramidite **8** (102 µmol, 2.5 eq) following the general procedure. Compound **S19** was obtained after column chromatography (DCM:Acetone, 1:1) in 65% yield (27 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 1:1)

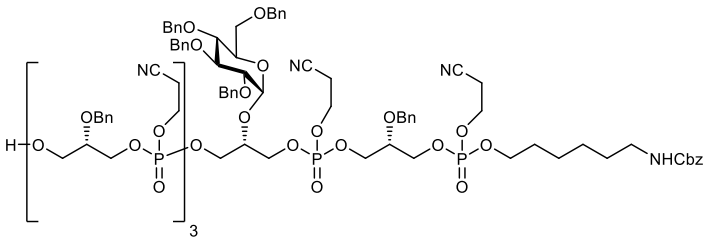
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.42-7.11 (40H, H_{arom}, m), 5.72-5.61 (1H, NH, b), 5.20-5.13 (1H, H_1, m), 5.02 (2H, CH_{2_Cbz}, s), 4.89-4.42 (14H, 7 x CH_{2_Bn}, m), 4.30-3.96 (24H, 4 x CH_{2_OCE}, 7 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.96-3.75 (4H, 2 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.15-3.00 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (8H, 4 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.7, 139.6, 139.5, 139.4 (C_q), 129.4 x 2, 129.3 x 2, 129.2, 129.1 x 2, 129.0, 128.9 x 2, 128.8 x 2,128.7, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.4 (C_1), 82.5 (C_3), 81.0 (C_2), 79.2 ($CH_{gltcerol}$), 78.7 (C_4), 78.0-77.8 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5, 72.7, 72.4 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.0 ($CH_{2\_Ospacer}$), 68.3-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.4-63.1 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.42, -0.40, -0.36, -0.34, -0.32, -0.14, -0.12.

HRMS: $C_{93}H_{113}N_5O_{28}P_4$ + H^+ required 1872.6595, found 1872.6598

(Protected) $(GroP)_3(GlcGroP)(GroP)$ -Spacer or Pentamer S20



Alcohol **S19** (20 µmol) was coupled with phosphoramidite **8** (50 µmol, 2.5 eq) following the general procedure. Compound **S20** was obtained after column chromatography (DCM:Acetone, 1:1) in 77% yield (15 µmol).

TLC analysis, R_f: 0.38 (DCM:Acetone, 4:6)

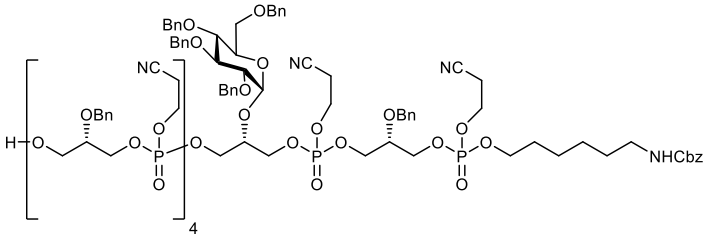
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.11 (45H, H_{arom}, m), 5.73-5.62 (1H, NH, b), 5.19-5.13 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.89-4.42 (16H, 8 x CH_{2_Bn}, m), 4.30-3.96 (30H, 5 x CH_{2_OCE}, 9 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.96-3.75 (4H, 3 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (6H, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.15-3.00 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (4H, 2 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.7, 139.6, 139.5, 139.4 (C_q), 129.4 x 2, 129.3 x 2, 129.2, 129.1 x 2, 129.0, 128.9 x 2, 128.8 x 2,128.7, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.4 (C_1), 82.5 (C_3), 81.0 (C_2), 79.2 ($CH_{gltcerol}$), 78.7 (C_4), 78.0-77.8 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5, 72.7, 72.4 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.0 ($CH_{2\_Ospacer}$), 68.3-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.4-63.1 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -0.41, -0.36, -0.34, -0.32, -0.30, -0.29, -0.14, -0.11.

HRMS: $C_{106}H_{129}N_6O_{33}P_5 + H^+$ required 2169.7361, found 2169.7363

(Protected) (GroP)$_4$(GlcGroP)(GroP) -Spacer or Hexamer 27

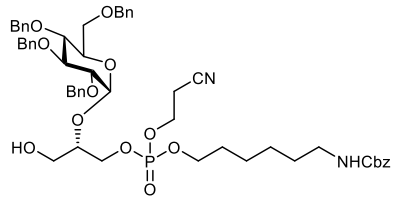


Alcohol **S20** (13 µmol) was coupled with phosphoramidite **8** (40 µmol, 2.5 eq) following the general procedure. Compound **27** was obtained after column chromatography (DCM:Acetone, 1:1) in 72% yield (9.4 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 4:6)

^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.11 (50H, H_{arom}, m), 5.73-5.62 (1H, NH, b), 5.19-5.13 (1H, H_1, m), 5.03 (2H, CH_{2_Cbz}, s), 4.89-4.42 (18H, 9 x CH_{2_Bn}, m), 4.30-3.96 (36H, 6 x CH_{2_OCE}, 11 x $CH_{2\_glycerol}$, $CH_{2\_Ospacer}$, m), 3.96-3.75 (6H, 4 x $CH_{\_glycerol}$, H_5, H_3, m), 3.74-3.42 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.15-3.00 (3H, OH, $CH_{2\_Nspacer}$, m), 2.77-2.53 (4H, 2 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).
^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 139.7, 139.6, 139.5, 139.4 (C_q), 129.4 x 2, 129.3 x 2, 129.2, 129.1 x 2, 129.0, 128.9 x 2, 128.8 x 2,128.7, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.4 (C_1), 82.5 (C_3), 81.0 (C_2), 79.2 ($CH_{gltcerol}$), 78.7 (C_4), 78.0-77.8 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5, 72.7, 72.4 (CH_{2_Bn}), 71.6 (C_5), 69.7 (C_6), 69.0 ($CH_{2\_Ospacer}$), 68.3-66.6 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.4-63.1 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25,7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).
^{31}P-NMR(162 MHz, CD_3CN), δ: -0.41, -0.36, -0.34, -0.32, -0.30, -0.29, -0.14, -0.11.
HRMS: $C_{119}H_{145}N_7O_{38}P_6$ + H^+ required 2466.8128, found 2466.8130

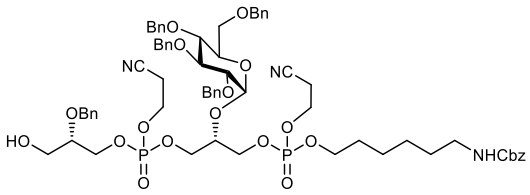
(Protected) GlcGroP-Spacer or Monomer S21



Alcohol spacer **9** (0.26 mmol) was coupled with phosphoramidite **7** (0.35 mmol, 1.3 eq) following the general procedure. Compound **S21** was obtained after column chromatography (DCM:Acetone, 7.5:2.5) in 81% yield (0.21 mmol). TLC analysis, R_f: 0.45 (DCM:Acetone, 7:3

^{1}H-NMR (400 MHz, CD_3CN), δ: 7.47-7.12 (25H, H_{arom}, m), 5.68-5.54 (1H, NH, b), 5.16 (1H, H_1, J=3.6 Hz, d), 5.03 (2H, CH_{2_Cbz}, s), 4.88 (1H, CH*H*$_{\_Bn}$, J=10.6 Hz, d), 4.82-4.61 (4H, 2 x CH_{2_Bn}, m), 4.58-4.43 (3H, CH*H*$_{\_Bn}$), 4.22-3.06 (4H, CH_{2_OCE}, $CH_{2\_glycerol}$), 4.05-3.80 (5H, $CH_{2\_Ospacer}$, H_5, $CH_{glycerol}$, H_3, m), 3.75-3.46 (6H, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.15-2.98 (3H, OH, $CH_{2\_Nspacer}$, m), 2.68-2.57 (2H,CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).
^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.6, 139.5, 139.4 (C_q), 129.4, 129.3 x 2, 129.2 x 2, 129.1, 128.9 x 2, 128.8 x 2,128.7 x 2, 128.5, 128.4 (CH_{arom}), 118.6 (C_q), 97.3 (C_1), 82.5 (C_3), 81.0 (C_2), 78.7 (C_4), 78.0-77.8 ($CH_{glycerol}$), 76.0, 75.6, 73.8, 73.5 (CH_{2_Bn}), 71.5 (C_5), 69.7 (C_6), 69.0 ($CH_{2\_Ospacer}$), 68.1-68.0 ($CH_{2\_glycerol}$), 66.6 CH_{2_Cbz}), 63.2 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).
^{31}P-NMR(162 MHz, CD_3CN), δ: -1.44, -1.37
HRMS: $C_{54}H_{65}N_2O_{13}P$ + H^+ required 981.4297, found 981.4296

(Protected) (GroP)(GlcGroP)-Spacer or Dimer S22



Alcohol **S21** (0.17 mmol) was coupled with phosphoramidite **8** (0.26 mmol, 1.5 eq) following the general procedure. Compound **S22** was obtained after column chromatography (DCM:Acetone, 6:4) in 82% yield (0.14 mmol).

TLC analysis, R_f: 0.32 (DCM:Acetone, 1:1).

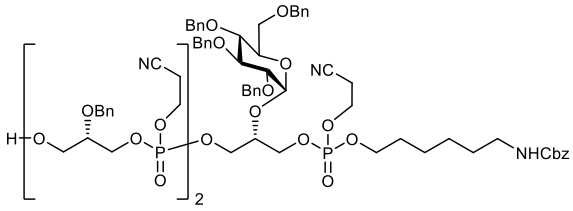
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.10 (30H, H_{arom}, m), 5.71-5.55 (1H, NH, b), 5.16 (1H, H_1, J=3.6 Hz, d), 5.02 (2H, CH_{2_Cbz}, s), 4.90-4.83 (1H, $CH\textit{H}_{\_Bn}$, m), 4.80-4.69 (3H, 3 x $CH\textit{H}_{\_Bn}$, m), 4.66-4.56 (3H, 3 x $CH\textit{H}_{\_Bn}$, m), 4.58-4.43 (3H, $CH\textit{H}_{\_Bn}$), 4.30-4.04 (11H,2 x CH_{2_OCE}, 3 x $CH_{2\_glycerol}$, $CH_{glycerol}$), 4.05-3.96 (2H, $CH_{2\_Ospacer}$, m), 3.95-3.86 (H_5), 3.86-3.76 (1H, H_3, m), 3.75-3.46 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.09-2.97 (3H, OH, $CH_{2\_Nspacer}$, m), 2.78-2.52 (4H,2 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.7, 139.6, 139.5 (C_q), 129.4 x3, 129.3 x 2, 129.2, 129.1 x 2, 129.0, 128.9 x 2, 128.8 x 3,128.7 x 2, 128.6 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.1-97.0 (C_1), 82.3 (C_3), 80.8 (C_2), 79.2-79.1 ($CH_{glycerol}$), 78.6 (C_4), 76.0, 75.6 (CH_{2_Bn}), 75.1-74.2 ($CH_{glycerol}$), 73.9, 73.1, 72.5 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.4 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).

^{31}P-NMR(162 MHz, CD_3CN), δ: -1.72, -1.70, -1.67, -1.66 -1.47, -1.41

HRMS: $C_{67}H_{81}N_3O_{18}P_2$ + H^+ required 1278.5063, found 1278.5067

(Protected) $(GroP)_2$(GlcGroP)-Spacer or Trimer S23



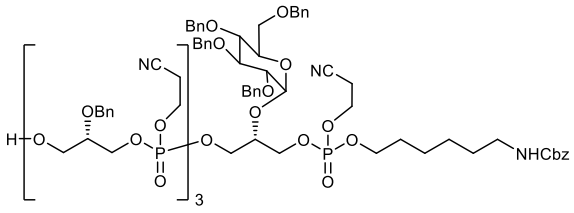
Alcohol **S22** (0.12 mmol) was coupled with phosphoramidite **8** (0.24 mmol, 2.0 eq) following the general procedure. Compound **S23** was obtained after column chromatography (DCM:Acetone, 1:1) in 77% yield (0.92 mmol).

TLC analysis, R_f: 0.38 (DCM:Acetone, 4:6)

^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.10 (35H, H_{arom}, m), 5.71-5.56 (1H, NH, b), 5.18-5.15 (1H, H_1, m), 5.02 (2H, CH_{2_Cbz}, s), 4.90-4.81 (1H, $CH\textit{H}_{\_Bn}$, m), 4.80-4.69 (3H, 3 x $CH\textit{H}_{\_Bn}$, m), 4.65-4.45 (8H, 8 x $CH\textit{H}_{\_Bn}$, m), 4.28-3.94 (17H,3 x CH_{2_OCE}, 5 x $CH_{2\_glycerol}$, $CH_{glycerol}$), 3.94-3.75 (5H, $CH_{2\_Ospacer}$, H_5, H_3, $CH_{glycerol}$, m), 3.75-3.46 (7H, $CH_{glycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.13-2.97 (3H, OH, $CH_{2\_Nspacer}$, m), 2.73-2.58 (6H,3 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

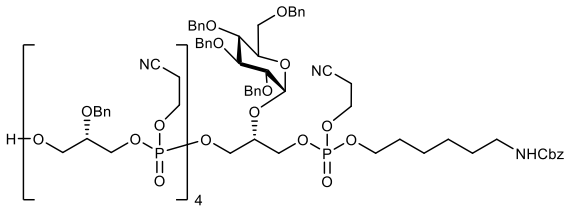
^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.7, 139.6, 139.5 (C_q), 129.4 x3, 129.3 x 2, 129.2, 129.1 x 2, 129.0, 128.9 x 2, 128.8 x 3,128.7 x 2, 128.6 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.1-97.0 (C_1), 82.3 (C_3), 80.8 (C_2), 79.2-79.1 ($CH_{glycerol}$), 78.6 (C_4), 76.0, 75.6 (CH_{2_Bn}), 75.1-74.2 ($CH_{glycerol}$), 73.9, 73.1, 72.5 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.4 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25.7 (CH_{2_spacer}), 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: $C_{80}H_{97}N_4O_{23}P_3$ + H^+ required 1575.5829, found 1575.5827

(Protected) $(GroP)_3$(GlcGroP)-Spacer or Tetramer S24



Alcohol **S23** (80 µmol) was coupled with phosphoramidite **8** (160 µmol, 2.0 eq) following the general procedure. Compound **S24** was obtained after column chromatography (DCM:Acetone, 1:1) in 81% yield (65 µmol).
TLC analysis, R_f: 0.33 (DCM:Acetone, 4:6)
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.10 (35H, H_{arom}, m), 5.74-5.56 (1H, NH, b), 5.18-5.15 (1H, H_1, m), 5.02 (2H, CH_{2_Cbz}, s), 4.90-4.81 (1H, $CH\mathit{H}_{\_Bn}$, m), 4.80-4.69 (3H, 3 x $CH\mathit{H}_{\_Bn}$, m), 4.65-4.45 (10H, 10 x $CH\mathit{H}_{\_Bn}$, m), 4.28-3.94 (23H,4 x CH_{2_OCE}, 7 x $CH_{2\_glycerol}$, $CH_{glycerol}$), 3.94-3.75 (6H, $CH_{2\_Ospacer}$, H_5, H_3,2 x $CH_{glycerol}$, m), 3.75-3.46 (7H, $CH_{gòycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.16-2.97 (3H, OH, $CH_{2\_Nspacer}$, m), 2.73-2.58 (8H,4 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).
^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.6 x 2, 139.5. 139.1 (C_q), 129.4 x3, 129.3 x 2, 129.2 x 2, 129.1 x 2, 129.0 x 3, 128.9 x 2, 128.8 x 3,128.7 x 2, 128.6 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.0-96.9 (C_1), 82.3 (C_3), 80.8 (C_2), 79.2-79.1 ($CH_{glycerol}$), 78.6 (C_4), 76.0, 75.6 (CH_{2_Bn}), 75.1-74.2 ($CH_{glycerol}$), 73.9, 73.1, 72.5 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.4 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25.7 (CH_{2_spacer}), 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: $C_{93}H_{113}N_5O_{28}P_4$ + H^+ required 1872.6595, found 1872.6601

(Protected) $(GroP)_4$(GlcGroP)-Spacer or Pentamer S25



Alcohol **S24** (48 µmol) was coupled with phosphoramidite **8** (120 µmol, 2.5 eq) following the general procedure. Compound **S25** was obtained after column chromatography (DCM:Acetone, 1:1) in 76% yield (36 µmol).

TLC analysis, R_f: 0.31 (DCM:Acetone, 4:6)

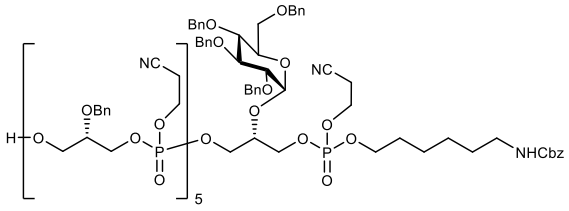
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.10 (35H, H_{arom}, m), 5.74-5.56 (1H, NH, b), 5.18-5.15 (1H, H_1, m), 5.02 (2H, CH_{2_Cbz}, s), 4.90-4.81 (1H, CH*H*$_{\_Bn}$, m), 4.80-4.69 (3H, 3 x CH*H*$_{\_Bn}$, m), 4.65-4.45 (12H, 12 x CH*H*$_{\_Bn}$, m), 4.28-3.94 (29H,5 x CH_{2_OCE}, 9 x $CH_{2\_glycerol}$, $CH_{glycerol}$), 3.94-3.75 (7H, $CH_{2\_Ospacer}$, H_5, H_3,3 x $CH_{glycerol}$, m), 3.75-3.46 (7H, $CH_{gòycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.16-2.97 (3H, OH, $CH_{2\_Nspacer}$, m), 2.73-2.58 (8H,4 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 157.3, 140.1, 139.6 x 2, 139.5. 139.1 (C_q), 129.4 x3, 129.3 x 2, 129.2 x 2, 129.1 x 2, 129.0 x 3, 128.9 x 2, 128.8 x 3,128.7 x 2, 128.6 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.0-96.9 (C_1), 82.3 (C_3), 80.8 (C_2), 79.2-79.1 ($CH_{glycerol}$), 78.6 (C_4), 76.0, 75.6 (CH_{2_Bn}), 75.1-74.2 ($CH_{glycerol}$), 73.9, 73.1, 72.5 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.4 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25.7 (CH_{2_spacer}), 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: $C_{106}H_{129}N_6O_{33}P_5$ + H^+ required 2169.7361, found 2169.7358

(Protected) $(GroP)_5$(GlcGroP)-Spacer or Hexamer 28



Alcohol **S25** (23 µmol) was coupled with phosphoramidite **8** (58 µmol, 2.5 eq) following the general procedure. Compound **28** was obtained after column chromatography (DCM:Acetone, 1:1) in 65% yield (15 µmol).

TLC analysis, R_f: 0.28 (DCM:Acetone, 4:6)

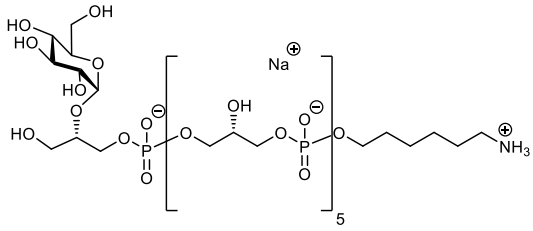
^{1}H-NMR (400 MHz, CD_3CN), δ: 7.48-7.10 (35H, H_{arom}, m), 5.74-5.56 (1H, NH, b), 5.18-5.15 (1H, H_1, m), 5.02 (2H, CH_{2_Cbz}, s), 4.90-4.81 (1H, CH*H*$_{\_Bn}$, m), 4.80-4.69 (3H, 3 x CH*H*$_{\_Bn}$, m), 4.65-4.45 (14H, 14 x CH*H*$_{\_Bn}$, m), 4.28-3.94 (35H, 6 x CH_{2_OCE}, 11 x $CH_{2\_glycerol}$, $CH_{glycerol}$), 3.94-3.75 (8H, $CH_{2\_Ospacer}$, H_5, H_3,4 x $CH_{glycerol}$, m), 3.75-3.46 (7H, $CH_{gòycerol}$, 2 x H_6, H_4, H_2, $CH_{2\_glycerol}$), 3.16-2.97 (3H, OH, $CH_{2\_Nspacer}$, m), 2.73-2.58 (8H,4 x CH_{2_OCE}, m), 1.65-1.49 (2H, CH_{2_spacer}, m), 1.45-1.14 (6H, 3 x CH_{2_spacer}, m).

^{13}C-NMR (101 MHz, CD_3CN), δ: 157.3, 140.1, 139.6 x 2, 139.5. 139.1 (C_q), 129.4 x3, 129.3 x 2, 129.2 x 2, 129.1 x 2, 129.0 x 3, 128.9 x 2, 128.8 x 3,128.7 x 2, 128.6 x 2, 128.4 (CH_{arom}), 118.6 (C_q), 97.0-96.9 (C_1), 82.3 (C_3), 80.8 (C_2), 79.2-79.1 ($CH_{glycerol}$), 78.6 (C_4), 76.0, 75.6 (CH_{2_Bn}), 75.1-74.2 ($CH_{glycerol}$), 73.9, 73.1, 72.5 (CH_{2_Bn}), 71.7 (C_5), 69.7 (C_6), 69.2 ($CH_{2\_Ospacer}$), 67.8-66.4 ($CH_{2\_glycerol}$, CH_{2_Cbz}), 63.5-63.3 (CH_{2_OCE}), 61.1 ($CH_{2\_glycerol}$), 41.4 ($CH_{2\_Nspacer}$), 30.7-30.4, 26.8, 25.7 (CH_{2_spacer}), 20.2-20.1 (CH_{2_OCE}).
^{31}P-NMR (162 MHz, CD_3CN), δ: -0.41, -0.36, -0.34, -0.32, -0.30, -0.29, -0.14, -0.11.
HRMS: $C_{119}H_{145}N_7O_{38}P_6 + H^+$ required 2466.8128, found 2466.8133

Final deprotections

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 by ^{1}H-NMR the disappearing of the cyanoethyl group, the residue is flushed over 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. $Ar_{(g)}$ is bubbled in the reaction mixture for 20 minutes while sonicating, Pd-black (≈10 mg) is added and after an additional 10 minutes of $Ar_{(g)}$ bubbling, the solution is left stirring under $H_{2(g)}$ 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).

(GlcGroP)(GroP)$_5$-Spacer or Hexamer (1)



Compound **23** (6 µmol) was deprotected following the general procedure. The final product **1** was obtained in 78% yield (4.7 µmol).

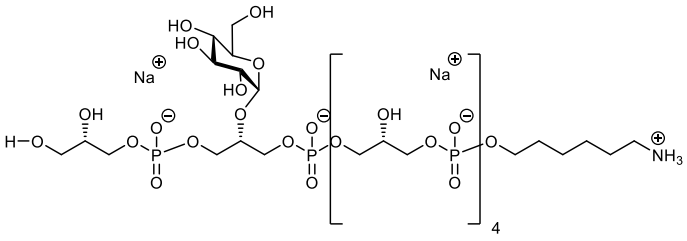
^{1}H-NMR (850 MHz, CD_3CN), δ: 5.07 (1H, H_1, J=3.8 Hz, d), 4.05-3.95 (7H, 5 x $CH_{glycerol}$, $CH_{2\_glycerol}$, m), 3.95-3.78 (24H, 10 $CH_{2\_glycererol,}$ $CH\mathit{H}_{glycerol}$, $CH_{2\_Ospacer}$, H_5, m), 3.77-3.64 (4H, 2 x H_6, H_3, $CH\mathit{H}_{glycerol}$, m), 3.49 (1H, H_2, J=3.8 Hz, J=9.9 Hz, dd), 3.38-3.30 (1H, H_4, m), 3.00-2.91 (2H, $CH_{2\_Nspacer}$, m), 1.69-1.56 (4H, CH_{2_spacer}, m), 1.45-1.34 (4H, CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 100.4 (C_1), 79.7 ($CH_{glycerol}$), 75.6 (C_3), 74.5 (C_5), 74.2 (C_2), 72.3 (C_4), 72.2-72.1 ($CH_{glycerol}$), 68.9-68.6 ($CH_{2\_glycerol}$), 67.8-67.7 ($CH_{2\_glycerol}$), 63.1 (C_6), 62.8 ($CH_{2\_glycerol}$), 42.1 ($CH_{2\_Nspacer}$), 32.0, 29.2, 27.7, 27.1 ($CH_{2spacer}$).

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

HRMS: $C_{30}H_{67}NO_{36}P_6$ + H^+ required 1204.1941, found 1204.1951

(GroP)(GlcGroP)(GroP)$_4$-Spacer or Hexamer (2)



Compound **24** (11 µmol) was deprotected following the general procedure. The final product **2** was obtained in 73% yield (8 µmol).

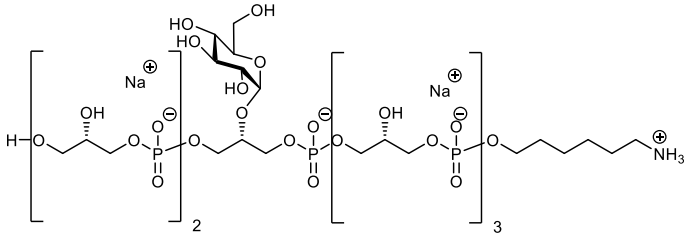
^{1}H-NMR (850 MHz, CD_3CN), δ: 5.14 (1H, H_1, J=3.8 Hz, d), 4.11-4.04 (1H, $CH_{glycerol}$, m), 4.05-3.95 (8H, 4 x $CH_{glycerol}$, 2 x $CH_{2\_glycerol}$, m), 3.95-3.78 (23H, 9 x $CH_{2\_glycererol,}$ H_6, $CH_{2\_Ospacer}$, H_5, $CH_{glycerol}$, m), 3.76-3.68 (2H, H_6, H_3, m), 3.64 (1H, $CH\mathit{H}_{glycerol}$, J=4.3 Hz, J=11.8 Hz, dd), 3.56 (1H, $CH\mathit{H}_{glycerol}$, J=6.1 Hz, J=11.8 Hz, dd) 3.50 (1H, H_2, J=3.8 Hz, J=9.9 Hz, dd), 3.35 (1H, H_4, J=9.6 Hz, t), 2.96 (2H, $CH_{2\_Nspacer}$, J=7.5 Hz, t), 1.69-1.56 (4H, CH_{2_spacer}, m), 1.45-1.34 (4H, CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 98.6 (C_1), 76.2-76.1 ($CH_{glycerol}$), 73.8 (C_3), 72.7 (C_5), 72.4 (C_2), 71.7-71.6 ($CH_{glycerol}$), 70.6 (C_4), 70.5-70.3 ($CH_{glycerol}$), 67.3-66.9 ($CH_{2\_glycerol}$), 66.1 ($CH_{2\_glycerol}$), 65.3 ($CH_{2glycerol}$), 62.8 ($CH_{2\_glycerol}$), 61.4 (C_6), 42.1 ($CH_{2\_Nspacer}$), 32.0, 29.2, 27.7, 27.1 ($CH_{2spacer}$).

^{31}P-NMR(162 MHz, CD_3CN), δ: 1.62, 1.84, 1.94, 2.04.

HRMS: $C_{30}H_{67}NO_{36}P_6$ + H^+ required 1204.1941, found 1204.1956

$(GroP)_2(GlcGroP)(GroP)_3$-Spacer or Hexamer (3)



Compound **25** (6 µmol) was deprotected following the general procedure. The final product **3** was obtained in 62% yield (3.7 µmol).

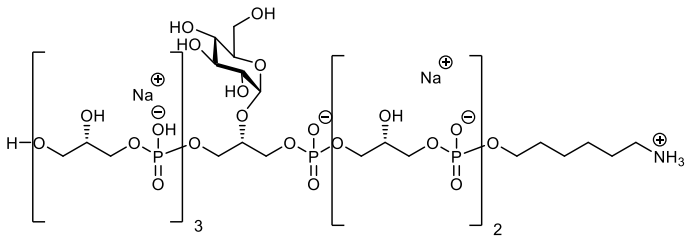
^{1}H-NMR (850 MHz, CD_3CN), δ: 5.14 (1H, H_1, J=3.8 Hz, d), 4.11-4.04 (1H, $CH_{glycerol}$, m), 4.05-3.95 (8H, 4 x $CH_{glycerol}$, 2 x $CH_{2\_glycerol}$, m), 3.95-3.78 (23H, 9 x $CH_{2\_glycererol,}$ H_6, $CH_{2\_Ospacer}$, H_5, $CH_{glycerol}$, m), 3.76-3.68 (2H, H_6, H_3, m), 3.64 (1H, $CH\mathit{H}_{glycerol}$, J=4.3 Hz, J=11.8 Hz, dd), 3.56 (1H, $CH\mathit{H}_{glycerol}$, J=6.1 Hz, J=11.8 Hz, dd) 3.50 (1H, H_2, J=3.8 Hz, J=9.9 Hz, dd), 3.35 (1H, H_4, J=9.6 Hz, t), 2.96 (2H, $CH_{2\_Nspacer}$, J=7.5 Hz, t), 1.69-1.56 (4H, CH_{2_spacer}, m), 1.45-1.34 (4H, CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 98.6 (C_1), 76.2-76.1 ($CH_{glycerol}$), 73.8 (C_3), 72.7 (C_5), 72.4 (C_2), 71.7-71.6 ($CH_{glycerol}$), 70.6 (C_4), 70.5-70.3 ($CH_{glycerol}$), 67.3-66.9 ($CH_{2\_glycerol}$), 66.1 ($CH_{2\_glycerol}$), 65.3 ($CH_{2glycerol}$), 62.8 ($CH_{2\_glycerol}$), 61.4 (C_6), 42.1 ($CH_{2\_Nspacer}$), 32.0, 29.2, 27.7, 27.1 ($CH_{2spacer}$).

^{31}P-NMR(162 MHz, CD_3CN), δ: 1.62, 1.84, 1.94, 2.04.

HRMS: $C_{30}H_{67}NO_{36}P_6$ + H^+ requires 1204.1941, found 1204.1951

$(GroP)_3(GlcGroP)(GroP)_2$-Spacer or Hexamer (4)



Compound **26** (9 µmol) was deprotected following the general procedure. The final product **4** was obtained in 81% yield (7.3 µmol).

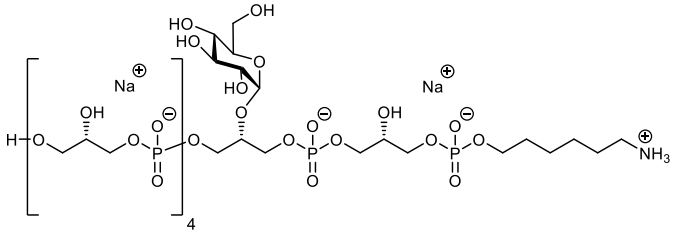
^{1}H-NMR (850 MHz, CD_3CN), δ: 5.14 (1H, H_1, J=3.8 Hz, d), 4.11-4.04 (1H, $CH_{glycerol}$, m), 4.05-3.95 (8H, 4 x $CH_{glycerol}$, 2 x $CH_{2\_glycerol}$, m), 3.95-3.78 (23H, 9 x $CH_{2\_glyceregerol,}$ H_6, $CH_{2\_Ospacer}$, H_5, $CH_{glycerol}$, m), 3.76-3.68 (2H, H_6, H_3, m), 3.64 (1H, $CHH_{glycerol}$, J=4.3 Hz, J=11.8 Hz, dd), 3.56 (1H, $CHH_{glycerol}$, J=6.1 Hz, J=11.8 Hz, dd) 3.50 (1H, H_2, J=3.8 Hz, J=9.9 Hz, dd), 3.35 (1H, H_4, J=9.6 Hz, t), 2.96 (2H, $CH_{2\_Nspacer}$, J=7.5 Hz, t), 1.69-1.56 (4H, CH_{2_spacer}, m), 1.45-1.34 (4H, CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 98.6 (C_1), 76.2-76.1 ($CH_{glycerol}$), 73.8 (C_3), 72.7 (C_5), 72.4 (C_2), 71.7-71.6 ($CH_{glycerol}$), 70.6 (C_4), 70.5-70.3 ($CH_{glycerol}$), 67.3-66.9 ($CH_{2\_glycerol}$), 66.1 ($CH_{2\_glycerol}$), 65.3 ($CH_{2glycerol}$), 62.8 ($CH_{2\_glycerol}$), 61.4 (C_6), 42.1 ($CH_{2\_Nspacer}$), 32.0, 29.2, 27.7, 27.1 ($CH_{2spacer}$).

^{31}P-NMR(162 MHz, CD_3CN), δ: 1.62, 1.84, 1.94, 2.04.

HRMS: $C_{30}H_{67}NO_{36}P_6$ + H^+ required 1204.1941, found 1204.1949

$(GroP)_4(GlcGroP)(GroP)$ -Spacer or Hexamer (5)



Compound **27** (16 µmol) was deprotected following the general procedure. The final product **5** was obtained in 78% yield (12 µmol).

^{1}H-NMR (850 MHz, CD_3CN), δ: 5.14 (1H, H_1, J=3.8 Hz, d), 4.11-4.04 (1H, $CH_{glycerol}$, m), 4.05-3.95 (8H, 4 x $CH_{glycerol}$, 2 x $CH_{2\_glycerol}$, m), 3.95-3.78 (23H, 9 x $CH_{2\_glyceregerol,}$ H_6, $CH_{2\_Ospacer}$, H_5, $CH_{glycerol}$, m),

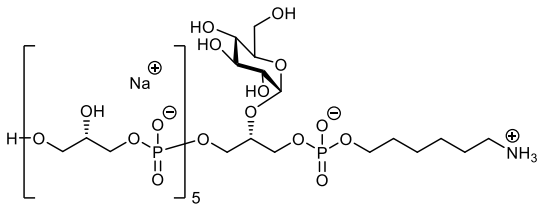
3.76-3.68 (2H, H_6, H_3, m), 3.64 (1H, CH$H_{glycerol}$, J=4.3 Hz, J=11.8 Hz, dd), 3.56 (1H, CH$H_{glycerol}$, J=6.1 Hz, J=11.8 Hz, dd) 3.50 (1H, H_2, J=3.8 Hz, J=9.9 Hz, dd), 3.35 (1H, H_4, J=9.6 Hz, t), 2.96 (2H, $CH_{2\_Nspacer}$, J=7.5 Hz, t), 1.69-1.56 (4H, CH_{2_spacer}, m), 1.45-1.34 (4H, CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 98.6 (C_1), 76.2-76.1 ($CH_{glycerol}$), 73.8 (C_3), 72.7 (C_5), 72.4 (C_2), 71.7-71.6 ($CH_{glycerol}$), 70.6 (C_4), 70.5-70.3 ($CH_{glycerol}$), 67.3-66.9 ($CH_{2\_glycerol}$), 66.1 ($CH_{2\_glycerol}$), 65.3 ($CH_{2glycerol}$), 62.8 ($CH_{2\_glycerol}$), 61.4 (C_6), 42.1 ($CH_{2\_Nspacer}$), 32.0, 29.2, 27.7, 27.1 ($CH_{2spacer}$).

^{31}P-NMR(162 MHz, CD_3CN), δ: 1.62, 1.84, 1.94, 2.04.

HRMS: $C_{30}H_{67}NO_{36}P_6$ + H^+ required 1204.1941, found 1204.1957

(GroP)$_5$(GlcGroP)-Spacer or Hexamer (6)



Compound **28** (21 µmol) was deprotected following the general procedure. The final product **6** was obtained in 68% yield (14 µmol).

^{1}H-NMR (850 MHz, CD_3CN), δ: 5.14 (1H, H_1, J=3.8 Hz, d), 4.11-4.04 (1H, $CH_{glycerol}$, m), 4.05-3.95 (8H, 4 x $CH_{glycerol}$, 2 x $CH_{2\_glycerol}$, m), 3.95-3.78 (23H, 9 x $CH_{2\_glycererol}$, H_6, $CH_{2\_Ospacer}$, H_5, $CH_{glycerol}$, m), 3.76-3.68 (2H, H_6, H_3, m), 3.64 (1H, CH$H_{glycerol}$, J=4.3 Hz, J=11.8 Hz, dd), 3.56 (1H, CH$H_{glycerol}$, J=6.1 Hz, J=11.8 Hz, dd) 3.50 (1H, H_2, J=3.8 Hz, J=9.9 Hz, dd), 3.35 (1H, H_4, J=9.6 Hz, t), 2.96 (2H, $CH_{2\_Nspacer}$, J=7.5 Hz, t), 1.69-1.56 (4H, CH_{2_spacer}, m), 1.45-1.34 (4H, CH_{2_spacer}, m).

^{13}C-NMR(101 MHz, CD_3CN), δ: 98.6 (C_1), 76.2-76.1 ($CH_{glycerol}$), 73.8 (C_3), 72.7 (C_5), 72.4 (C_2), 71.7-71.6 ($CH_{glycerol}$), 70.6 (C_4), 70.5-70.3 ($CH_{glycerol}$), 67.3-66.9 ($CH_{2\_glycerol}$), 66.1 ($CH_{2\_glycerol}$), 65.3 ($CH_{2glycerol}$), 62.8 ($CH_{2\_glycerol}$), 61.4 (C_6), 42.1 ($CH_{2\_Nspacer}$), 32.0, 29.2, 27.7, 27.1 ($CH_{2spacer}$).

^{31}P-NMR(162 MHz, CD_3CN), δ: 1.62, 1.84, 1.94, 2.04.

HRMS: $C_{30}H_{67}NO_{36}P_6$ + H^+ requires 1204.1941, found 1204.1948

Generation and Serum analysis of microarrays

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 containing 5% of Tween® 20 and PBS containing 1% of Tween® 20 subsequently. After removal of the PBS containing 1% of Tween® 20, the arrays were shaken with the primary antibody dilutions (0.25 ml, diluted with PBS containing 1% of Tween® 20 and 10 mg/ml of BSA) for 60 minutes. Serum obtained from rabbits immunized with native LTA isolated from *E. faecalis* strain **12030** was used at a 1:1000 dilution, while rabbit serum raised against the previously reported BSA-WH7 at a 1:500 dilution. The slides were flushed with PBS containing 5% of Tween® 20 and PBS containing 1% of Tween® 20 subsequently. After removal of the PBS containing 1% of Tween® 20, slides were shaken with anti-rabbit-IgG secondary antibodies, labeled with DyLight 550 reporter groups (0.25 ml, 0.5 µg/ml final dilution in PBS containing 1% of Tween® 20 and 10 mg/ml of BSA) for 30 minutes in the dark. The slides were flushed with PBS containing 5% of Tween® 20, PBS and MilliQ subsequently. The slides were dried by centrifugation and were analyzed on fluorescence on 532 nm and 635 nm using a G2565BA scanner. Data and image analyses were performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, USA) as described previously (J. Proteome Res., 8 (2009), pp. 4301–4310). Fluorescence intensities were quantified and corrected for background/non-specific antibody adhesion by subtracting the fluorescence at blank spots, where only spotting buffer was printed without GTA fragment. The average of the triplicate spots was normalized to the highest intensity on the array and visualized in bar graphs using Microsoft Excel.

References

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Chapter 5

Epitope recognition of a monoclonal antibody raised against a synthetic glycerol phosphate based teichoic acid

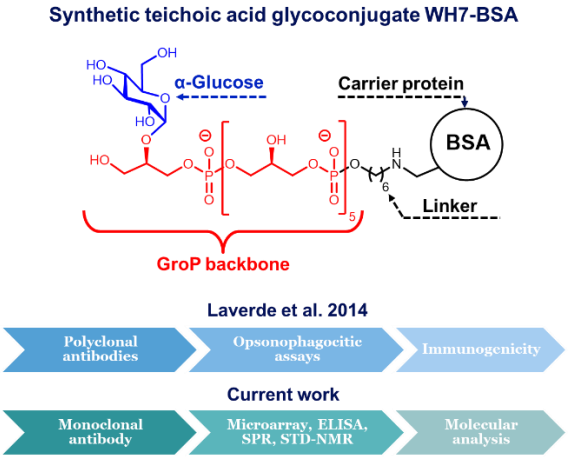
F. Berni,* E. Kalfopoulou,* A. C. Gimeno, F. Carboni, D. van der Es, F. Romero-Saavedra, D. Laverde, K. Miklic, S. Malic, T. Rovis, S. Jonjic, S. Ali, H. S. Overkleeft, C. H. Hokke, A. van Diepen, R. Adamo, J. Jiménez-Barbero, G. A. van der Marel, J. Huebner, J. D. C. Codée, *ACS Chem. Biol.*, **2021**, 16: 1344-1349.

INTRODUCTION

Teichoic acids are poly-alditolphosphate cell-wall components found in the majority of Gram-positive bacteria. They are divided into two main classes, differing in the way they are connected to the cell-wall: wall teichoic acids (WTAs) are covalently connected to the peptidoglycan, while lipoteichoic acids (LTAs) are inserted in the lipid bilayer through a lipid anchor via non-covalent, hydrophobic interactions.[1,] They are involved in various biological processes and as their structure extends towards the extracellular milieu, they have been appointed as possible antigen candidates for vaccine development. [2]

Staphylococci and enterococci are Gram-positive bacteria, associated with a variety of severe infections.[3] The prevalence of multidrug resistance strains in the clinical setting, such as methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE), has urged the development of alternative strategies, such as active or passive immunization.[4] LTAs from staphylococci and enterococci share a common structural motif composed of a glycerol phosphate (GroP) repeating unit.[5] The C-2-OH of the glycerol moiety can be decorated with D-alanine or carbohydrate appendages. Previously, a synthetic TA was generated, featuring an α-glucosyl substituent at the terminal unit of a GroP hexamer (**WH7**) and conjugated to the carrier protein Bovine Serum Albumin (BSA) in order to evaluate its immunogenicity and potential as a vaccine

Figure 1: *Schematic representation of the structure of the synthetic teichoic acid glycoconjugate WH7-BSA. Workflow of previous and current work.*



candidate (Figure 1).[6] It was observed that **WH7**-BSA was able to induce opsonic and protective cross-reactive antibodies against clinical isolates of *E. faecalis* **12030**, *E. faecium* E1162 and community acquired S. *aureus* MW2 (USA400), confirming the antigenic property of **WH7**. Microarray analysis showed that the polyclonal serum raised against the conjugate contained antibodies that preferentially bound α-glucosyl functionalized GroP-oligomers.[7] Simultaneously, Snapper and co-workers used a non-

glycosylated synthetic GroP-decamer to generate a TA-conjugate and showed that this could be used to raise protective antibodies against *S. aureus*.[8] These studies have revealed synthetic TA-fragments as attractive antigens in the generation of well-defined synthetic vaccines. The molecular basis of their immunogenicity however has not been elucidated and understanding how these fragments are recognized by the generated antibodies will provide valuable insights for the design of improved conjugate vaccines.[9] Therefore, in the current study, the aforementioned WH7-conjugate was employed for the generation of a monoclonal antibody (WH7.01 mAb) to characterize the binding with synthetic GroP based fragments at the molecular level using TA-microarrays, ELISA, SPR-binding studies and STD-NMR. This work represents the first case-study for a synthetic GroP based TA immunogen and the detailed binding studies have revealed that all structural components, the length and chirality of the GroP backbone, as well as the presence of the carbohydrate substituent, contribute to binding of the antibody.

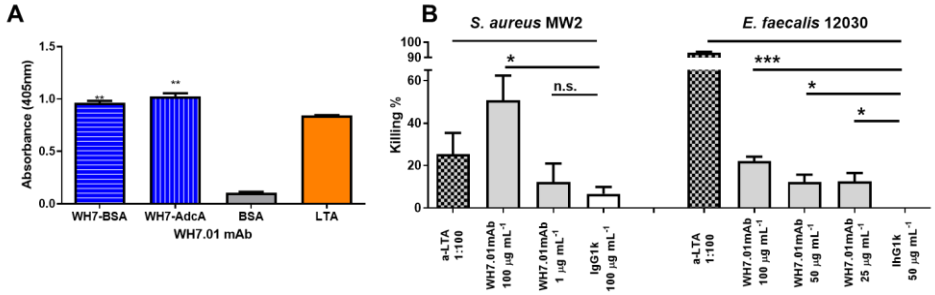
RESULTS AND DISCUSSION

First, the hybridoma technology was implemented in order to generate a mAb against **WH7**.[10] Briefly, mice were immunized with the conjugated form of **WH7** to the commonly used carrier protein BSA. The generated hybridomas were selected by ELISA against the conjugated form of **WH7** and by negative selection with BSA (see experimental section). The secreted mAb from the resulting hybridoma cell line, named WH7.01 mAb, was confirmed by ELISA to be of the IgG1 class for the heavy chain and kappa for the light chain (data not shown). WH7.01 mAb exhibited strong binding in ELISA against the **WH7**-BSA conjugate as well as towards a second conjugate that was constructed using the zinc ABC transporter substrate-binding lipoprotein AdcA[11] from *E. faecium* (Figure 2A). It also bound native LTAs from *S. aureus* (commercially available from Sigma) but it didn't recognize BSA neither in ELISA nor in Western blot (see experimental section). These results confirmed the development of a new mAb that specifically recognized the synthetic teichoic acid **WH7** antigen. This monoclonal was evaluated in an opsonophagocytic assay and it was observed that the WH7.01 mAb exhibited opsonic killing activity against *E. faecalis* **12030** and *S. aureus* MW2, with most killing of the latter species (Figure 2B).

Figure 2: *Generation of the monoclonal antibody against WH7-BSA. A) Antigen-specificity of the supernatant from the hybridoma cell line producing the WH7.01 mAb. The binding was evaluated by ELISA against the synthetic antigen WH7 (blue) conjugated to BSA (horizontal stripes) or to AdcA (vertical stripes), the unconjugated carrier protein BSA (grey) and the commercially available LTAs from S. aureus (orange), all coated in duplicates with 1 µg well–1. The concentration of the WH7.01 mAb in the supernatant was 35 µg mL–1. Bars represent mean data and the error bars represent the standard errors of the means. Significance was inferred by two tailed unpaired t-tests between WH7 conjugates and the carrier protein (**, P<0.01). B) Opsonophagocytic killing activity of the newly generated WH7.01 mAb against S. aureus MW2 on the left and E. faecalis* ***12030*** *on the right. The opsonophagocytic killing activity of the purified monoclonal from the hybridoma cells producing WH7.01 mAb (grey) at different dilutions was evaluated against S. aureus MW2, and E. faecalis* ***12030****. Polyclonal sera raised in a rabbit against the purified LTA from E. faecalis* ***12030*** *(black and grey squares) was used as a positive control and a mAb of the same isotype, IgG1κ, was*

*used as a negative control. Bars represent mean data and the error bars represent the standard errors of the means. n.s. (not significant), *, P < 0.05; ***, P < 0.001; all by one-way ANOVA with Dunnett's multiple comparison to the negative control (monoclonal antibody of the same isotype).*

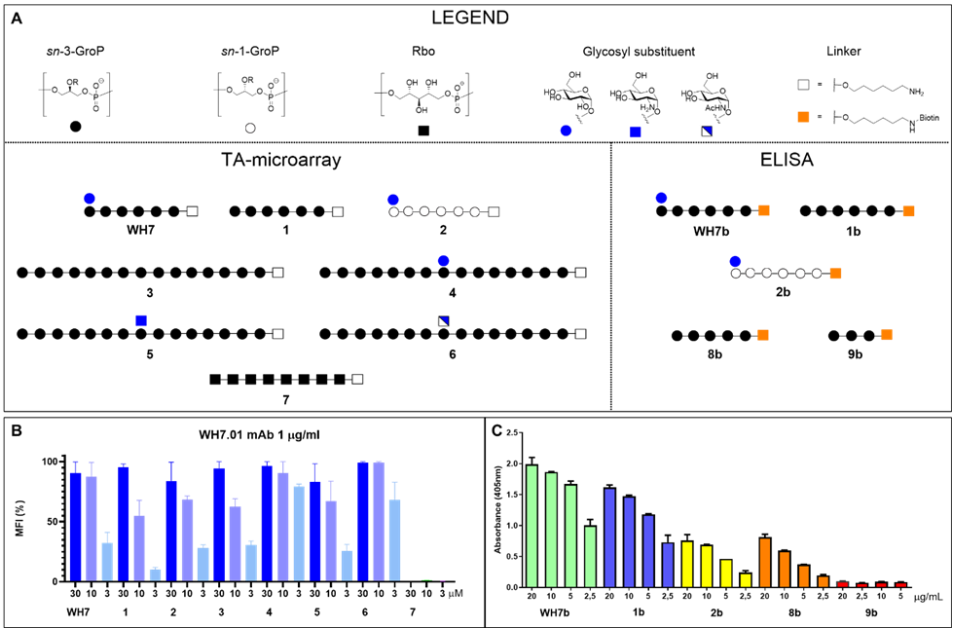
Next, the binding specificity of the newly generated monoclonal was qualitatively



assessed using a TA-microarray. This tool allows the simultaneous screening of the binding interaction of a designated protein with a number of substrates using only minute amounts of materials.[12] In line with previous TA-microarray studies (Chapters 3 and 4),[7,13] different synthetic GroP-fragments, each equipped with an aminohexanol linker, were covalently immobilized on an epoxide functionalized microarray glass slide. The compounds were printed on the slides in three different concentrations (30 µM, 10 µM and 3 µM) in triplicates. As shown in Figure 3A, the tested library comprises GroP-based fragments varying in length (hexamers or pentadecamers), nature of the glucosyl substituent (α-glucose, α-glucosamine or α-N-acetylglucosamine), the position of the carbohydrate on the chain (terminal or in the middle) and the stereochemistry of the glycerol unit (*sn*-1 vs. *sn*-3). Compound **7**, a ribitol phosphate chain resembling the structure of *S. aureus* WTAs,[14] was included as negative control. As can be observed from the results shown in Figure 3B, the WH7.01 mAb recognized all printed GroP-based TA fragments well, indicating that the GroP-backbone is the main recognition element for the mAb. The sugar appendage also seems to contribute to binding with WH7 being recognized slightly better than its non-glucosylated counterpart **1** and α-glucosyl pentadecamer **4** showing the strongest binding among the longer GroP-fragments. The ribitol phosphate-based S. aureus WTA fragment **7** was not recognized by the monoclonal. To further validate the binding, ELISA assays were performed using the biotinylated derivatives **WH7b**, **1b** and **2b** as well as the shorter pentamer **8b** and trimer **9b** (Figure 3A). The synthetic fragments, tagged with biotin, were immobilized at a concentration of 1 µM on streptavidin coated plates and different dilutions of the monoclonal were employed (20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml) in duplicates. These ELISA studies (Figure 3C) revealed that the binding of WH7.01 mAb is influenced by three structural elements: the length of the GroP chain (**1b** > **8b** >> **9b**), the presence of the glucosyl substituent (**WH7** > **1b**) and the stereochemistry[13] of the glycerol backbone (**WH7** >> **2b**). These results overall confirm the observations from the microarray analysis, that the GroP backbone is the major structural feature that is recognized by this monoclonal antibody. At the same time, it becomes more prominent that the number of

repeating units, the relative position of the C-2-OH (*sn*-1 vs *sn*-3 GroP) and the presence of the glucosyl substituent also have an impact. Previously it has been observed that the GroP backbone stereochemistry did play an important role in IgG binding of the polyclonal sera, raised against **WH7** antigen or native *E. faecalis* LTA.[23] In these cases the major epitope recognized was the glycosyl substituent, while binding of the monoclonal antibody generated here is dominated by the GroP backbone (**1b** and **8b**), likely driven by ionic interactions with the phosphate moieties. The preferential binding of the monoclonal to **WH7** compared to its diastereoisomer **2** is determined by the different GroP stereochemistry, but it also suggests a role of the glucosyl appendage to the antibody interaction. Without the glucosyl substituent, the influence of the chirality of the internal GroP residues of the *sn*-1-GroP and *sn*-3-GroP oligomers is lost.

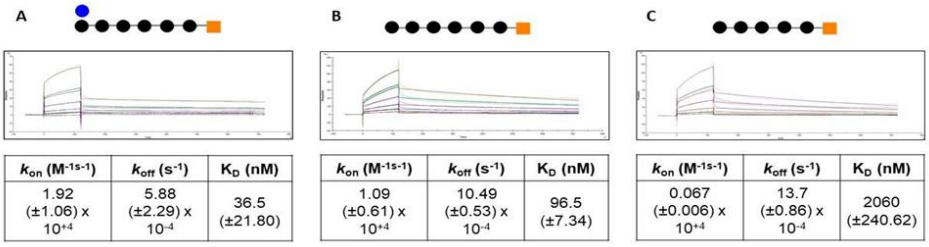
Figure 3: *TA-library overview and binding assays results from microarray and ELISA. A) Overview of the synthetic fragments used for the binding analysis of WH7.01 mAb. B) TA-microarray results with WH7.01 mAb at 1 µg ml–1. Compounds WH7, 1¬7 were immobilized on an epoxide functionalized glass slide in three different concentrations: 30 µM (dark blue), 10 µM (shade blue) and 3 µM (light blue). The average of the triplicate spots was normalized to the highest intensity on the array. C) ELISA results of WH7.01 mAb at different concentrations against WH7b (Light green), 1b (blue), 2b (yellow), 8b (orange) and 9b (red). All biotin-derivatives were coated at the same concentration (1 µM) on a streptavidin ELISA plate. Bars represent mean data and the error bars the standard errors of the means.*



In order to investigate binding in more detail, a quantitative analysis of binding parameters was performed by measuring the antibody-ligand interaction by SPR.[15] To this end, **WH7b**, non-glucosylated hexamer **1b** and pentamer **8b** were immobilized on a streptavidin functionalized sensor chip. Figure 4 reveals a higher affinity towards the

hexameric fragments (**WH7b** and **1b**) than to the pentamer (**8b**). The length of the TA backbone significantly affects the k_{on} with the value for compound **8b** being 16 and 28 times lower than **1b** and **WH7b**, respectively. While **WH7b** and **1b** have the same number of GroP repeating units, the affinity slightly increases when the glucosyl substituent is present. The carbohydrate appendage contributes positively to the binding by increasing the k_{on} and decreasing the k_{off} value.

Figure 4: *Binding kinetics and kinetic and affinity constants of WH7.01 mAb against WH7b (A), compound 1b (B) and 8b (C), respectively. Serial dilutions of the analyte, WH7.01 mAb (2000¬62.5 nM), were run. The numbers in parentheses represent the standard deviations of kon and koff. Results are representative of three independent experiments.*



k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
1.92 (±1.06) x 10^{+4}	5.88 (±2.29) x 10^{-4}	36.5 (±21.80)

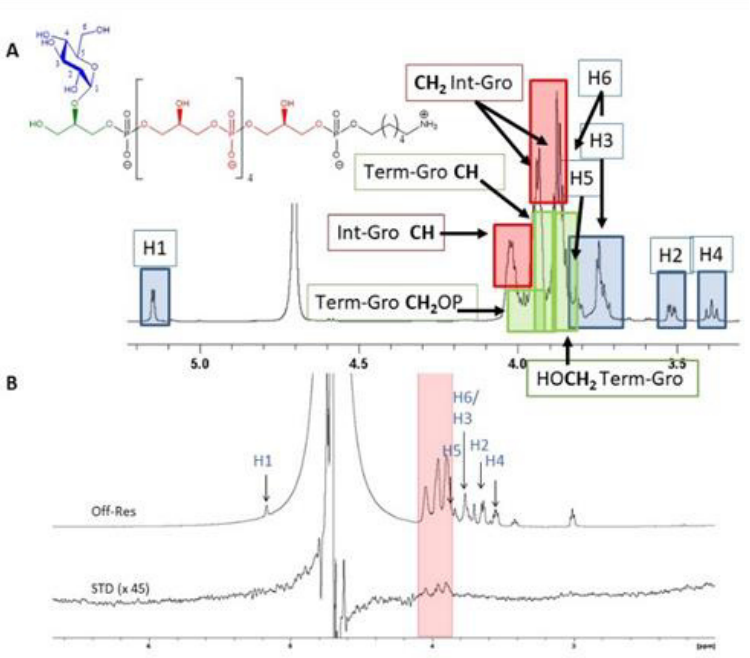
k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
1.09 (±0.61) x 10^{+4}	10.49 (±0.53) x 10^{-4}	96.5 (±7.34)

k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
0.067 (±0.006) x 10^{+4}	13.7 (±0.86) x 10^{-4}	2060 (±240.62)

STD-NMR spectroscopy has been widely used in the last decades for structural epitope-mapping.[16] Therefore, our binding study between the monoclonal and its ligand **WH7** was continued using this technique. The experiment was carried out using a 2.5 µM concentration of WH7.01 with a protein/ligand ratio of 1:100, at 303 K to avoid overlapping of the glucosyl anomeric proton with the HDO signal. Figure 5 shows the ^{1}H-NMR spectrum of the ligand alone (A) and the STD-NMR experiment (B). Because of the repetitive nature of the ligand there is significant signal overlap of the GroP repeating units, but the signals of the glucosyl H1, H2 and H4 can be clearly distinguished. STD effects can be observed for signals related to CH/CH_2 of the glycerol units while no significant involvement of the glucosyl substituent is detected. This indicates that the sugar substituent is not directly involved in the binding of WH7.01 mAb. Taking into account the SPR results, showing that the presence of the glucosyl moiety provides a better GroP binder for the case-study antibody, it may be speculated that the glucosyl moiety reduces the conformational freedom of the terminal glycerol unit and/or provides a conformation that enables stabilizing additional contacts with the mAb, which has previously been observed in the immunorecognition of other polysaccharides.[17]

Figure 5: *STD-NMR analysis. A) Assignment of the 1H-NMR spectrum of WH7 in D_2O at room temperature. H peeks for the intermediate GroP (Int-Gro CH/CH_2), terminal GroP (Term-Gro $CH/CH_2OP/HOCH_2$) and a-Glucose (H1 to 5) are assigned with red, green and blue, respectively. B)*

Off- (top) and on-resonance (bottom) of 1H-STD-NMR spectra between WH7.01 mAb (2.5 µM) and WH7 (0.5 mM) at 303K.



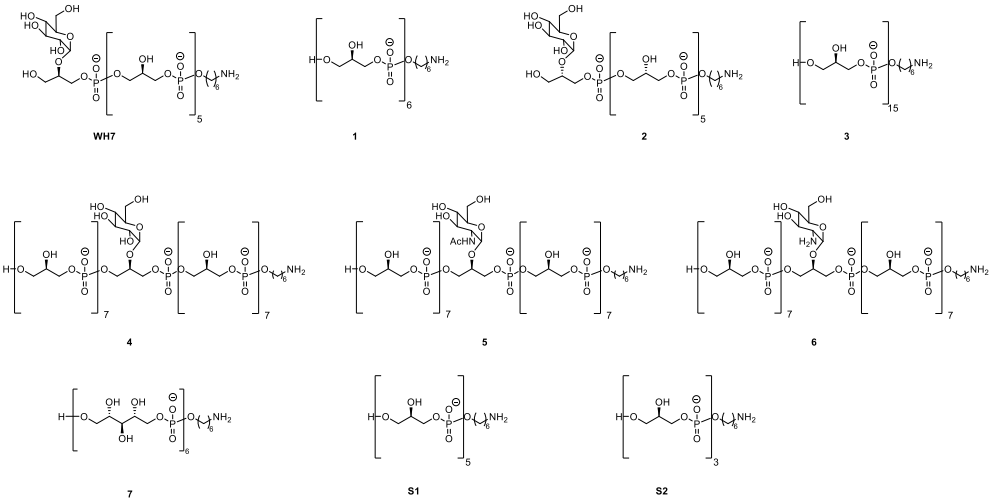
CONCLUSION

In conclusion, the first mAb raised against a synthetic glycosylated GroP based TA has been generated by hybridoma technology. This mAb showed high binding specificity towards both the synthetic antigen and the commercially available LTA from *S. aureus*. Different techniques were used to reveal the key structural elements for ligand recognition. A first screening using a TA-microarray showed the GroP backbone as the main recognition element. Further analysis using ELISA and SPR indicated the importance of the number of GroP residues as well as a role for the glucosyl substituent. STD-NMR spectroscopy revealed interactions with the GroP backbone, but no interaction of the glucose residue with the mAb was detected. This suggests a possible indirect contribution of the glucosyl substituent to the binding. The carbohydrate may impose a particular conformational geometry to the GroP residue or backbone, leading to increased binding of the glucosylated TA. Due to the high structural heterogenicity in TAs from native sources, the availability of more well-defined TA-fragments[18] can provide insights on the structural elements (the backbone, the stereochemistry, carbohydrates as well as D-Ala substituents) required for TA-antibody binding. In particular, the workflow here presented can be used in the future for the generation of a library of mAbs[15] with high specificity to a variety of different poly-alditolphosphate antigen candidates and to analyze the structure-immunogenicity relationship for future TA-based vaccine development.

EXPERIMENTAL SECTION

Overview synthetic fragments

***Figure 6**: Overview of the synthetic GroP-TA fragments used for the study of epitope recognition by WH7.01 mAb*



Synthesis of fragments WH7, 1-7, S1-S3 and WH7-BSA conjugate

The synthesis and the characterizations can be found to the corresponding references:

WH7, **1**: Hogendorf, W.F.J., Meeuwenoord, N., Overkleeft, H.S., Filippov, D.V., Laverde, D., Kropec, A., Huebner, J., Van der Marel, G.A., Codée, J.D.C. (2011) Automated solid phase synthesis of teichoic acids. Chem. Commun. 47, 8961-8963.

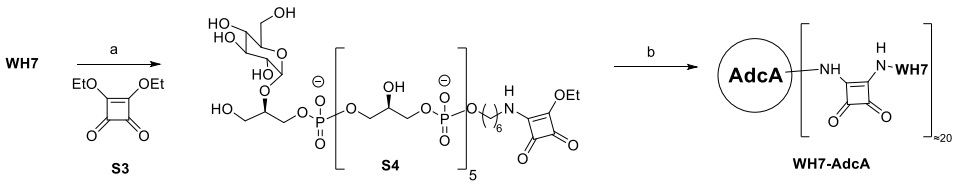
3-6: Van der Es, D., Berni, F., Hogendorf, W. F. J., Meeuwenoord, N., Laverde, D., van Diepen, A., Overkleeft, H.S., Filippov, D.V., Hokke, C. H., Huebner, J., van der Marel, G. A., Codée, J. D. C. (2018) Streamlined synthesis and evaluation of teichoic acid fragments. Chem.-Eur. J. 24, 4014–4018.

7: Hendriks, A., van Dalen, R., Ali, S., Gerlach, D., van der Marel, G. A., Fuchsberger, F. F., Aerts, P. C., de Haas, C. J. C., Peschel, A., Rademacher, C., van Strijp, J. A. G., Codée, J. D. C., van Sorge, N. M. (2021) Impact of Glycan Linkage to Staphylococcus aureus Wall Teichoic Acid on Langerin Recognition and Langerhans Cell Activation ACS Infect. Dis. 7, 624-635

2: Berni, F., Wang, L., Kalfopoulou, E., Nguyen, D. L., van der Es, D., Huebner,J., Overkleeft, H. S., Hokke, C. H., van der Marel, G. A., van Diepen, A., Codée, J. D. C. (2021) Generation of glucosylated sn-1-glycerolphosphate teichoic acids: glycerol stereochemistry affects synthesis and antibody interaction RSC Chem. Biol. 2, 187-191.

S1, S2: Hogendorf, W.F.J., Lameijer, L.N., Beenakker, T.J.M., Overkleeft, H. S., Filippov, D. V., Codée, J. D. C., Van der Marel, G. A. (2012) Fluorous linker facilitated synthesis of teichoic acid fragments. Org. Lett. 14, 848-851.
WH7-BSA conjugate: Laverde, D., Wobser, D., Romero-Saavedra, F., Hogendorf, W., van der Marel, G. A., Berthold, M., Kropec, A., Codee, J. D. C., Huebner, J. (2014) Synthetic Teichoic Acid Conjugate Vaccine against Nosocomial Gram-Positive Bacteria. PLoS ONE. 9(10): e110953.

Generation of WH7-AdcA conjugate

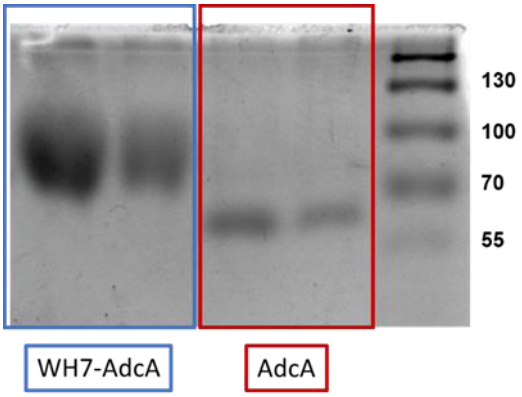


Scheme 1*: Generation of WH7-AdcA conjugate. a) S3, PBS (pH=8), EtOH, 92%; b) S4, borate buffer (pH=9).*

WH7-ethylsquarate (S4)
Hexamer **WH7** (10 mg, 8.3 µmol) was dissolved in a mixture of water (0,83 ml) and sodium phosphate buffer (1 M, pH=8, 42 µl). A solution of 3,4-Diethoxy-3-cyclobutene-1,2-dione (S3, 0.04 M in EtOH, 1.0 ml, 42 µmol) was added. The mixture was stirred for 24 hours and the EtOH was evaporated by flushing dry air over the reaction mixture. The mixture was purified by size-exclusion chromatography (HW40, dimensions: 16/60 mm, eluent: 0.15 M NH_4OAc). After repeated lyophilization, the product was eluted through a small column containing Dowex Na^+ cation-exchange resin (type: 50WX4¬200, stored on 0.5 M NaOH in H_2O, flushed with H2O and MeOH before use). Lyophilization gave squarate equipped hexamer S4 (10.2 mg, 7.7 µmol) in 92% yield. 1H NMR (500 MHz, D2O) δ: 5.18 (d, J = 3.9 Hz, 1H), 4.12 – 4.02 (m, 6H), 4.01 – 3.95 (m, 10H), 3.94 – 3.82 (m, 16H), 3.82 – 3.72 (m, 4H), 3.69 – 3.58 (m, 1H), 3.58 – 3.48 (m, 2H), 3.42 (t, J = 9.7 Hz, 1H), 3.35 (s, 2H), 1.93 (s, 6H), 1.71 – 1.59 (m, 4H), 1.50 – 1.37 (m, 7H); 31P NMR (202 MHz, D2O) δ 1.5, 1.4, 1.4.

WH7-AdcA conjugate
AdcA was overexpressed and purified as described previously.1 Squarate **S4** (1.6 mg, 1.3 µmol) was dissolved in borate buffer (0.5 M, pH=9.0, 100 µl) and transferred to a vial containing AdcAFM (2.8 mg, 0.05 µmol) in PBS (20 µl). The mixture was shaken for 16 hours and passed over a Micro Bio-Spin Column (Bio-Gel P-6, Bio-Rad, pretreated with PBS) yielding WH7-AdcA conjugate. SDS-PAGE was used to check the formation and purification of the conjugate, while with orcinol mediated glycoside quantification a loading of ~20 hexamers per protein was observed.

***Figure 7**: SDS-PAGE on WH7-AdcA conjugate (blue) and AdcA (red)*



General procedure for orcinol mediated glycoside quantification

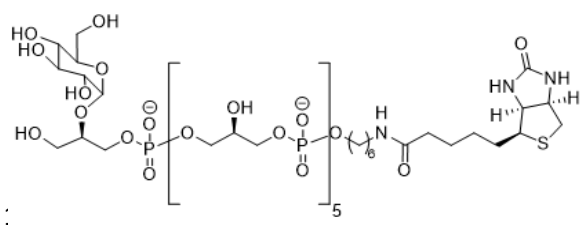
A series of defined glucose solutions (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg ml–1) was prepared. The solutions (100 µl) were gently mixed with a solution of orcinol (20 mg ml–1 in 25% H_2SO_4, 200 µl) in glass vials. H_2SO_4 (60%, 1.5 ml) was added forcefully and the mixtures were heated for 20 minutes at 80°C. The absorbance at 530 nm was measured for all samples and a calibration trend line was constructed. The conjugate was diluted to two concentrations (25 µM and 30 µM) and both dilutions (100 µl) were treated as described above. The absorbance at 530 nm was measured for both dilutions and they were plotted on the trend line to reveal the glucose concentration. Combined with the protein concentration, based on the initial protein concentration, loading was determined.

<u>Synthesis of biotinylated fragments</u>

General procedure

The synthetic fragment was dissolved in DMSO (2 mM), then DIPEA (1.5 eq) and Biotin-OSu (1.3 eq) were added and the mixture stirred overnight at room temperature. After centrifugation, the biotinylated fragment was purified by size exclusion chromatography (HW-40 column, dimensions: 16/60 mm, eluent 0.15 M NH_4OAc). After repeated co-evaporation with miliQ water to remove NH_4OAc, the product was eluted through a small column containing Dowex Na^+ cation-exchange resin (type 50WX8-50-100, stored on 0.5 M NaOH in H2O, flushed with H_2O and MeOH before use). Lyophilization yielded the product, which was characterized by ^{1}H-NMR.

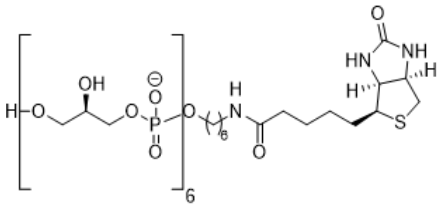
Compound WH7b



Starting from **WH7** (1 mg, 0.8 µmol), following the general procedure, compound **WH7b** (0.83 mg, 0.5 µmol) was isolated in 65% yield.

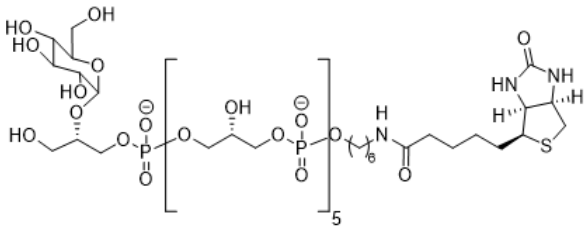
^{1}H NMR (500 MHz, D_2O) δ: 5.14 (d, J=3.9 Hz, 1H), 4.61¬4.54 (m, 1H), 4.44¬4.36 (m, 1H), 4.07¬3.66 (m, 36H), 3.55¬3.47 (m, 1H), 3.43¬3.26 (m, 2H), 3.20¬3.09 (m, 3H), 3.02¬2.93 (m, 1H), 2.75 (d, J=12.0 Hz, 1H), 2.28¬2.17 (m, 2H), 1.79¬1.26 (m, 14H)

Compound 1b



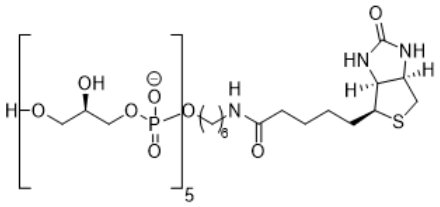
Starting from **1** (2.7 mg, 2.6 µmol), following the general procedure, compound **1b** (2.3 mg, 1.6 µmol) was isolated in 61% yield.
^{1}H NMR (500 MHz, D_2O) δ: 4.61¬4.52 (m, 1H), 4.44¬4.33 (m, 1H), 4.07¬3.71 (m, 28H), 3.69¬3.61 (m, 1H), 3.60¬3.50 (m, 1H), 3.34¬3.26 (m, 1H), 3.20¬3.06 (m, 2H), 3.00¬2.91 (m, 1H), 2.75 (d, J=12.0 Hz, 1H), 2.27¬2.16 (m, 2H), 1.78¬1.23 (m, 14H)

Compound 2b



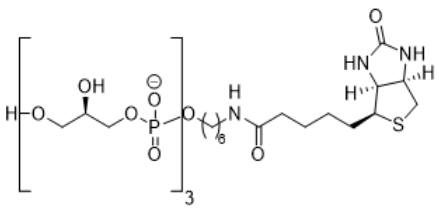
Starting from **2** (2.7 mg, 0.5 µmol), following the general procedure, compound **2b** (0.6 mg, 0.4 µmol) was isolated in 80% yield.
^{1}H NMR (500 MHz, D_2O) δ: 5.15 (d, J=3.9 Hz, 1H), 4.61¬4.54 (m, 1H), 4.44¬4.36 (m, 1H), 4.14¬3.79 (m, 30H), 3.79¬3.69 (m, 2H), 3.69¬3.61 (m, 1H), 3.61¬3.52 (m, 1H), 3.52¬3.45 (m, 1H), 3.41¬3.28 (m, 2H), 3.21¬3.09 (m, 3H), 3.01¬2.93 (m, 1H), 2.75 (d, J=12.0 Hz, 1H), 2.28¬2.17 (m, 2H), 1.80¬1.24 (m, 14H)

Compound 8b



Starting from **S1** (0.49 mg, 0.6 µmol), following the general procedure, compound **8b** (0.3 mg, 0.2 µmol) was isolated in 33% yield.
^{1}H NMR (500 MHz, D_2O) δ: 4.61¬4.52 (m, 1H), 4.44¬4.33 (m, 1H), 4.07¬3.71 (m, 25H), 3.69¬3.61 (m, 1H), 3.60¬3.50 (m, 1H), 3.34¬3.26 (m, 1H), 3.20¬3.06 (m, 2H), 3.00¬2.91 (m, 1H), 2.75 (d, J=12.0 Hz, 1H), 2.27¬2.16 (m, 2H), 1.78¬1.23 (m, 14H)

Compound 9b

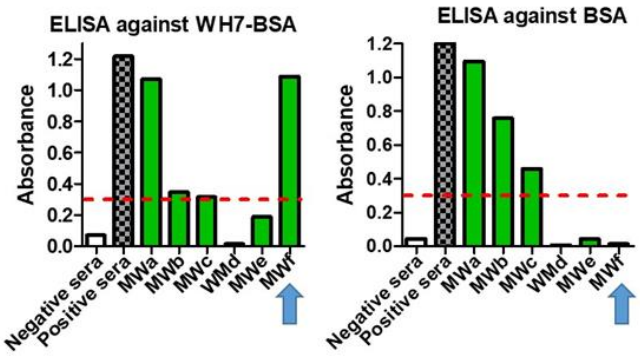


Starting from **S2** (0.8 mg, 1.4 µmol), following the general procedure, compound **9b** (0.6 mg, 0.7 µmol) was isolated in 50% yield.
^{1}H NMR (500 MHz, D_2O) δ: 4.61¬4.52 (m, 1H), 4.44¬4.33 (m, 1H), 4.07¬3.71 (m, 15H), 3.69¬3.61 (m, 1H), 3.60¬3.50 (m, 1H), 3.34¬3.26 (m, 1H), 3.20¬3.06 (m, 2H), 3.00¬2.91 (m, 1H), 2.75 (d, J=12.0 Hz, 1H), 2.27¬2.16 (m, 2H), 1.78¬1.23 (m, 14H)

Generation of the mouse monoclonal antibody

Three BALB/c mice were immunized by two subcutaneous injections of 50 µg, in protein content, of the glycoconjugate **WH7**-BSA with Freund's incomplete adjuvant given 2 weeks apart. The mouse sera were screened by ELISA for antibody titers against **WH7**-BSA and three weeks later, mice were subjected to a final intraperitoneal injection of 50 µg of glycoconjugate in PBS. Three days after the injection, mice were sacrificed and the splenocytes were isolated, red blood cells were eliminated by lysis and fusion of the splenocytes with SP2/O myeloma cells was performed at a 1:1 ratio. The cells were resuspended in RPMI supplemented with 20% fetal bovine serum (FBS) (PAN-Biotech) and hypoxanthine, aminopterin, and thymidine (Gibco) for the selection of the hybridomas and were equally distributed into six 96-well plates. The supernatants from the mother-wells (MWs) were selected by ELISA against **WH7**-BSA and by negative selection with ELISA against BSA. At the first selection point, from the three independent fusions, 1800 MWs were obtained and screened. From these MWs, only six were considered positive, having an absorbance of over 0.3 in ELISA against **WH7**-BSA. These six MWs were retested and the final screening is represented in Figure 3. The mother-well MWf that exhibited immune specificity to WH7 (Figure 3) was subjected to cloning by limiting dilution to obtain monoclonal cell populations. The obtained cell line was propagated and maintained in 10% FBS, 2 mM l-glutamine, 25 mM HEPES, and RPMI 1640 (Gibco). For the purification of the monoclonal antibody, the hybridoma cells were seeded in Nunclon Delta surface three layer flasks (Thermo scientific) and grown in ISF-1 medium (Biochrom). After one week the supernatants were collected and purified using Protein G Gravitrap purification columns (GE Healthcare) according to manufacturers' instructions. The elution buffer was exchanged to PBS for the antibody storage using 10kDa molecular weight cutoff Amicon Ultra centrifugal filters (Millipore).

***Figure 8**: Final selection of the immunoreactive hybridoma mother wells by ELISA screening against WH7-BSA conjugate and BSA. The supernatants from the mother-wells are indicated with MWa to f. Mouse sera before and after immunization with WH7 were used at 1:1000 ratio as a negative (white) and positive control (black and grey squares), respectively. The mother well with immunospecificity to WH7 is indicated with a blue arrow. Absorbance of 0.3, indicated here with a red dashed line, is used as a threshold for positive signal.*



Quantification of mouse IgG concentrations

The concentration of the purified mAbs and the rabbit sera were determined by sandwich ELISA as previously described by Salauze et al.3 Nunc-immuno Maxisorp MicroWell 96 well plates were coated with 0.1 µg per well of unlabeled a-mouse IgG antibody (SouthernBiotech) or a-rabbit IgG antibody (Sigma-Aldrich) in 0.2 M carbonate-bicarbonate. After an overnight incubation at 4°C, wells were washed three times with 200 µL washing buffer (0.9% sodium chloride, 0.1% Tween 20) and incubated with the same volume of blocking buffer (3% BSA in PBS) for 2 hours at RT. After blocking, wells were washed three times with 200 µL washing buffer and 100 µL of serial dilutions in blocking buffer of either the purified mAbs or the rabbit sera were plated in triplicate. In addition, 100 µL of dilutions in blocking buffer of either standard mouse IgG (SouthernBiotech) or rabbit IgG ranging from 31.2 ng mL–1 to 0.24 ng mL–1 were plated in triplicates. After 2 hours incubation, wells were washed three times with 200 µL washing buffer and 100 µL of the secondary antibody, AP conjugated a-mouse or a-rabbit IgG produced in goat (Sigma-Aldrich), at 1:1000 dilution were added. The incubation was carried out for 2 hours at RT, the wells were washed four times with washing buffer and detection was performed using 100 µL of p-nitrophenyl phosphate at 1 mg mL–1 in glycine buffer. After 30 min of incubation at RT in the dark, the absorbance was measured at 405 nm. For the calculation of the antibody concentration the calibration curves of the standard mouse or rabbit IgG dilutions were used.

Bacterial Strains and Rabbit Immunizations

The bacterial strains used in this study were the clinical strain E. faecalis **12030**,4 and the community-acquired S. aureus MW2 (USA 400).5 All strains were grown in tryptic soy

agar and broth (Carl Roth) at 37°C with and without agitation, for S. aureus MW2 and E. faecalis **12030**, respectively. a-LTA serum was obtained upon immunization of a New Zealand white rabbit with purified LTA from E. faecalis **12030** as described elsewhere.[6]

ELISA against WH7 conjugates and LTA from S. aureus

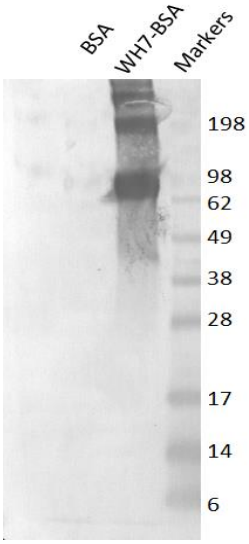
Nunc-immuno Maxisorp MicroWell 96 well plates were coated with 1 µg per well of BSA, **WH7**-BSA, **WH7**-AdcA and lipoteichoic acid from *Staphylococcus aureus* (Sigma) in 0.2 M carbonate-bicarbonate (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH=9.6) and incubated overnight at 4°C. The next day the plates were washed three times with 200 µL washing buffer (PBS, 0.05% Tween 20) and blocked with 3% BSA in PBS for 1 hour at 37°C. After blocking, plates were washed two times with 200 µL washing buffer and 100 µL of the serial dilutions of the supernatants from the hybridomas or the immune rabbit sera in blocking buffer (3% BSA in PBS) were plated in triplicate. After 1 hour incubation, the plates were washed three times with 200 µL washing buffer and 100 µL of the secondary antibody, alkaline-phosphatase-conjugated (AP conjugated) a-mouse or a-rabbit IgG produced in goat (Sigma-Aldrich), at 1:1000 dilution was added. The plates were incubated for 1 hour at room temperature (RT), washed three times with washing buffer and the detection was performed using 100 µL of p-nitrophenyl phosphate (Sigma-Aldrich) at 1 mg mL–1 in glycine buffer (0.1 M glycine, 1 mM MgCl2, 1 mM ZnCl2, pH=10.4). After 30 min of incubation at RT in the dark, absorbance was measured at 405 nm in an ELISA reader (Synergy H1 Hybrid reader, BioTek, USA). For the selection of the clones and the determination of the isotype ELISA was performed as described previously.[7]

Western blotting

BSA and conjugated BSA with **WH7** (**WH7**-BSA) were analyzed by SDS-PAGE and subsequently by western blotting as described before.7 After western blotting the membrane was blocked with blocking buffer (BB; 3% bovine serum albumin in PBS) overnight at 4°C. The next day, it was washed three times with washing buffer (WB; PBS, 0.05% Tween 20) and incubated for 1 hour with supernatant from the hybridoma cell line expressing WH7.01 mAb at 8.99 µg ml–1. The membrane was then washed again as described above and incubated for 1 hour with alkaline phosphatase-conjugated anti-mouse IgG (Sigma-Aldrich) diluted 1:1000 in BB. Finally, three washes with WB were performed and binding was detected by the colorimetric AP substrate reagent kit (Bio-Rad).

Figure 9: *Western blotting against **WH7**-BSA conjugate (right) and carrier protein BSA (left) using the supernatant from hybridoma expressing WH7.01 mAb. SeeBlue Plus2 prestained protein*

*standard (Markers) was used to assess the molecular weight of the samples, e.g., **WH7**-BSA is around 98 kDa as reported before.[8]*



Opsonophagocytic assay (OPA)

MAb activity was evaluated by OPA as previously described[9] using the bacterial strain, baby complement, white blood cells (WBCs) and purified mAb without antibiotics. Polyclonal rabbit sera raised against native LTA from *E. faecalis* **12030** was used as positive control while IgG1k mAb, a monoclonal of the same isotype as WH7.01 mAb, was used as negative control. Bacteria were grown at 37°C until the optical density at 650 nm reached 0.4 and adjusted to a final concentration of 2 X 107 CFU ml–1 in RPMI 1640 (Gibco) with 15% FBS (termed 15% RPMI). Rabbit complement (Cedarlane) was diluted at a final concentration of 6.7%, vol/vol, in 15% RPMI, incubated with the target strain for 60 min at 4°C with shaking, and filter sterilized. WBCs, freshly isolated from a healthy human donor, were prepared by mixing blood with an equal volume of heparin-dextran buffer. After incubation for 45 min at 37°C, the upper layer was collected and centrifuged (at 2.700 rpm for 10 min at 10°C) and the resulting pellet was washed with 15% RPMI. The erythrocytes in the pellet were lysed with 1% NH4Cl (Sigma-Aldrich) at RT for 20 min. WBCs were washed again and resuspended in 15% RPMI to yield a final concentration of ≈ 2 X 107 cells ml^{-1}. The four components were added in equal volumes and incubated on a rotor rack at 37°C for 90 min. The samples were plated in quadruplicates to enumerate the CFU. The percentage of killing was calculated by comparing the surviving CFU in the reaction WBCs (WBCpos) to the surviving CFU in the tubes lacking WBCs (WBCneg) using the following formula: % killing = 100 - [100 X (WBCpos mean CFU at 90 min)/(WBCneg mean CFU at 90 min)]. Negative controls lacking one, two or three of the components were included in the assay. In all experiments presented, no killing was observed for these four negative controls.

Microarray analysis

The amino-spacer equipped synthetic fragments (**WH7**, 1¬**7**) were dissolved in spotting buffer (Nexterion Spot, Schott Nexterion) with 10% DMSO in 384-wells V-bottom plates (Genetix, New Milton, UK). The fragments were printed in three final concentrations (30 µM, 10 µM and 3 µM) 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 (0.10 ml, 0.05 M in PBS containing 20 mg ml^{-1} of BSA). The slides were flushed with PBS containing 5% of Tween® 20 and then PBS and finally rinsed with PBS containing 1% of Tween® 20. After removal of the PBS containing 1% of Tween® 20, the arrays were shaken with WH7.01 mAb at 1 µg ml^{-1} (0.10ml, diluted with PBS containing 1% of Tween® 20 and 10 mg ml^{-1} of BSA) for 60 minutes. The slides were flushed with PBS containing 5% of Tween® 20 and PBS and rinsed with PBS containing 1% of Tween® 20 subsequently. After removal of the PBS containing 1% of Tween® 20, the arrays that were goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor® 555 conjugate (Invitrogen, A21422), (0.10 ml, 0.5 µg ml^{-1} final dilution in PBS containing 1% of Tween® 20 and 10 mg ml^{-1} of BSA) for 30 minutes in the dark. The slides were flushed with PBS containing 5% of Tween® 20, PBS and MilliQ subsequently. The slides were dried by centrifugation and were analyzed on fluorescence on 532 nm and 635 nm using a G2565BA scanner. Data and image analyses were performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, USA) as described previously.10 Fluorescence intensities were quantified and corrected for background/non-specific antibody adhesion by subtracting the fluorescence at blank spots, where spotting buffer was printed without GTA fragment. The average of the triplicate spots was normalized to the highest intensity on the array and visualized in bar graphs using GraphPad Prism 8.4.3.

ELISA against the synthetic fragments

The monoclonal antibody specificity to the synthetic teichoic acid fragments was evaluated by ELISA. Streptavidin coated plates (Thermo Scientific Pierce) were washed three times with 200 µL wash buffer (WB: 25 mM Tris, 150 mM NaCl, 0.1% BSA and 0.05% Tween 20, adjusted to pH=7.2). The wells were coated in duplicate with 1.0 µM of the synthetic teichoic acid fragments dissolved in WB. After 2 hours of incubation at 4°C, the plates were washed three times with 200 µL WB and incubated with 100 µL of WH7.01 mAb at the specified concentrations for 2 hours at RT with gentle shaking. Wells were washed three times with 200 µL WB and incubated for 30 min with gentle shaking at RT with 100 µL of alkaline-phosphatase-conjugated anti-mouse IgG produced in goat (Sigma, St. Louis, Mo.) at 1:1000 dilution in WB. After three washes with 200 µL of WB, the detection was performed with 100 µL of p-nitrophenyl phosphate (Sigma-Aldrich) at 1 mg ml^{-1} in glycine buffer. After 10 min of incubation at RT in the dark, the reaction was

stopped with 50 µL of 3 M sodium hydroxide and the absorbance was measured at 405 nm in a Synergy H1 hybrid reader (BioTek).

Surface plasmon resonance

Binding kinetics and affinities were determined by SPR using a Biacore X100 system as described elsewhere.[11] In order to achieve a 1:1 binding model, immobilizations of the biotinylated synthetic compounds were performed on high-affinity streptavidin (SA) sensor chips (GE Healthcare) starting from low amounts of the biotinylated compounds in water (0.1 nM) and increasing until a detectable interaction with the mAb was reported at 100 nM of the compound in water. Serial dilutions of the analyte, WH7.01 mAb (2000 ¬ 62.5 nM), were run. Sensorgram data were analyzed using BIAevaluation software (Biacore).

1H-STD-NMR

STD experiment was acquired using Bruker AVANCE 2 600 MHz spectrometer equipped with cryoprobe. The samples were prepared in deuterated phosphate-buffered saline (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH=7.4). WH7.01 mAb was exchanged in the working buffer using 10kDa molecular weight cutoff Amicon Ultra centrifugal filters (Millipore). After several washing the antibody concentration was determined by absorbance (ε=120500 $M^{-1}cm^{-1}$). The sample for the STD experiment was prepared using a mAb/ligand ratio of 1:100 with mAb concentration of 2.5 µM in a shigemi 5 mm NMR microtube. In order to avoid overlapping of the glucosyl anomeric proton with the H_2O signal, the temperature was set at 303 K. The on-resonance was tested both in the aromatic and aliphatic region but no difference in STD effects was observed. Moreover, a negative control spectrum was performed in the absence of the monoclonal antibody to ensure the interpretation of the result. Finally, the on-resonance frequency was set at 0.17 ppm, while the off-resonance frequency at 100 ppm. Protein saturation was achieved by using a train of 50 ms Gaussian-shaped pulses with a total saturation time of the protein of 2s and a spin-lock filter (10 ms) was used to remove the NMR signals of the macromolecule.

Ethics statement.

Mouse experiments were approved by the Ethics Committee of the Veterinary Department of the Ministry of Agriculture and Animal Welfare Committee of the University of Rijeka, Faculty of Medicine. This study was carried out in accordance with the recommendations of regulations on the protection of animals used for scientific purposes (Official Gazette of the Republic of Croatia, 55/2013).

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Chapter 6

Summary and future prospects

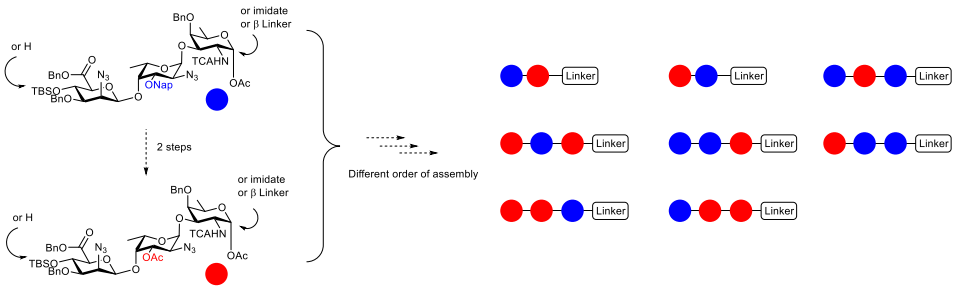
This thesis deals with the synthesis and antibody-recognition evaluation of well-defined fragments of carbohydrate-based cell-wall components from staphylococci and enterococci. The availability of libraries, comprising similar yet minutely diverse molecules, allows to evaluate the impact of all structural 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).

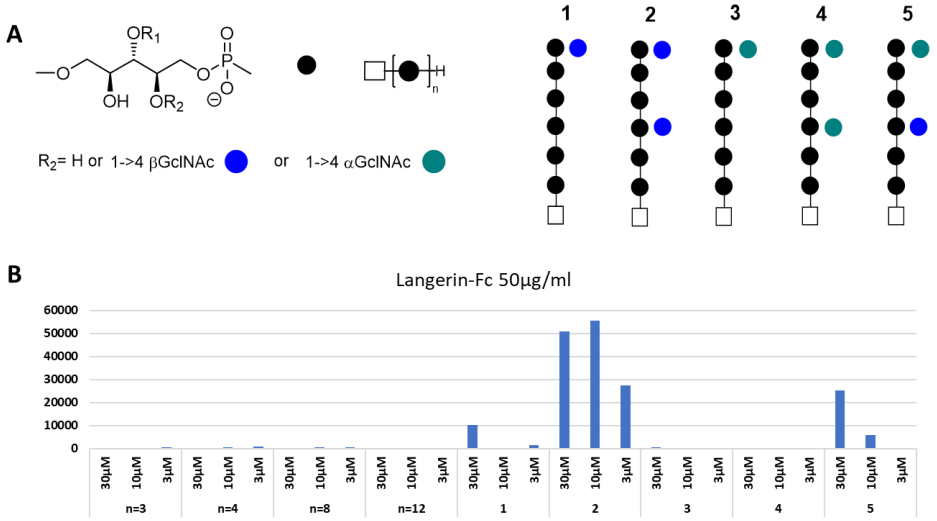
Figure 1: *Synthetic strategy to deliver hexamers and nonamers with different acetyl ester substitution patterns*



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*



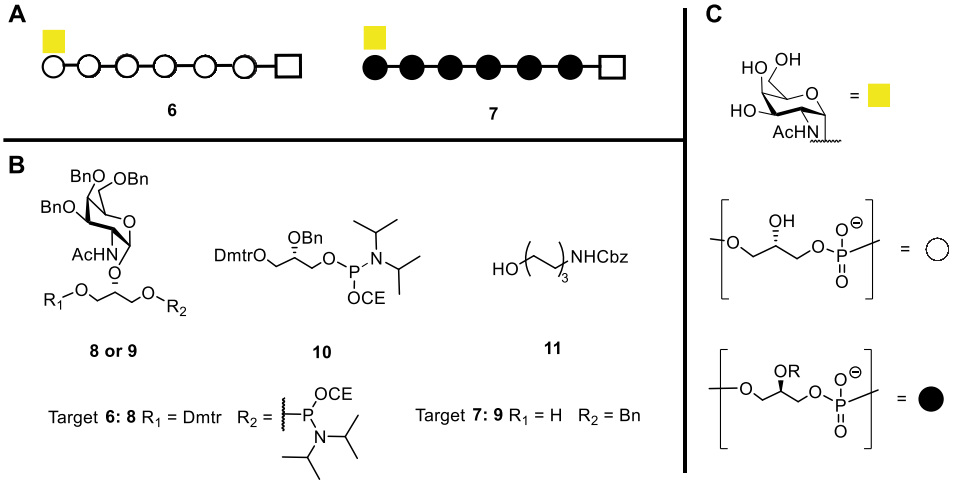
In **Chapter 4**, the synthesis of a new set of glucosylated GroP-LTA-fragments is reported.[19] The compounds differ from the previous generated library as the linker was attached to the side of the naturally occurring lipid anchor. Thus, the fragments feature a *sn*-Gro-1-P backbone with an α-glucosyl 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

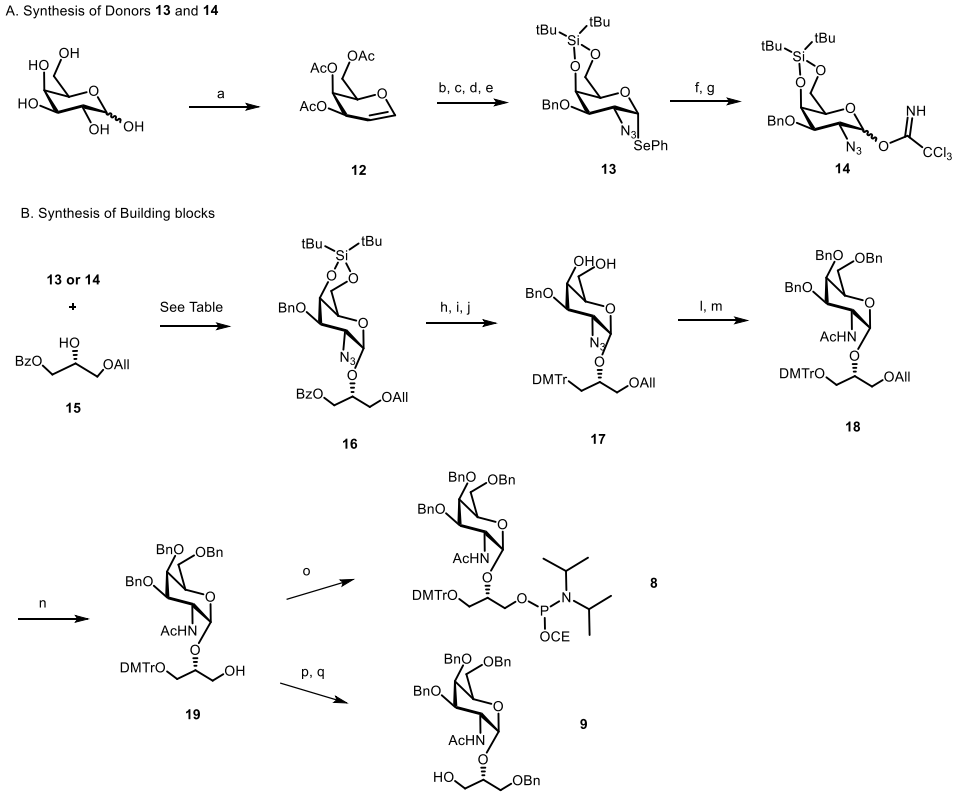
also the stereochemistry of the GroP backbone is pivotal for antibody binding. In Figure 3 a strategy is shown to generate well-defined structures in both the *sn*-1- and the *sn*-3-GroP series. The synthetic approach is based on the use of the same phosphoramidate strategy (Chapter 4) with opposite 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.

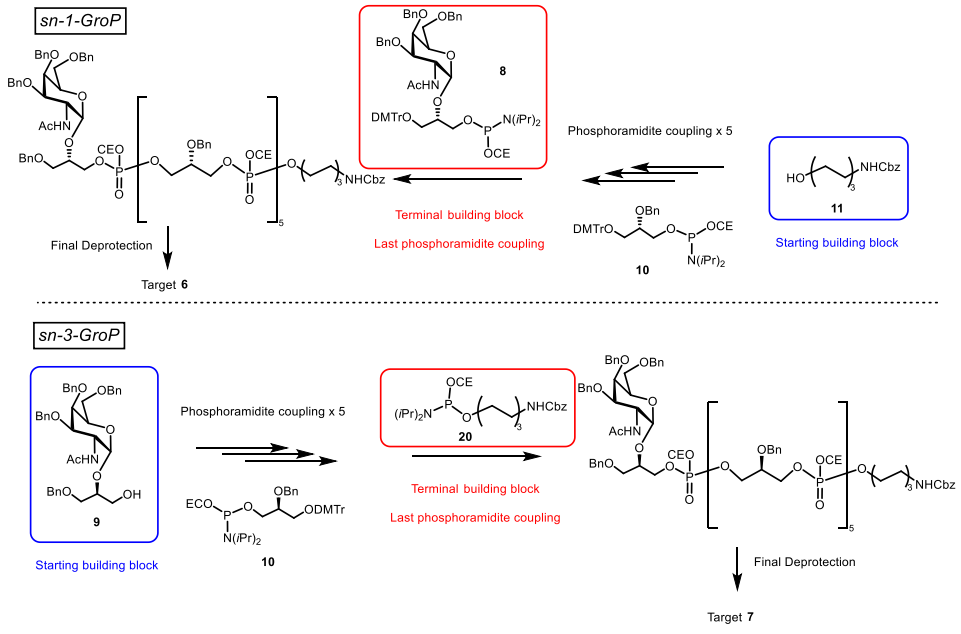
Table 1: *Reagents and conditions for coupling between donor* ***13*** *or* ***14*** *and acceptor* ***15***

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

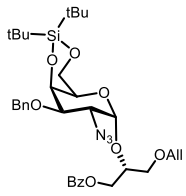
The amino-spacer equipped GTA-fragments were dissolved in spotting buffer (Nexterion Spot, Schott Nexterion) with 10% DMSO in 384-wells V-bottom plates (Genetix, New Milton, UK). The GTA-fragments were printed in three final concentrations (30μM, 10μM and 3μM) in triplicate on epoxysilane-coated glass 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).

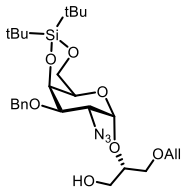
TLC analysis: R_f = 0.38 (Pentane:EtOAc; 95:5)

^{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).

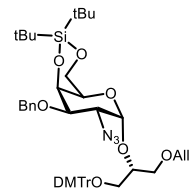
TLC analysis: R_f = 0.34 (Pentane:EtOAc; 8:2)

^{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)



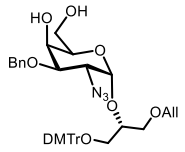
Alcohol **21** (2.74 mmol, 1.0 eq) was dissolved in dry DCM (9 ml, 0.3 M) and the reaction cooled to 0°C. Under inert atmosphere, 4,4'-dimethoxytrityl chloride (3.15 mmol, 1.15 eq) and Et_3N (4.11 mmol, 1.5veq) were added and the reaction mixture was left stirring, allowing to warm up to room temperature. 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

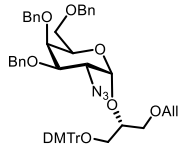
(2H, H_5, H_3,m), 3.51-3.47 (2H, $CH_{2\_glycerol}$, m), 3.23 – 3.13 (2H, $CH_{2\_glycerol}$, m), 1.06 (9H, 3xCH_{3_tBu}, s), 1.03 (9H, 3xCH_{3_tBu}, s).
^{13}C NMR (101 MHz, CD_3CN) δ: 136.0 (C_{allyl}), 131.0, 129.4 (C_q), 129.0 x2, 128.8, 128.7, 127.8 (C_{arom}), 117.0 (CH_{2_allyl}), 114.0 (C_{arom}), 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.

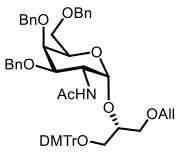
2-*O*-(2-azido-3,4,6-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-allyl-1-4,4'-dimethoxytrityl-*sn*-glycerol (23)



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.

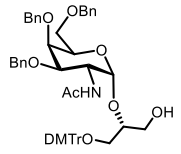
2-*O*-(2-*N*-acetylamine-3,4,6-*O*-benzyl-2-deoxy-α-d-galactopyranosyl)-3-*O*-allyl-1-4,4'-dimethoxytrityl-*sn*-glycerol(18)



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

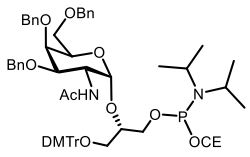
TLC analysis: R_f=0.28 -(Pentane:EtOAc; 7:3, 1% TEA) compound **19**

^{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)



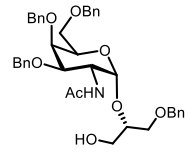
Galactose **19** (891 mg, 1.03 mmol) was in dry DCM (7 mL, 0.14 M) and Et_3N (1.545 mmol, 1.5 eq) was added At 0 °C 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.2 mmol, 1.2 eq) was added and the reaction was left for 2 hours after which TLC analysis (Pentane:EtOAc:Et_3N, 7:3:0,1) showed complete 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)



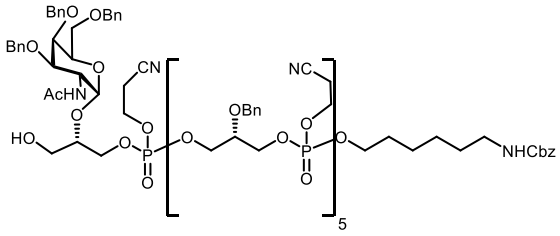
Galactose **19** (865 mg, 1.0 mmol) was dissolved in DMF (10 ml, 0.1 M) and the mixture was cooled to 0°C. Subsequently, BnBr (1,3 mmol, 1,3 eq) and NaH (1,3 mmol, 1 eq, 60% w/w) were added. The reaction mixture was stirred overnight reaching room temperature, after which TLC analysis (PE:EtOAc; 6:4, 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).

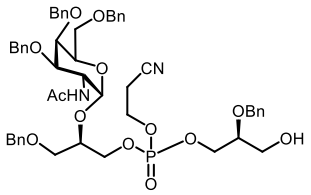
^{1}H NMR (400 MHz, CD_3CN) δ: 7.41 – 7.11 (45H, H_{arom}, m), 7.01-6.76 (1H, NH, m), 5.72-5.63 (1H, NH, bs), 5.03 (s, 2H, H-26), 4.90-4.35 (20H, H_1, 18x$CH_{Bn}H$, H_2, m), 4.32 – 3.97 (36H, H_5, H_4, 6xCH_{2_OCE}, $CH_{2\_OSpacer}$, 10x$CH_{2_glycerol}$, m), 3.97 – 3.80 (5H, 5x$H_{glycerol}$, m), 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).

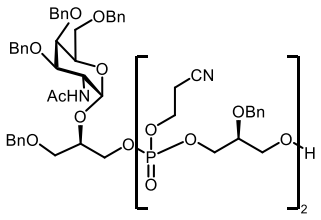
^{1}H NMR (400 MHz, CD_3CN) δ: 7.32 – 7.13 (25H, H_{arom}, m), 7.01 – 7.00 (1H, NH, m), 4.81-4.76 (1H, H_1, m), 4.75-4.66 (1H, CHH_{Bn}, m), 4.65-4.56 (1H, CHH_{Bn}, m), 4.55 – 4.49 (2H, CHH_{Bn}, m), 4.48 – 4.26 (7H, 3x CHH_{Bn}, H_2), 4.21 – 3.84 (8H, H_5, H_4, CH_{2_OCE}, 2x$CH_{2_glycerol}$, m), 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).

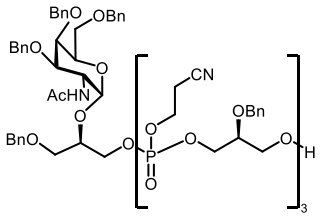
^{1}H NMR (400 MHz, CD_3CN) δ: 7.42 – 7.19 (30H, H_{arom}, m), 7.18 – 7.00 (1H, NH, m), 4.90-4.76 (2H, H_1, CHH_{Bn},m), 4.73-4.33 (12H, 11xCHH_{Bn}, H_2), 4.29 – 3.92 (14H, H_5, H_4, 2xCH_{2_OCE}, 4x$CH_{2_glycerol}$, m), 3.90 – 3.78 (2H, 2x$H_{glycerol}$, m), 3.71 – 3.47 (8H, H_3, $H_{glycerol}$, 2xH_6,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

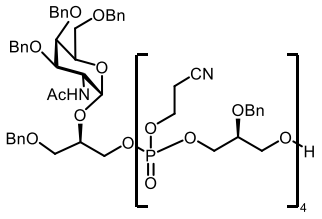
^{1}H NMR (400 MHz, CD_3CN) δ: 7.39 – 7.20 (35H, H_{arom}, m), 7.17 – 6.98 (1H, NH, m), 4.88-4.75 (2H, H_1, CHH_{Bn},m), 4.73-4.33 (14H, 13xCHH_{Bn}, H_2), 4.29 – 3.92 (20H, H_5, H_4, 3xCH_{2_OCE}, 6x$CH_{2_glycerol}$, m), 3.90 – 3.78 (3H, 3x$H_{glycerol}$, m), 3.69 – 3.47 (8H, H_3, $H_{glycerol}$, 2xH_6,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

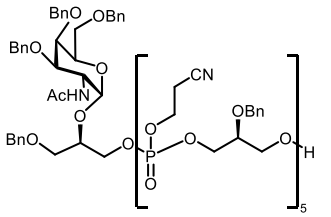
^{1}H NMR (400 MHz, CD_3CN) δ: 7.39 – 7.20 (40H, H_{arom}, m), 7.17 – 7.08 (1H, NH, m), 4.89-4.31 (18H, H_1, 16xCHH_{Bn}, H_2, m), 4.27 – 3.92 (26H, H_5, H_4, 4xCH_{2_OCE}, 8x$CH_{2_glycerol}$, m), 3.90 – 3.78 (4H, 4x$H_{glycerol}$, m), 3.69 – 3.47 (8H, H_3, 2xH_6, $H_{glycerol}$, 2x$CH_{2_glycerol}$),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

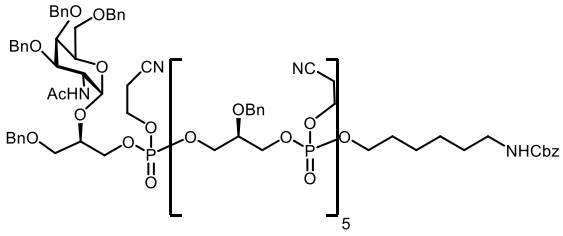
^{1}H NMR (400 MHz, CD_3CN) δ: 7.43 – 7.20 (45H, H_{arom}, m), 7.19 – 7.08 (1H, NH, m), 4.90-4.32 (18H, H_1, 16x$CH\mathit{H}_{Bn}$, H_2, m), 4.27 – 3.92 (26H, H_5, H_4, 4xCH_{2_OCE}, 8x$CH_{2_glycerol}$, m), 3.90 – 3.78 (4H, 4x$H_{glycerol}$, m), 3.69 – 3.47 (8H, H_3, 2xH_6, $H_{glycerol}$, 2x$CH_{2_glycerol}$),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

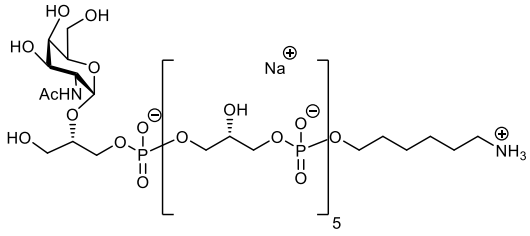
^{13}C NMR (101 MHz, CD_3CN) δ: 171.1, 171.0, 139.9x3, 139.8, 139.7, 139.5,139.4, 139.1 (C_q), 129.4x2, 129.3x2, 129.2, 129.0x2, 128.9x2, 128.8x3, 128.7x2, 128.6x3, 128.5x4 (CH_{arom}), 100.8, 100.6 (C_1), 79.1 ($CH_{glycerol}$), 78.3 (C_3), 77.6 ($CH_{glycerol}$), 76.7 ($CH_{glycerol}$), 75.4 (CH_{2_Bn}), 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

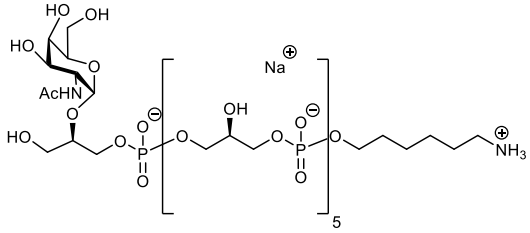
The oligomer is dissolved in dioxane (2mM) and upon the addition of ammonia solution in H_2O (33%) the reaction mixture turns turbid. Once the solution becomes transparent (1-3 hours) the reaction mixture is concentrated *in vacuo*. After checking the disappearing of the cyanoethyl group by ^{1}H-NMR, 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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Nederlandse samenvatting

Stafylokokken en enterokokken zijn Gram-positieve bacteriën die behoren tot onze natuurlijke bacteriële microbiota. Ze worden beschouwd als onschadelijke commensalen bij gezonde personen, maar onder bepaalde omstandigheden kunnen ze de oorzaak zijn van verschillende infecties en endocarditis, sepsis of peritonitis veroorzaken. Door het wijdverbreide en intensieve gebruik van antibiotica hebben deze bacteriën de afgelopen decennia een hoge resistentie tegen antibiotica ontwikkeld. Om deze reden richt het onderzoek, beschreven in dit proefschrift, zich op de ontwikkeling van alternatieve mogelijkheden om deze bacteriën te bestrijden, waaronder de ontwikkeling van vaccins en monoklonale antilichamen. Het werk gepresenteerd in dit proefschrift richt zich op de synthese van koolhydraatstructuren die kenmerkend zijn voor de celwand van deze bacteriën en de toepassing van methoden om de interactie van deze mogelijke kandidaat-antigenen met geschikte antilichamen te bestuderen.

Hoofdstuk 1 geeft een algemeen beeld van de belangrijkste polysacchariden die aanwezig zijn op de celwand van *Staphylococcus aureus*. Voor elk van deze worden gebruikte synthesemethoden beschreven om goed gedefinieerde structuren te verkrijgen en, indien aanwezig, de geraporteerde immunologische evaluatie. In het bijzonder worden de syntheses van fragmenten van het capsulaire polysaccharide van *S. aureus* type 5 (CP5) gepresenteerd en worden de teichoïnezuren, anionische polyfosfaten die aanwezig zijn in vrijwel alle Gram-positieve bacteriën maar die voorkomen met verschillende moleculaire structuren, geïntroduceerd.

Hoofdstuk 2 beschrijft de synthese van zowel een trimeer als een hexameer van CP5. Voor de synthese van het hexamneer wed een [3+3] benadering gebruikt. Het gebruikte trisaccharide was zo ontworpen dat het een acetylgroep op de anomere positie heeft om het overeenkomstige imidaat te vormen, en een naftylgroep op de C3-positie van het L-fucose 189residu, om na glycosylering een acetylgroep te introduceren, alsmede een silylgroep op de C3-positie van D-mannose voor verlenging. De ontwikkelde strategie maakte het mogelijk om het gewenste trimeer en hexameer te verkrijgen met en zonder de acetylsubstituent op C3-positie van de L-fucose eenheid. Met de aldus verkregen moleculen zal het mogelijk zijn om het effect van de acetyl groep en de lengte van de oligosacchariden op de immunogenicitieit te evalueren.

Lipoteichoïnezuren die in de celwand van stafylokokken en enterokokken worden aangetroffen, zijn opgebouwd uit glycerolfosfaat of

ribitolfosfaat ketens, waarvan de alcohol groepen van de glycerol en ribitol eenheden gebonden kunnen zijn aan enkelvoudige of complexe suikers. Met behulp van eerder gesynthetiseerde teichoïnezuur structuren, op basis van glycerol of ribitolfosfaat eenheden, die verschillen in lengte en type, aantal en positie van de koolhydraat decoratie, is in **Hoofdstuk 3** microarray-technologie ontwikkeld om de interactie van deze moleculen met monoklonale antilichamen of sera efficiënt te evalueren. De toepasbaarheid van deze techniek werd eerst geverifieerd met behulp van een commercieel verkrijgbaar monoklonaal antilichaam, opgewekt tegen de teichoïnezuren van *S. epidermidis*. De verkregen resultaten bevestigden de toepasbaarheid van de techniek, maar lieten ook zien dat het alleen gebruikt kan worden voor kwalitatieve doeleinden, aangezien de gemeten binding niet altijd evenredig was met de verschillende hoeveelheid antigeen die begruikt was om de antigenen aan te brengen op de microarray objectglaasjes. Vervolgens is de kwalitatieve analyse beschreven van meer complexe biologische monsters zoals konijnensera verkregen door immunisatie met geïsoleerde teichoïnezuren van *Enterococcus faecalis* of met een synthetisch conjugaat alsmede met sera van gezonde menselijke proefpersonen.

De eerder verkregen glycerolfosfaat lipoteichoïnezuur fragmenten zijn gefunctionaliseerd met een linker die gepositioneerd is aan de kant van de oligomeren die formeel uit de bacteriewand steekt om zodoende een reeks te genereren met de niet-natuurlijke *sn*-3-GroP-stereochemie. In **Hoofdstuk 4** wordt de bibliotheek uitgebreid door zes hexameren te synthetiseren met de natuurlijke *sn*-1-GroP-stereochemie, die varieren in de positie van de glucose substituent langs de keten. Vanuit synthetisch oogpunt zijn verbeteringen aangebracht in het maken van de geglycosyleerde glycerol bouwsteen. Er is een glycosylering toegepast, waarbij trifenylfosfine oxide en trimethylsilyl iodie zijn gebruikt om de glucosyl donor te activeren en er is waargenomen dat de α/β-selectiviteit van de koppeling wordt beïnvloed door zowel de stereochemie als de beschermgroepen van de glycerol bouwsteen. De binding van de moleculen aan antilichamen werd vervolgens geanalyseerd met behulp van het microarray-systeem dat in het vorige hoofdstuk is beschreven, met behulp van het konijnenserum dat werd gegenereerd door immunisatie met LTA van *E. faecalis* en met het conjugaat vaccin dat was gegenreerd met een geglucosyleerd *sn*-3-GroP hexameer (WH7-BSA). Er werd waargenomen dat de interactie met de antilichamen sterk wordt beïnvloed door de stereochemie van de glycerolfosfaat keten.

Uit eerdere studies is gebleken dat een sn-3-GroP-hexameer met een glucose substituent aan het einde van de keten (WH7) in staat is om opsonische

antilichamen tegen zowel stafylokokken als enterokokken op te wekken. Dit synthetische conjugaat werd gebruikt om via het hybridomaproces een monoklonaal antilichaam te genereren. Nadat het monoklonale antilichaam WH7.01mAB was geselecteerd en geproduceerd, werden verschillende technieken gebruikt om de binding aan het doel epitoop te karakteriseren. Microarray-technologie werd gebruikt om de interactie van WH7.01mAB met een relatief grote en gevarieerde bibliotheek van glycerolfosfaat teichoïnezuren, die in de voorgaande hoofdstukken is gepresenteerd, te onderzoeken. Omdat zelfs structuren die geen glucose substituent droegen werden herkend is de evaluatie van de antilichaam-ligand-interactie verdiept met behulp van analyse methoden zoals ELISA, SPR en STD-NMR. De resultaten van deze analyses lieten zien dat het belangrijkste epitoop dat wordt herkend door het monoklonale antilichaam de glycerolfosfaatketen is en dat de glucose substituent een kleinere rol speelt, maar wel van belang is. In het bijzonder wanneer het glucose residu aanwezig is op de terminale positie van een sn-3-GroP-keten, zoals in het geval van WH7, is de interactie groter dan met het substituentvrije ligand. De hypothese is dat de aanwezigheid van het glucose residu een conformatie van de glycerol fosfaat keten kan induceren die meer lijkt op de vrom die ligand aanneemt in complex met het antilichaam. In de toekomst kan de ontwikkelde methodologie gebruikt worden om een bibliotheek van monoklonale antilichamen te genereren en te evalueren.

Het laatste hoofdstuk beschrijft kort het in dit proefschrift gepresenteerde werk met een overzicht van mogelijke toepassingen en toekomstige implicaties. Met de strategie die in hoofdstuk 2 wordt gepresenteerd, zal het mogelijk zijn om de bibliotheek van fragmenten gerelateerd aan *S. aureus* CP5 uit te breiden, waardoor de synthese van gedefinieerde moleculen met verschillende graden van acetylering mogelijk wordt. De toepassing van de teichoïnezuur microarray kan worden verbreed door gebruik te maken van receptoren van het aangeboren immuunsysteem zoals DC-SIGN en Langerin. Ten slotte is een strategie gepresenteerd voor de synthese van glycerolfosfaten met α-*N*-acetylgalactosamine substituenten op de keten.

CURRICULUM VITAE

Francesca Berni was born in 1991 in Guastalla, Italy. She attended high school at Liceo Scientifico G. Passerini in Guastalla. In 2010, after graduating with 100 *cum laude* with specialization in Science and Languages, she started the bachelor in Chemistry and Material Chemistry at Alma Mater Studiorum in Bologna, which was finilized (*cum laude*) three years later with a thesis disserting on the synthesis of enantiomerically enriched tripodes based on pyrrol and phenol under the superivision of Prof. Cozzi. In the same year she moved to Milan to start a Master in Chemistry with focus on Organic chemistry and Biochemistry at Università degli Studi di Milano. She graduated (*cum laude*) in 2015 with a dissertation on the synthesis of benzofuran modulators of Hsp90 activity under the supervision of Prof. Bernardi.

She finally moved to the Netherlands in 2016 to start the research presented in this thesis under the supervision of Prof. Jeroen Codeé and Prof. Gijs van der Marel, joining the group as Marie Curie ESR of the Glycovax network. The work has been presented in different conferences: NWO Chains n Veldhoven (2016, poster), ICS in Lisbon (2018, flash presentation) and EuroCarb in Leiden (2019, presentation). Since September 2021, she is working as Clinical Research Associate at Hippocrates Research.

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