Synthesis of oligosaccharide libraries from GBS capsular polysaccharides for structure-based selection of vaccine candidates

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Chapter 2

Group B Streptococcus

Group B *Streptococcus* epidemiology overview

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), is one of the major causes of sepsis, pneumonia and meningitis in neonatal and infants in the first three months after birth[1]. It is a Gram-positive beta-hemolytic coccus which appears in pairs or chains, that colonizes the urogenital and gastrointestinal tracts of more than 30% of the healthy population and, in particular, the vagina of 25-40% of healthy women[2, 3]. It is an etiologic agent also in adults, in particular elderly or immunocompromised persons, diabetics and patients affected by others chronic pathology of liver and kidney.

GBS clinical isolates are classified into ten serotypes, according to the chemical nature of capsular polysaccharides (PS): Ia, Ib, II, III, IV, V, VI, VII, VIII and IX[4-6]. However, around 8-14% of the clinical isolates in the Europe and USA are non-typeable strains because they cannot be distinguished on the basis of PS antigenicity. All GBS serotypes contain, in different combinations, these carbohydrates: galactose, glucose, rhamnose, *N*-acetylglucosammine and sialic acid (*N*-acetylneuraminic acid) (Figure 1).

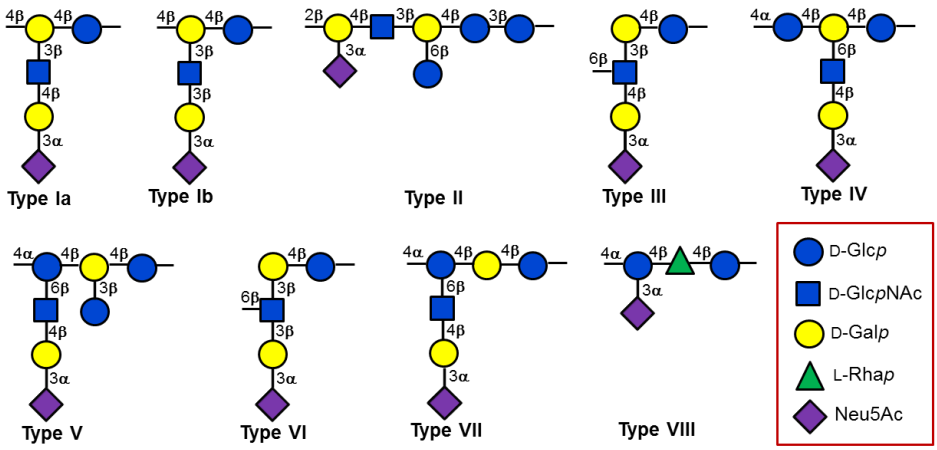


Figure 1. Chemical structure of GBS strains capsular polysaccharides

In most cases infection is transferred vertically from asymptomatic mothers and every year 8000-12000 infected babies and 2000 deaths are recorded in the US alone. Infection by contact of throat, ear, nostril, umbilicus and respiratory ways with amniotic liquid or infected vaginal fluids occurs in uterus or at delivery. After the aspiration/ingestion of bacteria, neonatal lungs become the starting focus of infection. From here it can rapidly arrive to blood flow, continue towards other tissues and organs like blood-brain barrier.

Neonatal disease can occur in two different forms: **Early-Onset Disease** (EOD) and **Late-Onset Disease** (LOD).

EOD occurs during the first seven days of life, with the vast majority of cases (approximately 90%) happening during the first 24 hours of life[7]. Neonates with EOD present respiratory disease, sepsis without focus and meningitis. Serotype Ia and III account together for more than 70% of EOD.

LOD occurs beyond seven days of life and can develop up to three months of age. In 50% of the cases it has a maternal origin, while in the other half of the cases infection is acquired nosocomially or from community sources. Neonates with LOD present sepsis, meningitis, urinary infection, osteoarthritis, respiratory disease and cellulitis. Over 20% of survivors of GBS meningitis have permanent sequelae, including hearing loss, mental retardation, cortical blindness and seizures[8]. LOD is mostly caused by serotype III, with an incidence until beyond 70% depending on the geographic area.

GSB can cause diseases also in non-pregnant adults, and studies performed on non-pregnant adults with GBS associated invasive disease revealed that GBS serotypes Ia, III and V accounted for more than two-third of cases. In particular, more than 25% of the subjects had invasive GBS disease caused by type V strains (Table 1)[9, 10].

Table 1. Disease spectrum and serotype distribution of Group B streptococcal infections according to the age of the patient[9]

	Common manifestation	**Risk factors**	**Case fatality rate**	**Serotype distribution**	
Early-onset diseases	Sepsis with unknown source (80-85%) Pneumoniae (10%) Meningitis (7%)	Maternal obstetric complications, maternal genital GBS colonization, GBS bacteriuria	5-10%	Ia (27.7%) Ib (7.3%) II (4.2%) III (46.2%) IV (1.5%) V (12.9%)	
Late-onset diseases	Primary bacteremia (65%) Meningitis (25-30%) Bone and joint infection (5%) Cellulitis and/or adenitis (4%)	Prematurity	2-6%	Ia (11.3%) Ib (5.4%) II (0.7%) III (76.0%) IV (0%) V (6.6%)	
Pregnant women	Genitourinary tract infection (50%) Chorioamnionitis (4%) Endometritis (8%) Primary bacteremia (31%) Pneumonia (2%) Puerperal sepsis (2%)	Genital GBS colonization	Not available	*Infection* Ia (32.7%) Ib (4.0%) II (14.9%) III (28.7%) IV (0%) V (19.8%)	*Colonization* Ia/Ib (36.0%) II (14.5%) III (24.0%) IV (1.2%) V (202%)
Non-pregnant adults	Primary bacteremia (24%) Skin and soft tissue infection (20%) Respiratory tract infection (12%)	Central nervous system infection, old age, diabetes mellitus, liver cirrhosis, stroke, cancer,	3.7% (up to 15% in old adults)	Ia (24.3%) Ib (12.2%) II (11.9%) III (16.5%) IV (0.3%) V (27.5%)	

	Urinary tract infection (10%) Bone and joint infection (5%) Endocarditis (4%)	neurogenic bladder, decubitus ulcer		

GBS prevention strategies

Intrapartum Antibiotic Prophylaxis (IAP) is the most widely used strategy for GBS prevention in developed countries and is applied after the identification of GBS carriers through a screening-based approach or by identification of clinical risk factors, such as prematurity (<37 weeks' gestation), prolonged rupture of membrane, intraamniotic infection, temperature (>38°C) or previous births of baby with GBS disease. The screening approach recommends that all pregnant women should be screened between 35 and 37 weeks gestation for GBS vaginal and rectal colonization. Both approaches recommend antibiotics administration during labor to women who were GBS positive at the screening time and/or presenting one of the risk factors[8, 11]. Although this approach led to decrease in EOD infections, no impact on LOD disease was observed. Furthermore, the IAP approach presents a number of drawbacks that prevent its further implementation: the screening method for early identification of GBS carriers is complex in terms of speed/accuracy of prenatal screening culture, women delivering preterm or showing adverse antibiotic events can't be subjected to antimicrobial prophylaxis and concerns about resistance to β-lactam antibiotic are raised[12].

For this reason, vaccination against GBS is an appealing alternative to IAP. Effective vaccines would stimulate the production of functionally active antibodies that could cross the placenta and provide protection against neonatal GBS infection.

GBS capsular polysaccharide represents a main virulence factor, which helps GBS to escape host defense mechanism by interfering with phagocytic clearance.

The first evidence of the protective nature of CPS-specific antibodies was in 1930s when Rebecca Lancefield *et al.* demonstrated that polyclonal antibodies from rabbit sera, able to recognize PS epitopes, conferred protection against GBS infection in animal models[13, 14]. During the last two decades, plain GBS polysaccharides have been extensively studied as vaccines in preclinical and clinical human studies. However, the first human clinical trial conducted in the 1980s showed that the purified native PS from serotype III was not sufficient to induce a robust IgG response in adults and induced an insignificant response in neonates. Subsequently, conjugation of PSs to immunogenic proteins, such as tetanus toxoid (TT)[15, 16], was shown to dramatically increase the immune response in children, eliciting the differentiation of memory cells associated to a long-term protection. Following these findings, PS-TT conjugate vaccines prepared with GBS type-specific CPS (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) were produced and tested pre-clinically in animal models. These studies showed the higher immunogenicity of CPS-TT compared to the uncoupled CPS. This success constituted the rationale to proceed with the clinical studies in humans.

Although the immunogenicity in humans of GBS type-specific CPS antigens was successfully increased through conjugation to TT, the immune responses were serotype-specific.
Further studies demonstrated that glycoconjugate vaccines constituted by serotypes Ia, Ib, II, III, and V PS linked to TT were safe, well-tolerated and highly immunogenic in human adults, but cross-protection between serotypes was still lacking[17]. Therefore, a multivalent vaccine was required to obtain a broad coverage of the vaccine against the prevalent circulating GBS serotypes. A tetravalent combination of PS–TT conjugates (serotypes Ia, Ib, II, and III) was successfully tested in a mouse model and further human trials were performed using the combination of two PS TT-conjugates (serotypes II and III). Results showed that the combination had the same immunogenicity and reactogenicity of each monovalent PS vaccine[17].
However, although recent epidemiological studies[18] suggest that a tetravalent combination of serotypes Ia, Ib, III and V would be sufficient to achieve a coverage against the majority of GBS strains circulating in Europe and North America, there are other geographical areas where such combination would not be effective due to a different serotypes distribution.
An additional obstacle to the licensure of vaccine against GBS is the difficulty of conducting efficacy clinical trials in human: large sample size would be required, but the use of intrapartum antibiotic prophylaxis (IAP) reduces the incidence of neonatal disease.
To overcome this difficulty, Lin and coworkers suggested a ways to estimate the maternal GBS-PSs antibody levels needed to give protection to neonates from EOD[19]. This work demonstrated that it is possible to define thresholds of anti-GBS PSIa specific IgG levels in the mothers which are predictive for the protection of newborns, since the probability of developing EOD declined with increasing maternal levels of anti-PSIa IgG. More recently, thresholds have also been set for the levels of maternal anti GBS-PSIII IgGs required to protect newborns from EOD caused by GBS serotype III[20]. These results suggest that complex clinical trials required for a GBS vaccine registration might be replaced with an *in vitro* correlate of protection based on the quantification of vaccine induced antibodies.

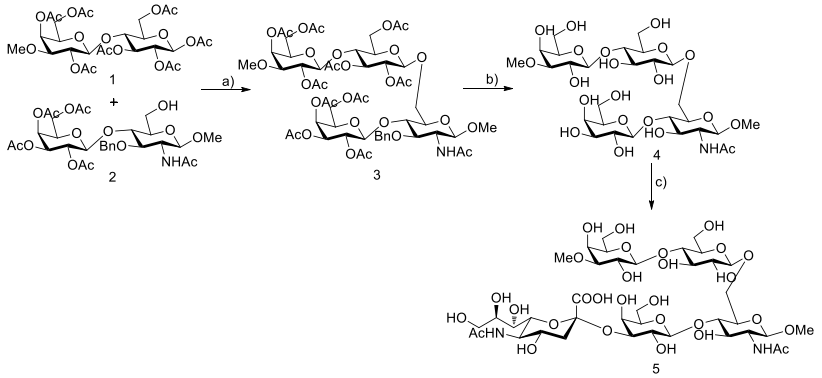
GBS glycans synthesis

GBS capsular polysaccharides represent promising targets for vaccine development. When long polysaccharides are used, correlation between chemical structure and immunogenicity is difficult to establish. Indeed, natural polysaccharides used for vaccine manufacturing are heterogeneous mixtures, and the impact of variables like length or substitution pattern cannot be easily determined[21]. Moreover, conjugation implies chemical manipulations of sugar residues, or the downstream terminal aldehyde, which are coupled to the protein. The understanding of the parameters impacting their immune-activity is better achieved by means of synthetic carbohydrates, because of their defined length and composition of glycoconjugates. Furthermore, synthetic glycans are coupled to the protein using a linker preinstalled at the downstream end of the oligosaccharides, allowing preservation of the integrity of sugar epitopes. Thus, synthetic

glycans are not only used to elucidate the biological function of CPSs but also to develop fully synthetic or semi-synthetic vaccines[22].

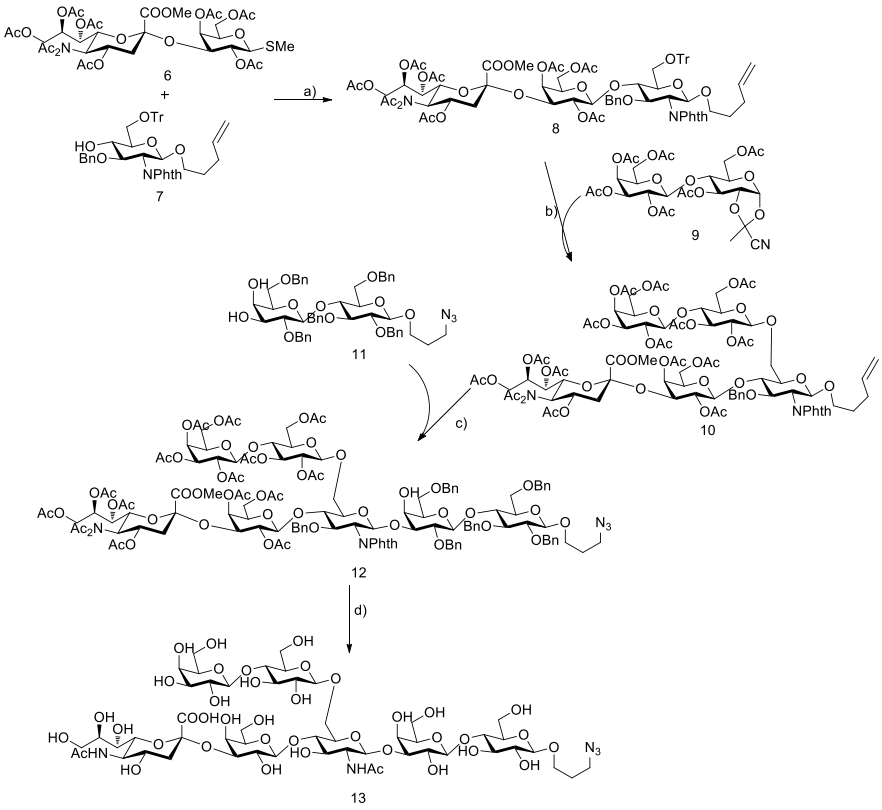
The availability of short defined oligosaccharides related to GBS PS repeating units is an important prerequisite to explore interactions between CPS and specific antibodies and to map the corresponding GBS PS epitopes. For this reason, different approaches have been proposed for the synthesis of GBS oligosaccharide fragments. Serotype III is by far the most studied GBS strain and approaches to produce short PSIII fragments have been based on either depolymerization or chemical synthesis.

Among the synthetic approaches, Poszgay et al. reported the chemical synthesis of desialylated tetrasaccharide fragment **4**[23], that was subsequently sialylated with a specific rat liver sialyltransferase to obtain the branched repeating unit **5** of GBS type III CPS (Scheme 1).



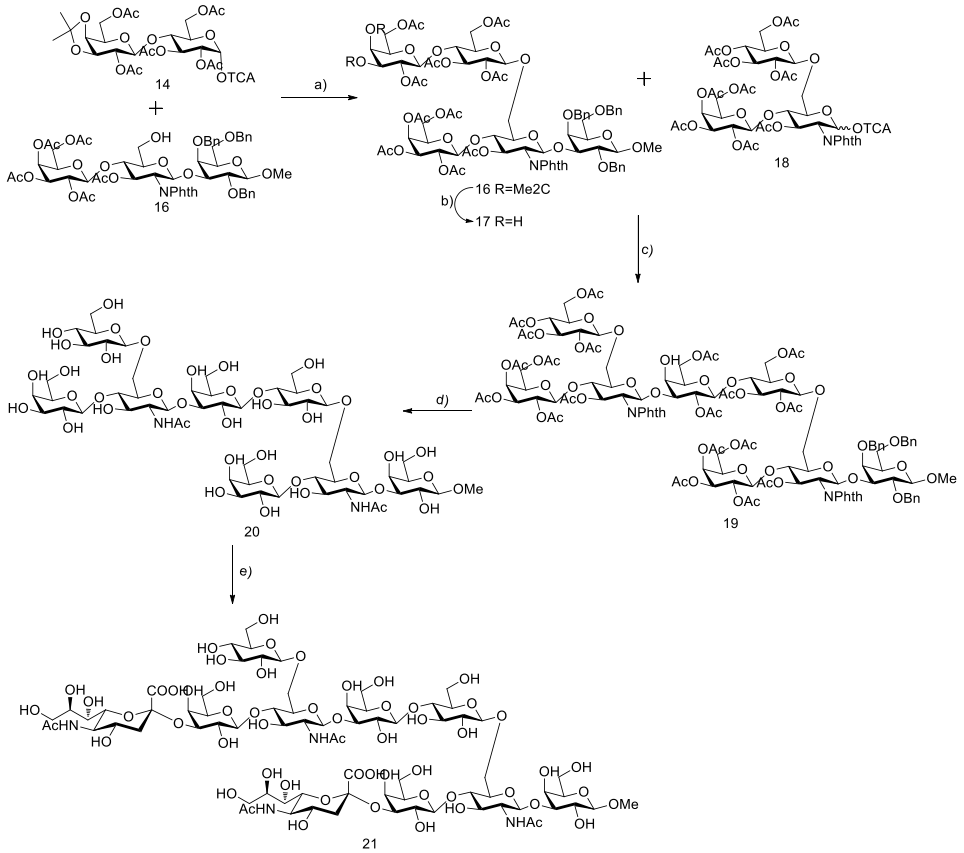
Scheme 1. Chemo-enzymatic synthesis of GBS type III repeating unit[23]. *Reagents and conditions*: a) TMSOTf, DCM, 24h, 25°C, 42.7%; b) NaOMe, MeOH, 24h, 25°C; H_2, Pd-C, EtOH, AcOH, 48h, 25°C, 77.9%; c) CMP-Neu5Ac, Galβ1,3(4)GlcNAc-α(2,3)-sialyltransferase, pH 6.5, 24h, 37°C, 33.8%.

Fully chemical assembly of a heptasaccharide related to PSIII was described by Demchenko et al.[24] that used a highly convergent strategy based on incorporation of the protected α-Neu5Ac-(2→3)-Galp methylthioglycoside **6** previously reported[25] to the GlcpNAc acceptor **7**. Further glycosylation of the 6-OH position of this residue by lactose donor **9** gave the pentenyl pentasaccharide **10**, which was in turn used as donor for glycosylation of the lactose acceptor **11** affording the target protected fragment **12** (Scheme 2). The related desialylated hexasaccharide fragment was also described by the same authors[26].



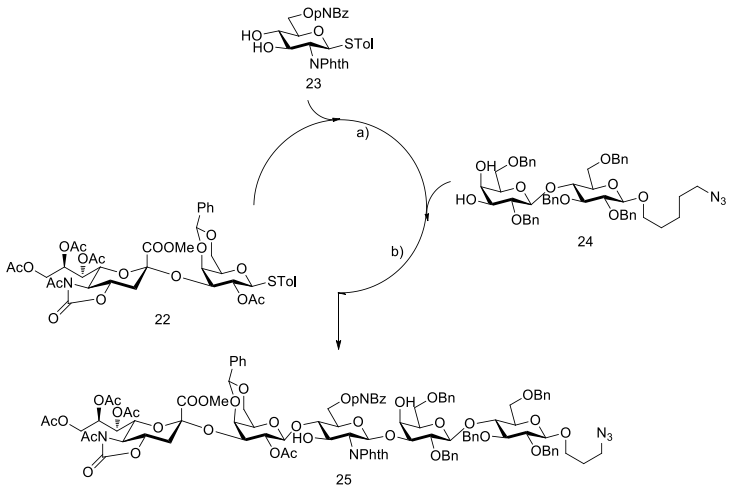
Scheme 2. Synthesis of GBS III heptasaccharide **13**. *Reagents and conditions*: a) MeOTf, MS 3Å, DCM, 96%; b) $TrCO_4$, DCM, 82%; c) NIS/TMSOTf, MS 3Å, DCM, 62%; d) LiI, Pyridine; $(NH_2CH_2)_2$, *n*-BuOH; Ac_2O/MeOH; Pd-C, EtOH/H_2O; 1N aq NaOH, 31%.

A chemo-enzymatic approach was also used by Zou et al. to assemble a decasaccharide, representing two repeating units, of GBS III[27]. The octasaccharide precursor **20** was obtained from the reaction between the acetylated trisaccharide donor **18** and the pentasaccharide acceptor **17** and subsequently enzymatically sialylated by reaction with α-(2→3)-sialyltransferase and CMP-Neu5Ac as donor (Scheme 3). Using the same octasaccharide, a *N*-propionyl substituted sialic acid analog of the GBSIII dimer was also prepared.

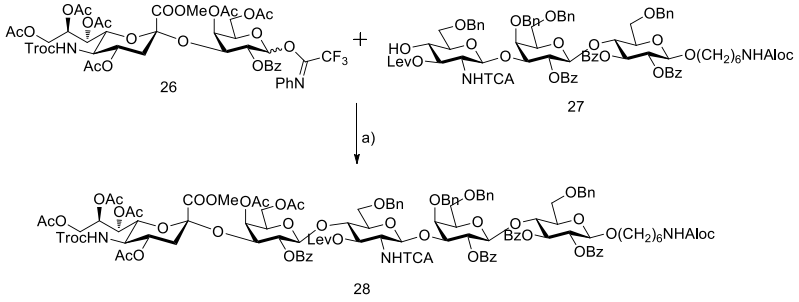


Scheme 3. Chemo-enzymatic route to GBS III decasaccharide. *Reagents and conditions*: a) TMSOTf, DCM, -45°C, 2h, 49%; b) 1:10 TFA/DCM, 0°, 2h, 89%; c) TMSOTf, DCM, -45°C, 2h, 69%; d) $NH_2NH_2 \cdot H_2O$ in 95% EtOH, reflux, 16h; Ac_2O, MeOH/H_2O, overnight, 35%; e) CMP-Neu5Ac, α(2→3)-sialyltransferase, 80%.

A similar enzymatic approach has been also utilized to transform oligosaccharides from S. pneumoniae type 14 to PSIII counterparts[28]. Syntheses of structures related to the linear repeating unit of GBS III, a glycan that is commonly found in nature[29], have also been reported. For instance, Hsu et al. used a 5-*N*,4-*O*-oxazolidinone α-Neu5Ac-(2→3)-Gal*p* tolylthioglycoside donor to achieve the one-pot assembly of oligosaccharide **25** (Scheme 4)[30], whereas the *N*-Trichloroethoxycarbonyl protected α-Neu5Ac-(2→3)-Galp trifluoroacetimidate donor **26** was utilized by Hanashima et al. for glycosylation of the trisaccharide acceptor **27**[31] (Scheme 5).

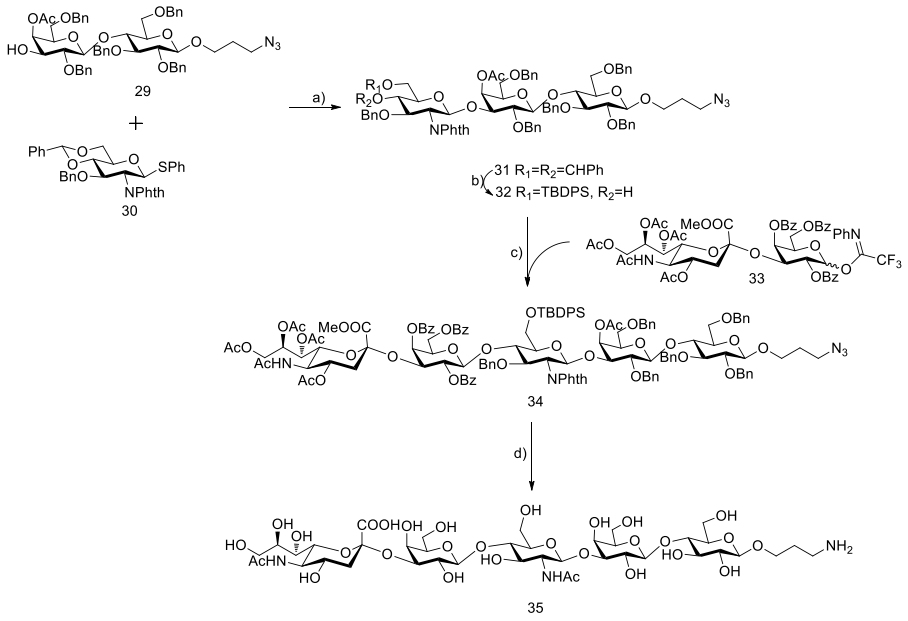


Scheme 4. Reactivity-based one-pot glycosylation of pentasaccharide **25**. *Reagents and conditions*: a) NIS, TfOH, MS 4Å, DCM, -78°C; b) NIS, TfOH, -20°C to rt, 48% over two steps performed one-pot.



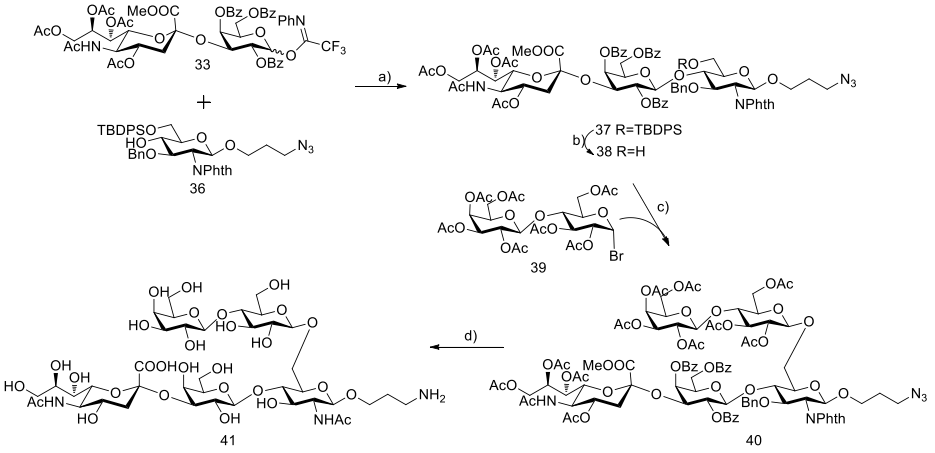
Scheme 5. Hanashima's synthesis of the linear repeating unit of GBS III. *Reagents and conditions*: a) TMSOTf, DCM, 89%.

Finally, in 2017 Cattaneo et al. described the synthesis of the three different frameshifts (linear pentasaccharide **35**, branched pentasaccharide **41** and "Y-shape" pentasaccharide **47**) of GBS PSIII repeating unit bearing a linker at the end-terminal sugar for conjugation to carrier proteins[32]. All pentasaccharide fragments were synthesized through a [3+2] convergent approach employing the sialogalactoside imidate donor **33**, obtained by simple manipulation of a commercially available building block. This disaccharide was used to glycosylate the linear trisaccharide acceptor **32** to obtain the protected linear repeating unit **34**, which after standard deprotection reactions afforded the target structure **35** (Scheme 6).



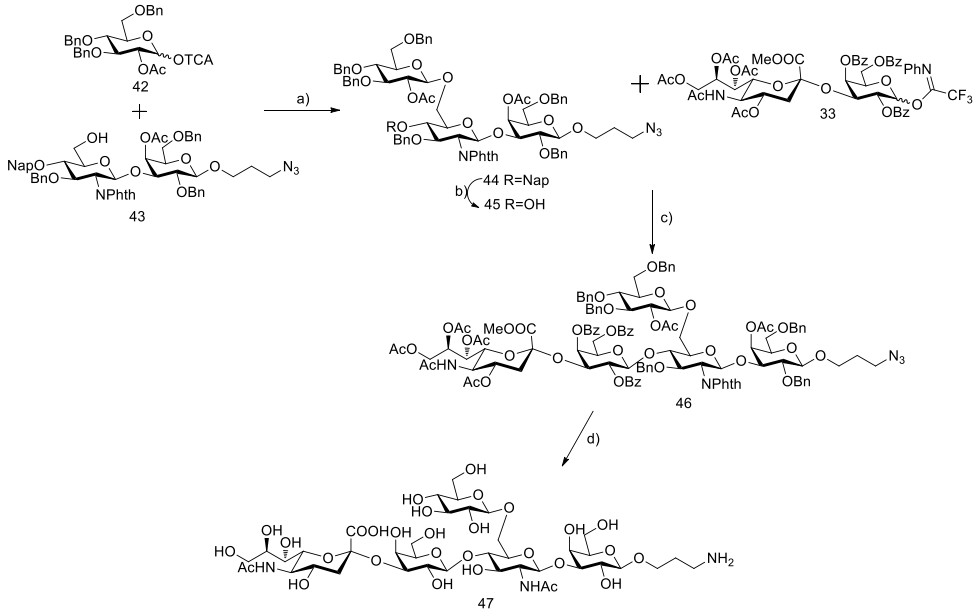
Scheme 6. Synthesis of the GBS III linear repeating unit. *Reagents and conditions:* a) NIS, TfOH, -20°C, 72%; b) 4:1 AcOH/H_2O, 70°C; TBDPS-Cl, DMAP, Py, 60°C, 80% over two steps; c) TMSOTf, DCM, 55%; d) LiI, Pyridine; $(NH_2CH_2)_2$, n-BuOH; Ac_2O/Py; NaOMe, MeOH; Pd-C, EtOH/H_2O, 33%.

The branched repeating unit **41** was again prepared with a [3+2] convergent route, where the sialylated trisaccharide acceptor **38** was glycosylated with peracetylated lactose bromide **39** to obtain the protected pentasaccharide **40**, precursor of the target oligosaccharide **41** (Scheme 7).



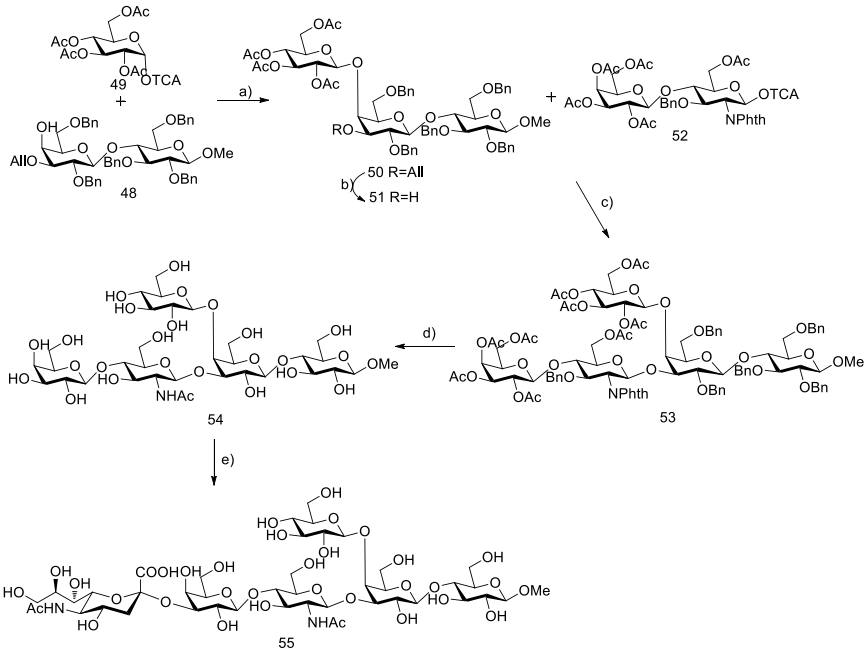
Scheme 7. Synthesis of the GBS III branched repeating unit. *Reagents and conditions*: a) TMSOTf, DCM, 70%; b) HF·Py, 4:1 THF/Py, 0°C to rt, 78%; c) AgOTf, DCM, 68%; d) LiI, Pyridine; $(NH_2CH_2)_2$, n-BuOH; Ac_2O/Py; NaOMe, MeOH; Pd-C, EtOH/H_2O, 42%.

Finally, synthesis of the Y-shape repeating unit **47** was accomplished starting from the trisaccharide acceptor **45**, obtained after Nap removal from compound **44**, which in turn derived from the glycosylation of disaccharide **43** with the glucose imidate donor **42**. Trisaccharide **45** was then glycosylated with the known disaccharide donor **33**, affording the pentasaccharide **46**, tha was deprotected to give target **47** (Scheme 8).



Scheme 8. Synthesis of the GBS III Y-shape repeating unit. *Reagents and conditions*: a) TMSOTf, DCM, 72%; b) DDQ, 4:1 DCM/MeOH, 85%; c) TMSOTf, DCM, 65%; d) LiI, Pyridine; $(NH_2CH_2)_2$, n-BuOH; Ac_2O/Py; NaOMe, MeOH; Pd-C, EtOH/H_2O, 55%.

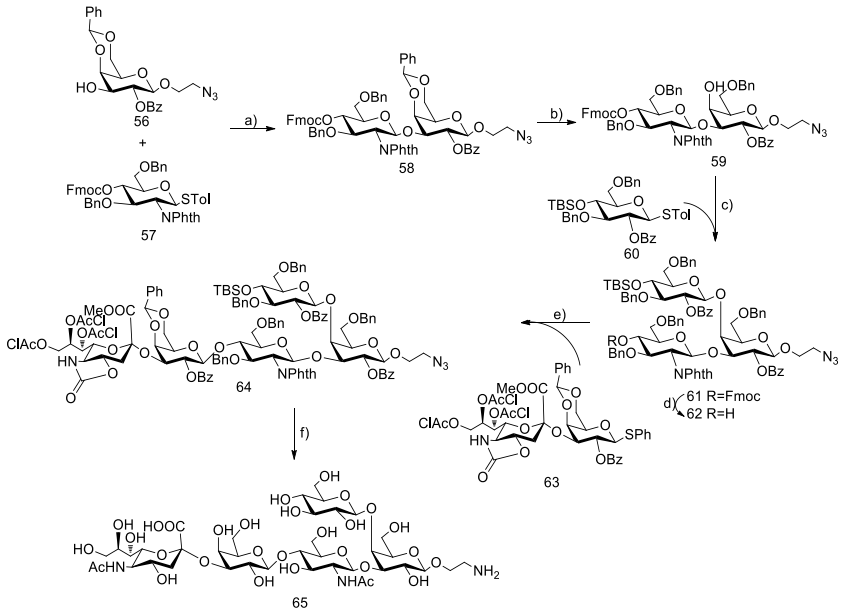
In recent years, syntheses of glycans from other GBS serotypes have been reported, with a particular attention to serotype Ia. A chemo-enzymatic synthesis was described by Zou and Jennings to deliver a GBS Ia related hexasaccharide starting from a synthetic pentasaccharide, which was sialylated with a recombinant α-(2→3)-sialyltransferase using CMP-Neu5Ac as donor (Scheme 9)[33].



Scheme 9. *Reagents and conditions*: a) TMSOTf, DCM, -45°C, 75%; b) $PdCl_2$, MeOH, 71%; c) TMSOTf, DCM, -45°C, 41%; d) H_2NNH_2, 95% EtOH, reflux, 16h; Ac_2O/ Py, overnight; NaOMe, MeOH, 2h; H_2, Pd-C, 79%; e) CMP-Neu5Ac, α(2→3)sialyltransferase, 59%.

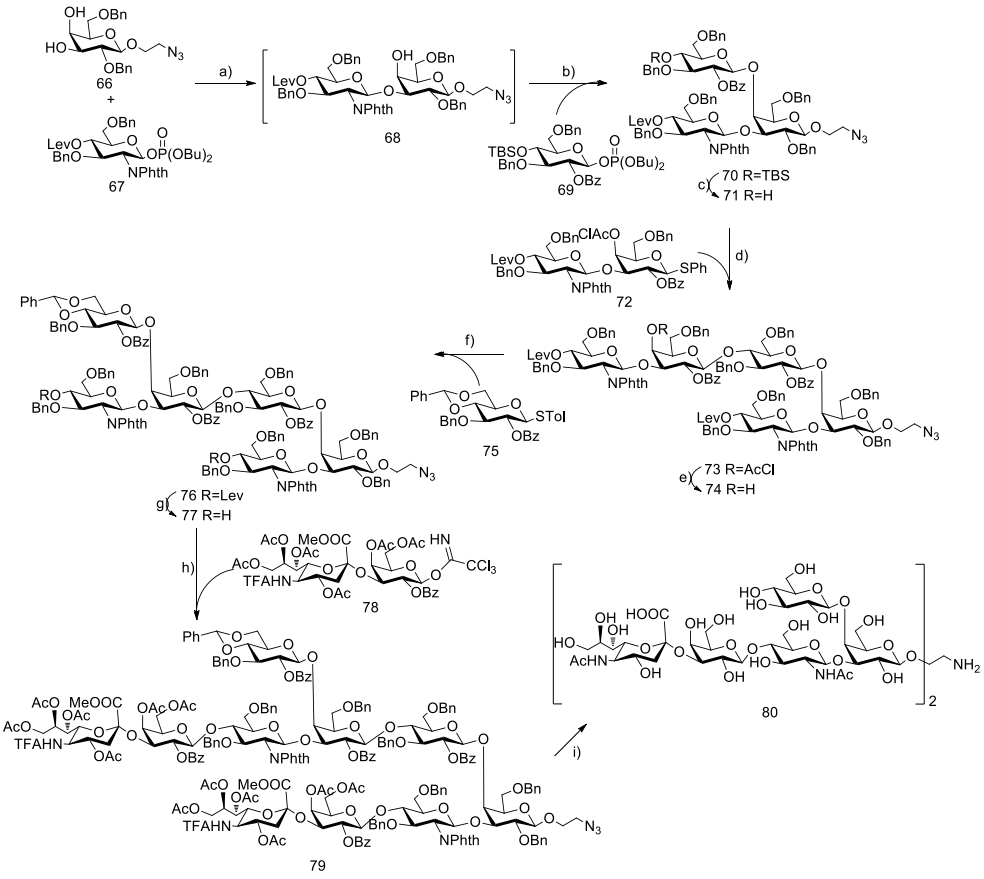
In 2015 the total synthesis of pentasaccharide **65** corresponding to the branched repeating unit of GBS Ia has been accomplished by Guo and coworkers, using a convergent [2+3] route between a sialo-galactose thioglycoside donor and a branched trisaccharide acceptor (Scheme 10)[34].

An investigation on the reactivity of the galactose acceptor in the glycosylation reaction showed that the presence of a sugar at the 4-*O*-position had a big impact on the reactivity of the 3-hydroxy group, since it made the 3-*O* position inaccessible for further glycosylation. Glycosylating the 3-*O*-position had a smaller impact on the 4-*O*-position; for these reasons the proper sequence for installation around galactose 3-/4-OH was to glycosylate the 3-*O*-position prior to 4-*O*-glycosylation. To this purpose, galactose **56** bearing a benzylidene protective group to block the C4 and C6 positions was used in the first glycosylation to obtain the fully protected Glc*N*Ac-β-(1→4)-Gal disaccharide **58**. At this point, the regioselective opening of the benzylidene ring afforded the disaccharide acceptor **59** with the 4-hydroxy group available for the second glycosylation to obtain the branched trisaccharide **61**. Deprotection of the Fmoc protecting group yielded trisaccharide **62**, which was glycosylated with the sialogalactoside donor **63** to afford pentasaccharide **64**. Full deprotection using a six steps procedure gave the target compound **65**, corresponding to the repeating unit of GBS serotype Ia CPS.



Scheme 10. Synthetic route to GBS serotype Ia repeating unit. *Reagents and conditions*: a) NIS, AgOTf, MS 4Å, -30°C to -25°C, DCM, 75%; b) $NaBH_3CN$, HCl, MS 4Å, DCM, 0°C to rt, 95%; c) NIS, AgOTf, MS 4Å, -40°C to -10°C, DCM, 72%; d) Et_3N/DCM 1:1, rt, 100%; e) NIS, AgOTf, MS 4Å, -30°C to -25°C, DCM, 55%; f) TEA-3HF, THF, rt, 48h; LiOH, MeOH/H_2O (1:1), NH_2NH_2-H_2O, reflux, 6d; Ac_2O/Py, rt, 12h; NaOMe/MeOH, rt, 12h; H_2, Pd-C, MeOH, 24h, 67%.

Recently, this approach was extended to the synthesis of GBS Ia decasaccharide **80** corresponding to two repeating units, using a highly convergent strategy (Scheme 11). First, the key intermediate trisaccharide **70** was constructed via a one-pot iterative glycosylation. This trisaccharide was elongated to provide hexasaccharide **77** to which two side chains were attached by dual glycosylation with the sialogalactoside donor **78**[35].



Scheme 11. Synthesis of GBS type Ia dimer. *Reagents and conditions*: a) TMSOTf, DCM, -78°C to -40°C; b) TMSOTf, DCM, -78°C to -40°C, 88%; c) $NEt_3 \cdot HF$, DCM, rt, 12h, 95%; d) NIS, TfOH, MS 4Å, DCM, -40°C, 1h, 85%; e) Et_3N, MeOH, rt, 10 min, 88%; f) NIS, TfOH, MS 4Å, DCM, -40°C, 1h, 92%; g) NH_2NH_2, DCM/MeOH, rt, 10 min, 89%; h) TMSOTf, MS 4Å, DCM, 0°C, 1h, 55%; i) LiOH, MeOH, H_2O, 12h; NH_2NH_2, EtOH, reflux, 24h; Ac_2O, Py, 12h; NaOMe, MeOH, 6h; H_2, Pd-C, 24h, 56%.

The synthesis of the repeating unit of other GBS serotypes which are not the topic of this thesis have also been described. It is worth to mention that Gao et al. described the first synthesis of GBS type V repeating unit, consisting of an heptasaccharide fragment, which was prepared by a preactivation-based one-pot [2+1+4] glycosylation strategy which not only resulted in the reduction of the number of steps to the final oligosaccharide, but also in avoiding many manipulations to the glycosyl donor[36]. In 2017, the same group described the synthesis of GBS type II branched heptasaccharide corresponding to a frameshift of the repeating unit, which was achieved by a convergent [4+2+1] glycosylation strategy[37]. Finally, recently Guo and coworkers assembled both a hexasaccharide repeating unit of GBS type VII, and the corresponding dimer. The synthesis was achieved by means of a preactivation-based iterative one-pot glycosylation protocol, which was made possible by optimization of the challenging stereoselective α-glucosylation reaction[38].

These synthetic efforts have paved the way towards preparation of more complex GBS oligosaccharides from different serotypes. These structurally defined oligosaccharides can be used either to map the relevant epitopes expressed on the capsular polysaccharides or as potential antigens in GBS glycoconjugate vaccines.

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