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Chemical Synthesis and Immunological Evaluation of Fragments of the Multiantennary Group-Specific Polysaccharide of Group B *Streptococcus*

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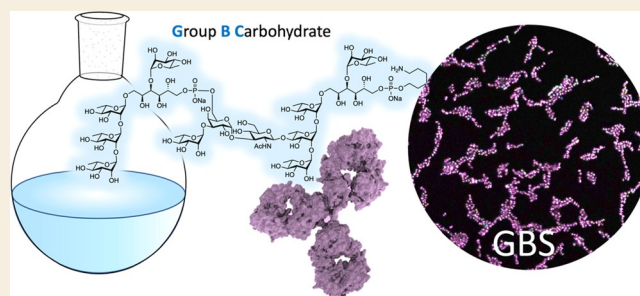
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ABSTRACT: Group B *Streptococcus* (GBS) is a Gram-positive bacterium and the most common cause of neonatal blood and brain infections. At least 10 different serotypes exist, that are characterized by their different capsular polysaccharides. The Group B carbohydrate (GBC) is shared by all serotypes and therefore attractive to be used in a glycoconjugate vaccine. The GBC is a highly complex multiantennary structure, composed of rhamnose rich oligosaccharides interspaced with glucitol phosphates. We here report the development of a convergent approach to assemble a pentamer, octamer, and tridecamer fragment of the termini of the antennae. Phosphoramidite chemistry was used to fuse the pentamer and octamer fragments to deliver the 13-mer GBC oligosaccharide. Nuclear magnetic resonance spectroscopy of the generated fragments confirmed the structures of the naturally occurring polysaccharide. The fragments were used to generate model glycoconjugate vaccine by coupling with CRM197. Immunization of mice delivered sera that was shown to be capable of recognizing different GBS strains. The antibodies raised using the 13-mer conjugate were shown to recognize the bacteria best and the serum raised against this GBC fragment-mediated opsonophagocytic killing best, but in a capsule dependent manner. Overall, the GBC 13-mer was identified to be a highly promising antigen for incorporation into future (multicomponent) anti-GBS vaccines.

KEYWORDS: carbohydrate synthesis, glycoconjugates, synthetic vaccines, glycosylation, phosphoramidites



INTRODUCTION

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is a β -hemolytic Gram-positive bacterium, and the most common cause of neonatal septicemia and meningitis, which are life-threatening for newborn babies.^{1–6} A recent report estimates that 147,000 stillbirths and infant deaths annually are caused by GBS.⁷ It was identified as a human pathogen in 1933, and is a crucial cause of severe disease for susceptible individuals, such as pregnant women, immunocompromised patients, and the elderly.^{8,9} Until now, there is no vaccine commercially available to prevent GBS infections, although clinical trials are ongoing.^{10,11} Bacterial cell surface-coated carbohydrates play an important role in binding events and recognition by the host immune system^{12,13} and bacterial cell wall polysaccharides (CWPs) are excellent targets to be used in carbohydrate-based antibacterial vaccines.^{14–16} As early as in 1938, Rebecca Lancefield demonstrated that the infection of mice by GBS could be prevented using CWP-specific rabbit sera.^{17,18} At least 10 different GBS serotypes can be distinguished on the basis of their capsular polysaccharide (CPS) structure, including type Ia, Ib, II, III, IV, V, VI, VII,

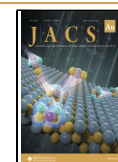
VIII, and IX, although there is a relevant number of GBS strains which do not belong to the classified serotypes.¹⁹ Different CPSs have been explored in conjugate vaccines, but none have shown cross reactivity to other serotypes, even though the structure of some of the CPSs are highly similar. GBS strains also express another carbohydrate-based epitope, that is common to all strains: the Group B carbohydrate (GBC), originally used by Rebecca Lancefield to define the species. The GBC is anchored to the peptidoglycan layer and its complex multiantennary structure has been elucidated by Jennings and co-workers.^{20,21} Four different subunits make up the structure of the GBC, which are composed of L-rhamnose, D-galactose, D-N-acetylglucosamine, and D-glucitol, with phosphate diesters joining the different subunits between a

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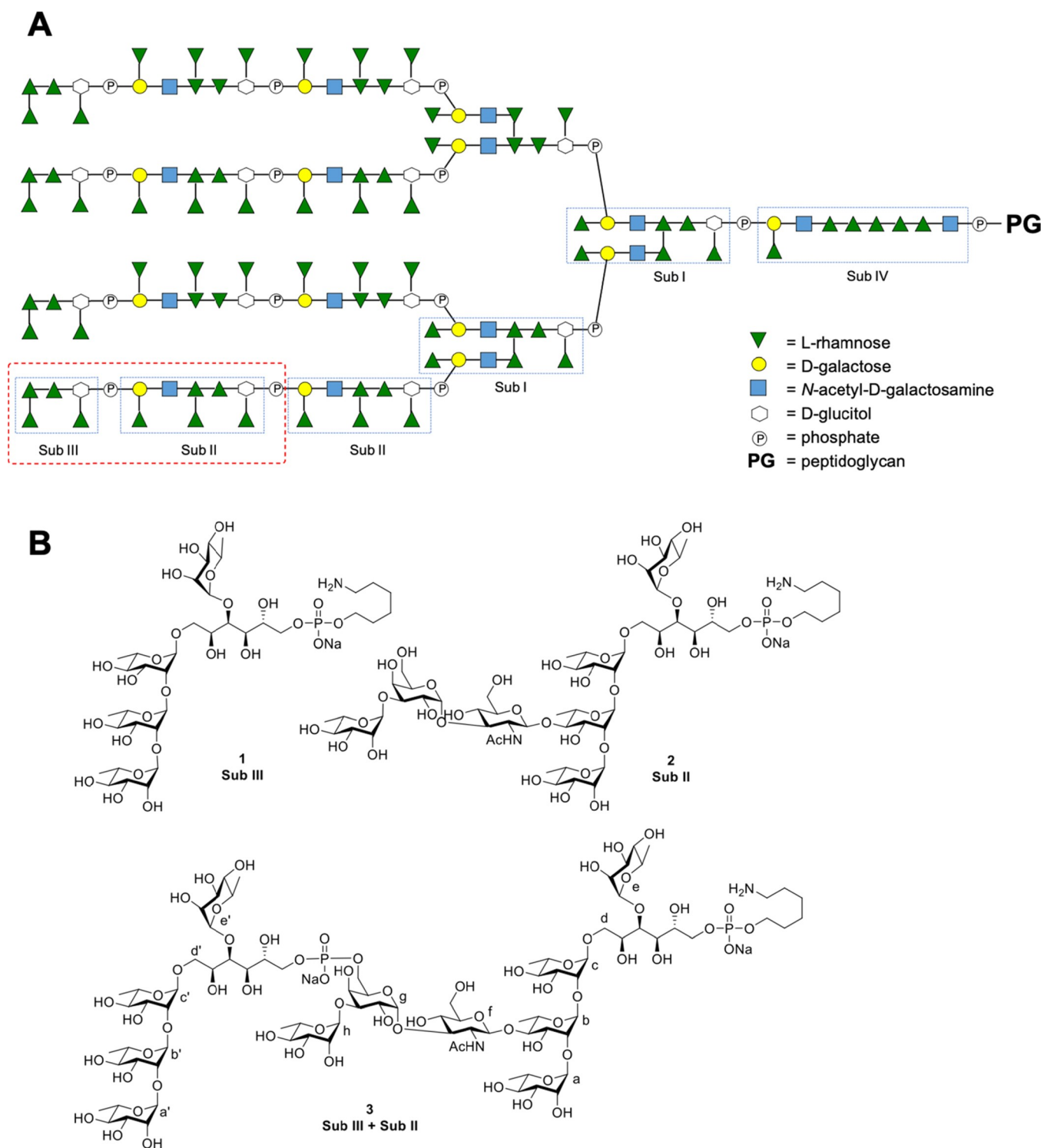


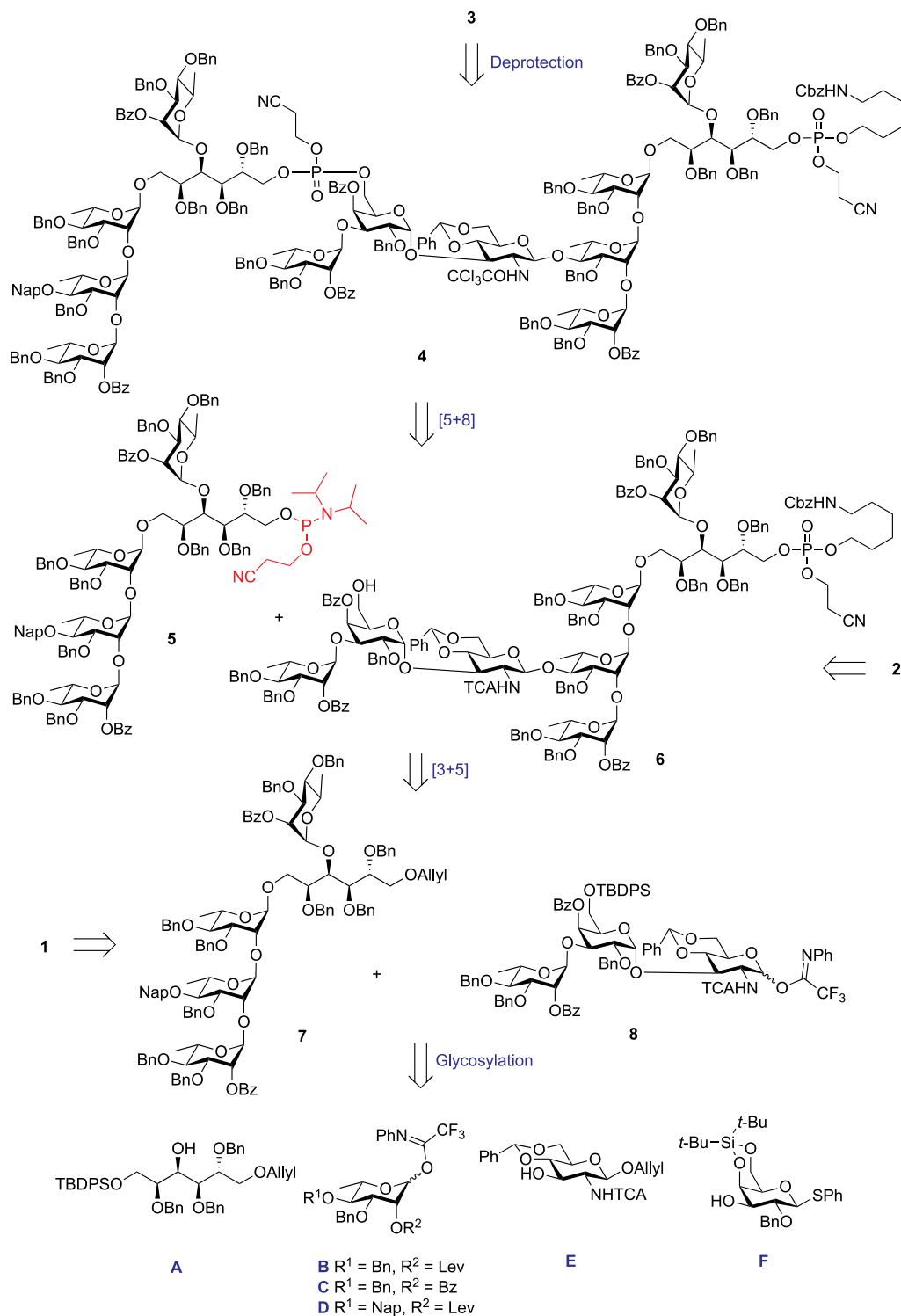
Figure 1. (A) Structure of the complex multiantennary structure of the GBC. P, phosphate; PG, peptidoglycan (B) structures targeted in this study.

glucitol residue and the O-6 of the α -galactose residue (see Figure 1A). While GBC seems to play a major role in the growth of GBS cells, the immunological properties of this polysaccharide and its potential as vaccine antigen are poorly understood.^{18,22}

Because of their immunological significance and due to the fact that bacterial polysaccharides often cannot be obtained in sufficient purity and quantity from natural sources, the chemical synthesis of bacterial oligosaccharides for vaccine

purposes has drawn considerable attention.^{14–16,23–29} The generation of well-defined oligosaccharide fragments also allows for binding studies (for example with monoclonal antibodies) at the atomic level and the mapping of relevant epitopes. Chemical syntheses of the repeating units of serotypes Ia,^{30–32} Ib,³² II,³³ III,³⁴ and V,³⁵ have been published in the last 10 years. As GBC is isolated as a contaminant from GBS CPS, the availability of pure and well-defined GBC structures is critical to elucidate its immunological potential.²²

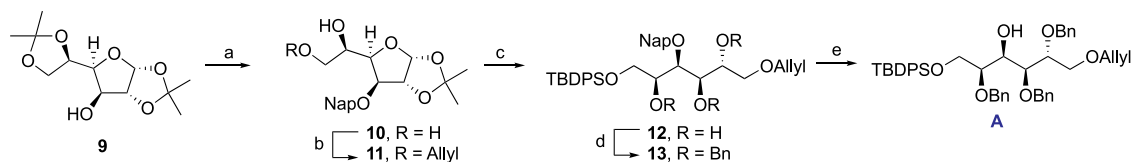
Scheme 1. Retrosynthetic Analysis of the Target Structures 1–3



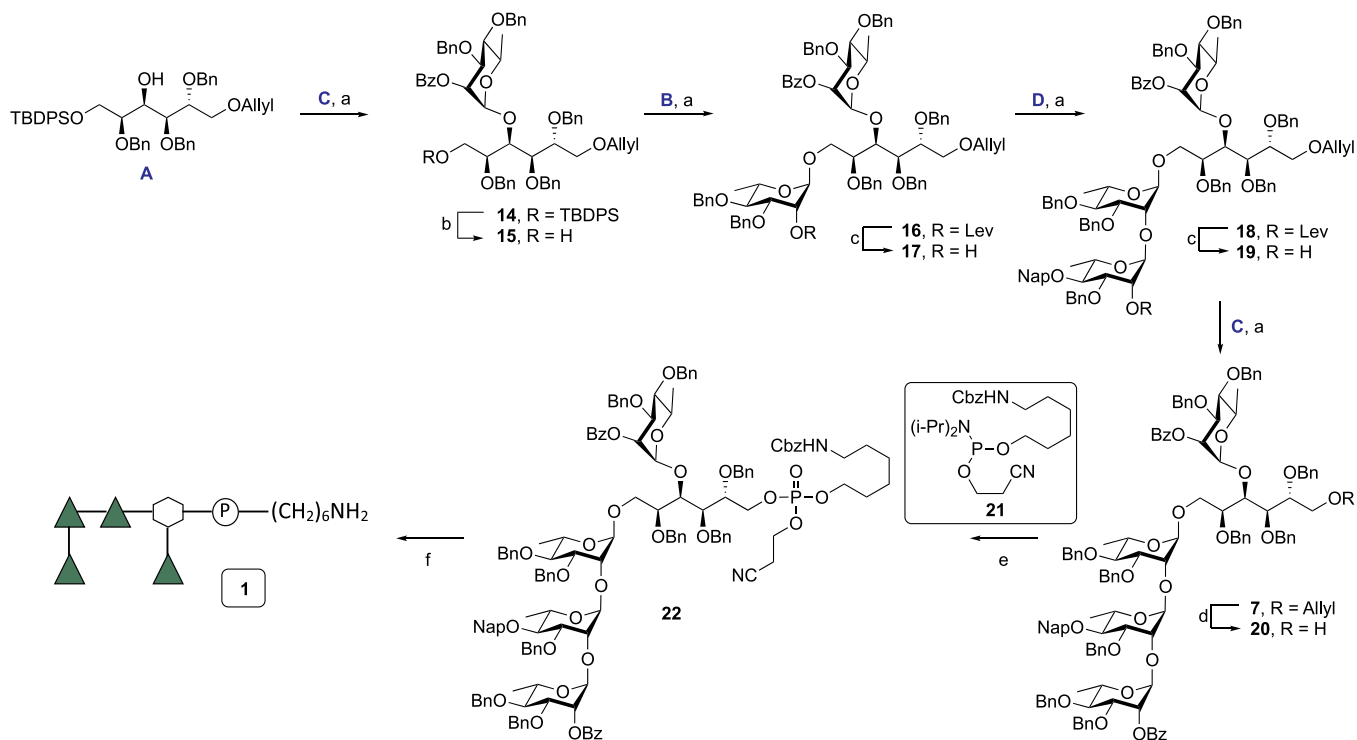
Even though the structure of GBC has been known for a long time, at present, only a trisaccharide³⁶ and tetrasaccharide³⁷ of the rhamnose moiety of the common antigen have been synthesized, and the role of this unique carbohydrate structure remains to be established.

To make well-defined fragments of the GBC available for further studies and potential applications in conjugate vaccines, we set out to develop a synthetic methodology to generate these fragments. As described above, the GBC is built up from

different substructures, and we decided to target the structures representing the termini of the tetra-antennary structure, as these are most exposed and likely to most prominently interact with the host. The boxed structure in Figure 1A represents a tridecasaccharide, containing most components of the complete GBC, therefore representing an attractive structure for immunological evaluation. It is built up from a pentasaccharide (Substructure III) and an octasaccharide (Substructure II), which are interconnected through a

Scheme 2. Assembly of Building Block A^a

^aReagents and conditions: (a) i, NapBr, NaH, DMF, 0 °C, 3 h; ii, 70% AcOH, rt., overnight, 92% (over 2 steps). (b) 2-Aminoethyl diphenylborinate, KI, K₂CO₃, AllylBr, MeCN, 60 °C, 24 h, 91%. (c) i, 80% AcOH, reflux, 2 h; ii, NaBH₄, H₂O, EtOH-CHCl₃; iii, TBDPSCl, imidazole, DMF, 0 °C, 87% (over 3 steps). (d) NaH, BnBr, TBAI, DMF, rt., 94%. (e) DDQ, DCM/H₂O, 95%.

Scheme 3. Assembly of Pentasaccharide 1^a

^aReagents and conditions: (a) TBSOTf, 4 Å MS, DCM, 0 °C, **14**, 97%; **16**, 82%; **18**, 71%; **7**, 87%. (b) TBAF, THF, 0 °C—rt., 93%. (c) N₂H₄•H₂O, Py/AcOH, 0 °C—rt., **17**, 97%; **19**, 93%. (d) Ir(COD)(Ph₂MeP)₂•PF₆, THF, H₂, 1 h, then NIS and H₂O, 1 h, 90%. (e) **21**, DCI, MeCN, 3 Å MS, 1 h, then CSO, 15 min, 90%. (f) i, ammonium hydroxide, 1,4-dioxane; ii, NaOMe, MeOH/1,4-dioxane; iii, Pd(OH)₂/C, H₂, *t*-BuOH/H₂O, 3 days, 80%.

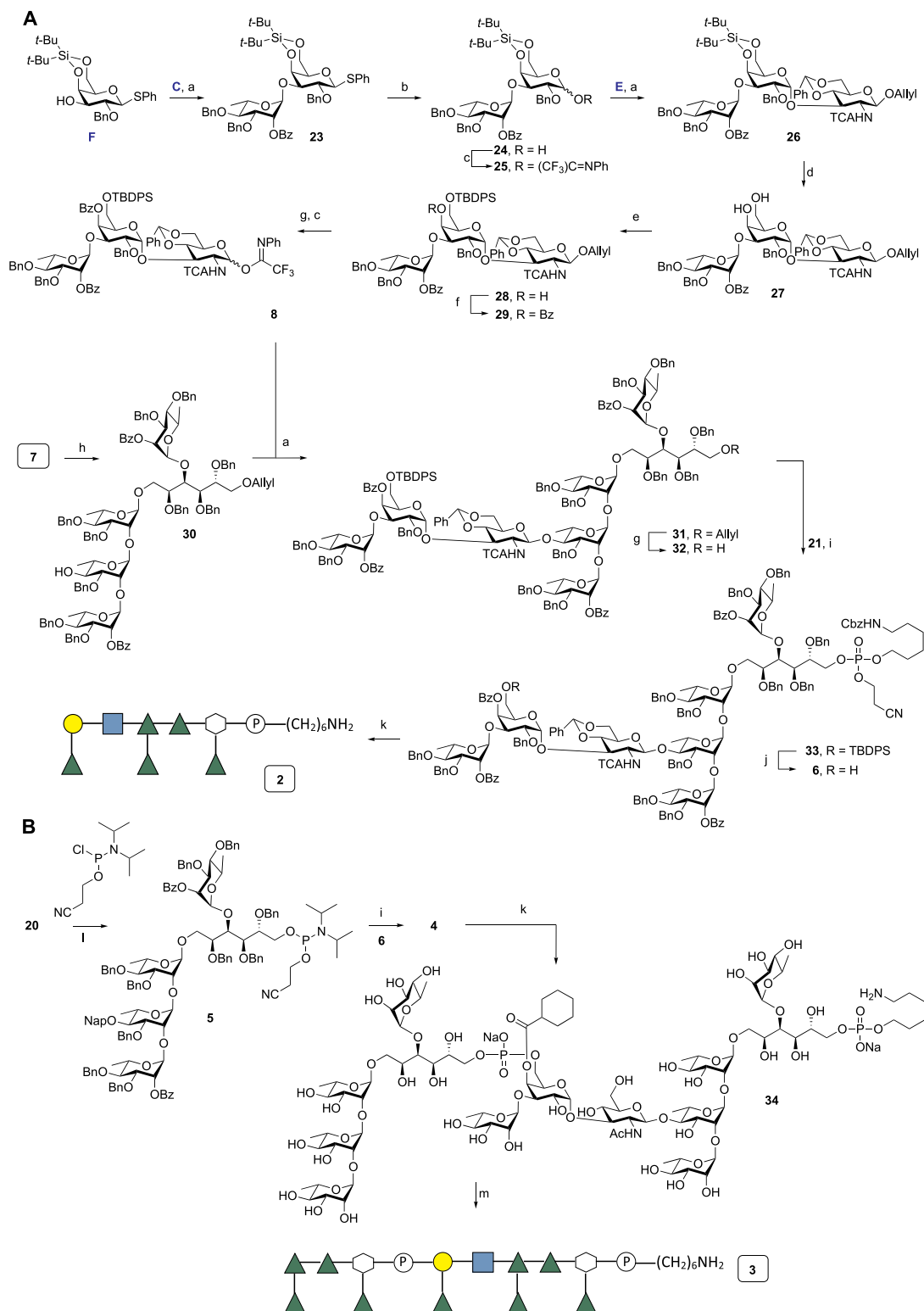
phosphate diester bridge. We here describe the synthesis of conjugation-ready GBC fragments **1** (the Sub III structure), **2** (the Sub II fragment), and **3** (the Sub II + Sub III oligomer). Because of the phosphate joints in the natural compound, a phosphate spacer was chosen to be coupled to the three different targets as shown in Figure 1B, terminating in a primary amine, to allow for conjugation to a carrier protein. The generated structures were used to construct neo-glycoconjugate vaccines by coupling with CRM197. These were used for immunization studies to generate anti-GBC sera, which were tested in ELISA showing cross reactivity with the HSA-conjugates of fragments **1**, **2**, and **3**. Antibodies in the raised sera were evaluated by confocal microscopy and an opsonophagocytic killing assay to assess their ability to bind and kill bacterial cells belonging to different GBS strains and serotype classes.

RESULTS AND DISCUSSION

A retrosynthetic analysis toward the three targets is shown in Scheme 1. It was reasoned that the target tridecasaccharide **3**

could be obtained from the protected tridecasaccharide **4** after a sequence of deprotection steps, including basic hydrolysis of the cyanoethyl (CE) and benzoyl (Bz) groups and hydrogenation of the benzyl (Bn), 2-methylnaphthyl (Nap), and benzyloxycarbonyl (Cbz) groups and transformation of the trichloroacetamide (TCA) to the corresponding acetamide. Compound **4** could be constructed via a convergent [5 + 8] phosphate coupling strategy using pentasaccharide phosphoramidite **5** and the branched octasaccharide with a free galactosyl C-6-OH **6**. The key octasaccharide intermediate **6** was assembled by a [3 + 5] glycosylation strategy, which employed the trisaccharide **8** as the donor and pentasaccharide **7** as the acceptor. The latter pentasaccharide is also the precursor to the pentasaccharide phosphoramidite **5**. The Nap-ether at the C-4 of the central rhamnose moiety in **7** and **5** thus serves both as a permanent and a temporary protecting group. Both the tri- and pentasaccharide **7** and **8** were prepared via glycosylations using monosaccharide building blocks **A** to **F**.

In the above-described strategy, neighboring group participation is expected to control the anomeric selectivity in the

Scheme 4. (A) Assembly of Octasaccharide 2 and (B) Tridecasaccharide 3⁴

⁴Reagents and conditions: (a) TBSOTf, DCM, 0 °C, 1 h, 4 Å MS, **23**, 93%; **26**, 81%; **31**, 75%. (b) NIS, TFA, DCM, 0 °C, 0.5 h, 94%. (c) *N*-Phenyltrifluoroacetimidoyl chloride, Cs₂CO₃, acetone, overnight, **25**, 93%; **8**, 72% (2 steps). (d) HF•Py, Py, THF, 87%. (e) TBDPSCI, DMF, imidazole, 94%. (f) BzCl, Py, DMAP, 3 days, 95%. (g) Ir(COD)(Ph₂MeP)₂•PF₆, THF, H₂, 1 h, then NIS and H₂O; trisaccharide hemiacetal, 90%; **32**, 87%. (h) DDQ, DCM/H₂O, 85%. (i) DCI, ACN, 3 Å MS, then, CSO, **33**, 92%; **4**, 82%. (j) HF•Py, THF/Py, 93%. (k) i, ammonium hydroxide, 1,4-dioxane; ii, NaOMe, MeOH/1,4-dioxane; iii, Pd(OH)₂/C, H₂, AcOH, *t*-BuOH/H₂O, 3 days, **2**, 70%. (l) DIPEA, DCM, 3 Å MS, 84%. (m) 1 M NaOH, H₂O, 52%.

glycosylations forming 1,2-*trans*-linkages. For the construction of the *cis*-galactosyl linkage, a silylidene group was to be used

as stereochemistry controlling functionality.^{38,39} Due to their high reactivity, convenient manipulation, and facile purification

tion, glycosyl *N*-phenyltrifluoroacetimidates were adopted as donors for all the glycosylation reactions. Of the listed building blocks (Scheme 1), rhamnosyl imidate donor **B**,⁴⁰ glucosamine acceptor **E**,⁴¹ and galactose acceptor **F**²³ were prepared following reported procedures, while rhamnosyl imidate donors **C** and **D** were synthesized specifically for this study following adapted literature methods as described in the Supporting Information. A detailed description of glucitol acceptor **A** is shown in Scheme 2. 1,2-*O*-Isopropylidene-3-*O*-naphthylmethyl- α -D-glucopyranose **10**,⁴² synthesized from diacetone-D-glucose **9**, was transformed into allyl-protected alcohol **11** in excellent yield via a borinic acid-catalyzed regioselective alkylation.⁴³ Subsequently, the ketal in **11** was removed in refluxing 80% acetic acid, which was followed by the sodium borohydride mediated reduction of the resulting hemiacetal and selective silylation using the bulky *tert*-butyldiphenylsilyl (TBDPS) group of the primary alcohol to afford triol **12** in 87% over three steps, without purification of the intermediate products. Benzoylation of the three hydroxyls, required careful optimization as silyl migration and hydrolysis were observed during this transformation (see the SI for probed conditions). Eventually the triple benzoylation was achieved in 94% yield using a large excess of benzyl bromide in conjunction with three equivalents tetrabutylammonium iodide. Finally, the D-glucitol building block **A** was obtained by removal of the Nap group in an oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ).

With all the six building blocks in hand, the assembly of the first target molecule **1** was undertaken as shown in Scheme 3. After an initial [3 + 2] model glycosylation showed that the stereochemistry of rhamnose-glucitol linkage was generated with poor selectivity,⁴⁴ the construction of the key pentasaccharide intermediate **7** was explored through a stepwise approach using the monosaccharide building blocks. The first glycosylation between glucitol acceptor **A** and rhamnosyl donor **C** in the presence of *tert*-butyldimethylsilyl triflate (TBSOTf) as a promotor gave glucitol glycoside **14** in excellent yield. The selective deprotection of the TBDPS protecting group was performed utilizing tetrabutylammonium fluoride (TBAF) in THF, and the subsequent glycosylation with donor building block **B** provided the trisaccharide **16** in 82% yield. Deprotection of the levulinoyl ester using $N_2H_4 \bullet H_2O$ furnished trisaccharide acceptor **17** in 97% yield, which was glycosylated with building block **D** under the promotion of TBSOTf to deliver tetrasaccharide **18** in 71% yield. Selective removal of the levulinoyl group and subsequent glycosylation with building block **C** using the above-mentioned conditions provided the key intermediate pentasaccharide **7** in 87% yield. To complete the synthesis of target pentasaccharide **1**, de-allylation was performed using an isomerization reaction employing a catalytic amount of $Ir(COD)(Ph_2MeP)_2$, which was activated using H_2 . The resulting enol ether was cleaved by treatment with NIS and H_2O to provide the alcohol **20** in 90% yield.⁴⁵ Subsequently, the attachment of the spacer was achieved using phosphoramidite functionalized spacer **21** and dicyanoimidazole (DCI) as an activator followed by the oxidation of the intermediate phosphite to the corresponding phosphate triester using (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) to give the fully protected pentasaccharide **22** in 90% yield over two steps. Finally, treatment of the pentasaccharide **22** with concentrated ammonia in dioxane led to the removal of the CE group after which the compound was treated with NaOMe in MeOH/dioxane to remove the Bz

esters. The subsequent palladium hydroxide mediated hydrogenation was performed in a mixture of water/*tert*-butanol under slightly acidic conditions⁴⁶ at 1 atm for 3 days to give the target pentasaccharide **1** in 80% over the last 3 steps.

With the key pentasaccharide **7** in hand, and according to the retrosynthetic analysis, attention was turned to the trisaccharide donor **8** (Scheme 4A). First, the glycosylation of imidate donor **C** with acceptor **F** resulted in desired disaccharide **23** in 93% yield. Transformation of the thiophenyl donor into the corresponding imidate disaccharide donor gave **25** in excellent yield. Subsequently, the stereoselective formation of trisaccharide **26** was achieved through the condensation of **25** and acceptor **E** in 81% yield in the presence of a catalytic amount of TBSOTf. To facilitate the phosphoramidite coupling to provide tridecasaccharide at a later stage, and prevent multiple protecting group manipulations on far-advanced intermediates, the silylidene in **26** was removed using hydrogen fluoride•pyridine (HF•Py) in 87% yield after which the primary C-6 alcohol of the galactoside moiety in **27** was selectively masked with a bulky TBDPS group to give **28**. The benzoylation of the remaining galactoside C-4 hydroxy proved to be challenging, which can be attributed to the low reactivity of this alcohol. Several methods were tried, including the combination of BzCl and Et_3N . However, because of the acidic N-H of the glucosamine moiety, a side-product was generated in which the amide was also benzoylated. The desired product **29** was finally obtained by stirring the substrate with BzCl in pyridine at RT for 3 days. Deprotection of the allyl group of the trisaccharide employing an iridium catalyst and subsequent NIS-mediated hydrolysis of the formed enol ether was followed by the installation of the imidate at the anomeric hydroxyl, to give the trisaccharide donor **8** in good yield.

To assemble the octasaccharide **2**, pentasaccharide **7** was first transformed into an acceptor by the selective removal of the Nap group using DDQ in DCM/ H_2O (Scheme 4A). Next, the [3 + 5] glycosylation with trisaccharide donor **8** using TBSOTf as a promotor gave octasaccharide **31** in 75% yield. As described for the synthesis of **22**, octasaccharide **33** was produced after de-allylation, and reaction with the phosphoramidite spacer and subsequent oxidation, to give the fully protected product in high yield. Deprotection of the octasaccharide **33** started with the removal of the TBDPS group using HF•Py in 93% yield to provide **6**, having a free galactosyl C-6-OH. No migration of the neighboring benzoate was observed under these conditions. Next, the same sequence of reactions was performed as described for the deprotection of pentasaccharide **1**, to generate target octasaccharide **2**. Thus, first the CE group and benzoate esters were removed, after which the reduction of all benzyl ethers, the benzylidene acetal, and the benzyl carbamate, and the concomitant transformation of the TCA into the corresponding acetamide delivered the GBC-octasaccharide **2** in 70% yield.

To assemble the tridecasaccharide **3**, the pentasaccharide phosphoramidite **5** was synthesized from **20** using 2-cyanoethyl *N,N*-di-*iso*-propylchlorophosphoramidite and di-*iso*-propylethyl amine (DIPEA, Scheme 4B). The key coupling of amidite **5** with octasaccharide **6** in the presence of DCI and in situ oxidation of the formed phosphite by CSO gave the fully protected tridecasaccharide **4** in 82% yield. When the global deprotection of this large oligosaccharide was performed using the procedures to generate **1** and **2**, undesired product **34**, containing a cyclohexyl ester, was formed as the major

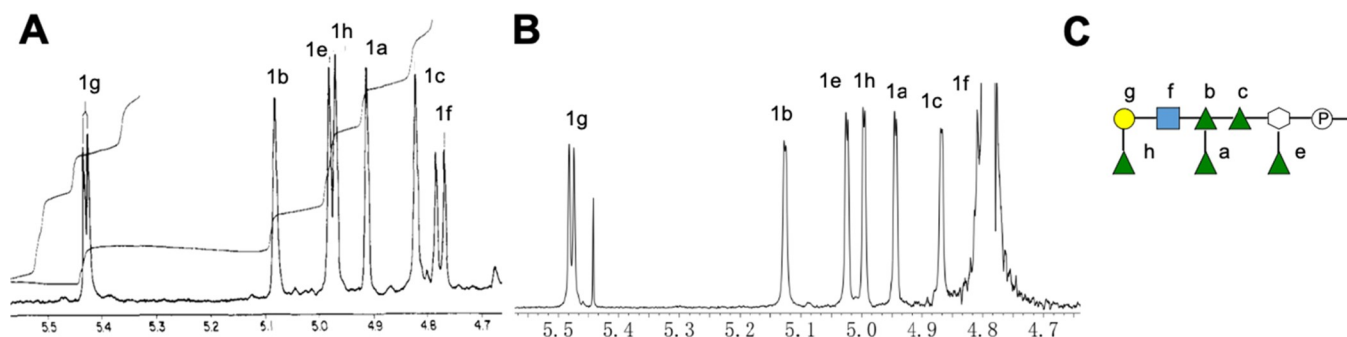


Figure 2. Partial ^1H NMR spectrum (anomeric proton region) of (A) a GBC-Sub II octasaccharide, isolated by degradation of the natural GBC in D_2O [500 MHz] and (B) synthesized octasaccharide **2** in D_2O [500 MHz]. (C) Sugar residue labeling according to [refs 21, 36].

product. This again underlines the low reactivity of the axial C-4 position of the galactose moiety. The cyclohexyl ester could be cleaved from **34** by treatment with 0.2 M NaOH for 24 h, delivering the final compound **3** after size exclusion chromatography in 52% over four steps and completing the set of target compounds.

The structural integrity of all compounds was fully ascertained by nuclear magnetic resonance (NMR) spectroscopy and the NMR data compounds **1**, **2**, and **3** closely matched the spectroscopic data reported for the GBC fragments generated from the natural polysaccharide, lacking the aminohexylphosphate spacer, as can be seen from Figure 2 and Tables 1 and 2.

Table 1. ^1H NMR Data (in D_2O) Comparison of GBC Fragments Obtained from the Natural Polysaccharide and the Synthetic Fragments **1**, **2**, and **3**

anomeric proton	tridecasaccharide 3		octasaccharide 2		pentasaccharide 1	
	natural	synthetic	natural	synthetic	natural	synthetic
1a	4.99	4.99	4.97	4.96		
1b	5.11	5.13	5.12	5.13		
1c	4.87	4.89	4.87	4.87		
1e	5.03	5.05	5.04	5.02		
1f	4.87	4.83	4.84	4.84		
1g	5.53	5.57	5.44	5.48		
1h	5.03	5.04	5.02	5.00		
1a'	4.98	4.97			4.97	4.99
1b'	5.12	5.14			5.10	5.14
1c'	4.89	4.90			4.87	4.90
1e'	5.03	5.02			5.03	5.05

With the structures **1–3** in hand, we generated a set of protein conjugates, to generate anti-GBC sera in mice (Figure 3). To this end, **1–3** were conjugated to CRM₁₉₇, a clinically approved carrier protein,⁴⁷ by functionalization of the amino spacer in the GBC fragments with an active ester through coupling with di-(*N*-succinimidyl)-adipate (SIDEA). The activated GBC fragments were then incubated with the CRM-carrier protein in sodium phosphate buffer at pH 7.2 with a 75:1 saccharide/protein ratio to provide, after purification by filtration, the three conjugates CRM-1, CRM-2, and CRM-3 carrying 28 pentasaccharides, 25 octasaccharides, or 17 tridecasaccharides, respectively, as assessed by CE-MS, while the protein content was estimated by microBCA. We also generated the corresponding human serum albumin (HSA) conjugates, to coat ELISA plates to characterize the serum. Conjugation of the GBC fragments to HSA proceeded

Table 2. ^{13}C NMR Data (in D_2O) Comparison of Natural and Synthetic Fragments of GBC

anomeric carbon	tridecasaccharide 3		octasaccharide 2		pentasaccharide 1	
	natural	synthetic	natural	synthetic	natural	synthetic
1a	103.1	103.3	103.0	102.3		
1b	101.3	101.5	101.2	100.5		
1c	99.6	99.7	99.6	98.8		
1e	102.5	102.4	102.4	101.4		
1f	102.1	102.4	102.0	101.4		
1g	99.1	99.3	99.6	98.8		
1h	103.1	103.3	103.0	102.4		
1a'	103.0	103.2			102.9	102.3
1b'	101.7	101.8			101.6	100.9
1c'	99.6	99.7			99.6	98.8
1e'	102.1	102.3			102.4	101.4

uneventfully and provided the conjugates HSA-1, HSA-2, and HSA-3 having 31, 30, and 19 saccharides attached, respectively.

The CRM conjugates were tested *in vivo* to assess their capability to elicit functional antibodies, that are able to bind and mediate opsonophagocytic killing (OPK) of GBS strains expressing different types of capsule. To this aim, BALB/c female mice (4–6 weeks old) were immunized with three intraperitoneal injections of CRM-1, CRM-2, and CRM-3 at a saccharide dose of 0.5 μg , formulated with alum hydroxide. Two weeks after the third immunization, the sera were collected and serum antibodies were measured by ELISA, where the capability of the elicited IgGs to cross-react with the homologous and heterologous synthetic antigens was assessed. It was demonstrated that each conjugate stimulated the production of a similar level of IgGs, which were able to bind to the homologous oligosaccharide structure as well as the other two synthetic oligosaccharides conjugated to HSA, used as a coating of the ELISA plate. No statistically significant differences were observed among the three groups (Figure 4). The absence of differences among the three groups might suggest the presence of a common immunodominant epitope present in all three fragments, against which most IgGs, elicited by the semisynthetic glycoconjugates, are directed.

To ascertain the recognition of the GBS Lancefield polysaccharide expressed on bacterial cells by the immune sera, we analyzed three GBS strains belonging to different serotypes with immunofluorescence confocal microscopy: strain H36B (serotype Ib), DK21 (serotype II), and CJB111 (serotype V). The binding of serum antibodies to these bacteria was detected by the localization of green fluorescence

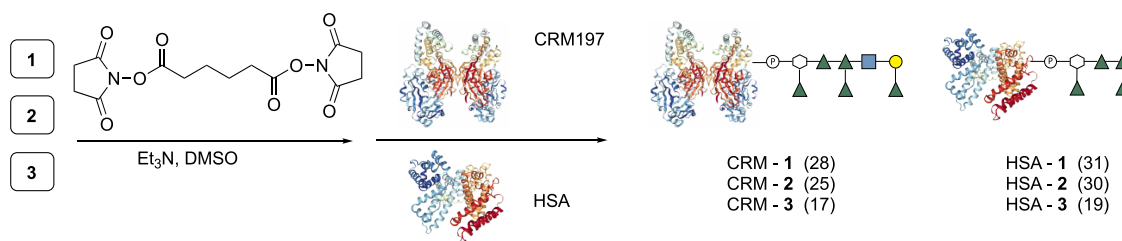


Figure 3. Generation of GBC-conjugates.

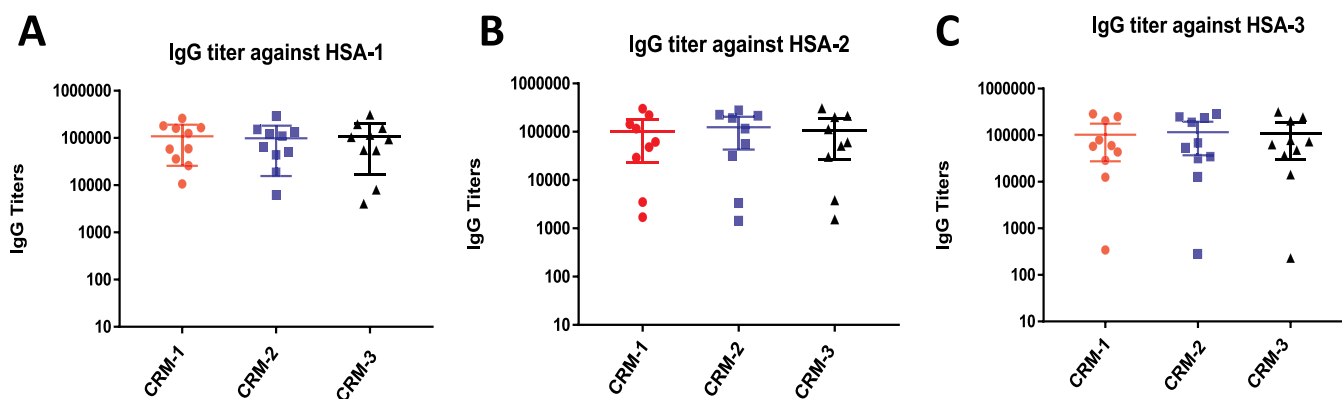


Figure 4. ELISA IgG titers of the sera elicited against CRM conjugates after the third immunization. Dots indicate individual mice. Statistical analysis was performed with Kruskal–Wallis and Dunn multiple comparisons test. (A) IgG titers of CRM conjugates against HSA-1. (B) IgG titers of CRM conjugates against HSA-2. (C) IgG titers of CRM conjugates against HSA-3.

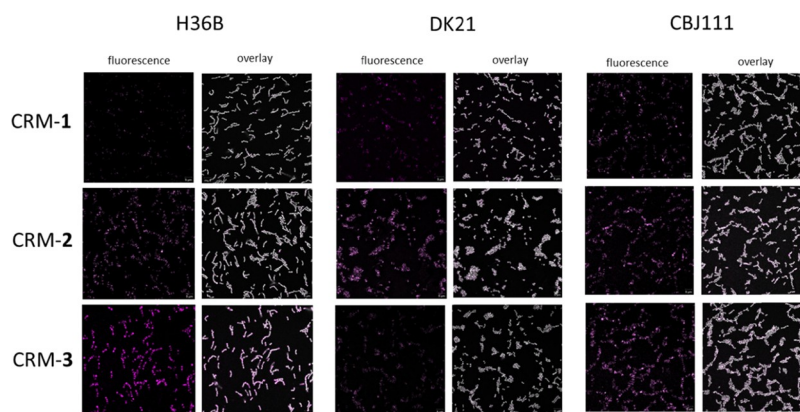


Figure 5. Recognition of different GBS strains by the sera raised against CRM-1, CRM-2, and CRM-3. Fluorescence shown in pink on left panels, overlay with bacterial DNA in right panels.

Table 3. OPKA Titer Obtained Testing Pools of Serum Samples Collected after Three Immunizations with CRM Conjugates CRM-1, CRM-2, and CRM-3^a

serotype	strain	OPKA titers					
		group CRM-1		group CRM-2		group CRM-3	
		preimmune	POST 3	preimmune	POST 3	preimmune	POST 3
Ib	H36B	<LLOD	71	<LLOD	179	<LLOD	336
II	DK21	34	171	<LLOD	258	<LLOD	198
V	CJB111	80	129	73	367	80	703

^aOPKA titers are expressed as the reciprocal serum dilution leading to 50% killing of bacteria. Post three values represent these titers after the third immunization as the mean of two independent experiments. <LLOD means below lower limit of detection.

after incubation with a secondary antimouse IgG antibody carrying an Alexa Fluor 488 dye. As shown in Figure 5, the sera bound to the surface of the different GBS strains. For H36B, the sera raised against the larger fragments seemed to bind best to the bacterial surface, with the highest fluorescence intensity

be observed for the anti-3 serum. For DK21, most binding was detected for the anti-2 serum, while the CBJ111 bacteria were recognized equally well by the sera raised against the octamer 2 and tridecamer 3. No recognition was detected for the preimmune sera (see SI).

In order to assess the functionality of the raised antibodies, the generated sera were analyzed by an *in vitro* opsonophagocytic killing assay (OPKA), to mimic the *in vivo* process of GBS killing by effector cells in the presence of complement and specific antibodies.^{48,49} In fact, we previously found that an OPKA for GBS polysaccharides is more sensitive in discriminating protective differences among conjugates as compared to the *in vivo* challenge model.⁵⁰ On the GBS cell surface, the GBC is co-expressed with CPS. To assess the influence of capsule expression on the access of the anti-GBC antibodies to the bacterial cell surface, pooled sera of the three groups were tested on several GBS strains belonging to different serotypes: strain H36B, DK21, CJB111 (tested in the confocal microscopy study above), COH1 (serotype III), and 515 (serotype Ia). These correspond to relevant GBS strains that have been used to evaluate the functionality of serum antibodies elicited by GBS CPS-conjugate vaccine candidates, tested in Phase I clinical trials, and they are characterized by different levels of capsule expression. The OPKA results (Table 3) show that the antibodies were functional against strains H36B, DK21, and CJB111. Notably, increasing titers of functional antibodies were observed going from the shortest (CRM-1) to the longest conjugated oligosaccharide (CRM-2 and CRM-3) for the H36B and DK21 strains. In contrast, no OPK titer could be measured against strains COH1 and 515 (data not shown), which are known to have a higher extent of CPS expression as compared to strain H36B and DK21. Although no information is available regarding the level of CPS expression of CJB111, the effective opsonophagocytosis indicates the GBC to be well accessible. Interestingly, we observed a good correlation between the intensity of the fluorescence signal measured in the confocal studies above and the OPK results, showing that immune sera raised against the conjugates with the longest GBC oligomers show more effective functionality in inducing phagocytic killing. Although there is no significant difference in the level of IgGs (Figure 4), the sera raised against the longer fragments show better binding and are more opsonophagocytic. This may indicate that IgGs directed against an immunodominant epitope, present in all three oligosaccharides, are less capable of binding GBS bacterial surfaces and contribute less to sera functionality. The longer fragments may harbor longer epitopes and they may induce the production of higher affinity antibodies.

CONCLUSIONS

GBS is a leading cause of pneumonia, septicemia, and meningitis in infants and newborns, but currently, there are no vaccines available. GBS CPSs, which determine the serotype classification, are considered promising targets for vaccine development. GBS CPS-conjugates have been tested in clinical trials in monovalent and multivalent formulations. The GBC is shared among all GBS serotypes and despite being a potential immunogen, it has never been investigated as a potential antigen in vaccine conjugates. We therefore synthesized three conjugation-ready structures, derived from the outer parts of the branches of tetra-antennary GBC, to characterize the immune potential of these antigens. The structures were conjugated to the CRM197 carrier protein and their immunogenicity was assessed *in vivo* in mice. ELISA showed that each conjugate elicited an immune response directed to the homologous antigen, which was also cross reactive with the other two GBC oligos. Using confocal

microscopy and OPK assays, we revealed the capacity of the raised antibodies to bind GBS cells and mediate opsonic killing in the presence of complement effectors. The antibodies were capable of recognizing various GBS strains with low levels of capsule expression. Immunization with the conjugates of the largest GBC fragment (CRM-3) elicited the production of antibodies that bound the bacteria best. Overall, this study has highlighted the potential of GBC antigens as components of a GBS vaccines. The synthetic structures allowed to investigate for the first time whether shorter oligosaccharides used as mimics of the GBC polysaccharide can elicit a functional immune response against relevant GBS serotypes. This result paves the way for the use of GBC oligosaccharide antigens in a multivalent vaccine against GBS: indeed, a GBC antigen, which is cross-protective across multiple strains, could expand the coverage of the GBS vaccine currently in clinical development and composed of the CPSs from serotypes Ia, Ib, II, III, IV, and V, against noncapsulated strains or against serotypes that at the moment are not included in the vaccine but that could become a potential threat in the future as result of a vaccine-induced serotype replacement.

METHODS

Synthetic Procedures

All reagents were of commercial grade and used as received. All reactions were carried out dry, under an argon or nitrogen atmosphere, at ambient temperature, unless stated otherwise. DCM used in the glycosylation reactions was dried with flamed 4 or 5 Å molecular sieves before being used. Reactions were monitored by TLC analysis on HPTLC aluminum sheets (Merck, silica gel 60, F245) with detection by UV absorption (254 nm) and where applicable by spraying with 20% sulfuric acid in EtOH or with a solution of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ (25 g/L) and $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4\cdot 2\text{H}_2\text{O}$ (10 g/L) in 10% sulfuric acid (aq.) followed by charring at ~ 150 °C. Column chromatography was performed on silica gel 60 (40–63 μm). ^1H , ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AV 400, a Bruker AV 500, a Bruker AV 600 or a Bruker AV 850. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as an internal standard (^1H NMR in CDCl_3) or the residual signal of the deuterated solvent. Coupling constants (J) are given in Hz. All ^{13}C NMR spectra are proton decoupled. NMR peak assignments were made using COSY and HSQC experiments, where applicable Clean TOCSY, HMBC, and GATED experiments were used to further elucidate the structure. High-resolution mass spectrometry (HRMS) was performed on a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electrospray ion source in positive ion mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) resolution $R = 60,000$ at m/z 400 (mass range of 150–4000) and dioctylphthalate ($m/z = 391.28428$) as lock mass, or on a Waters Spynat G2-Si(OTF) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV) and LeuEnk ($m/z = 556.2771$). Optical rotation measurements ($[\alpha]_D^{20}$) were performed on an Anton Paar Modular Circular Polarimeter MCP 100/150 with a concentration of 10 mg/mL (c 1), unless stated otherwise.

For all details on the synthesis of the building blocks, intermediates, and target oligosaccharides, see the [Supporting Information](#).

Conjugation to CRM197 and HSA

Triethylamine (3.0 equiv) was added to a 9:1 DMSO/water solution of hexasaccharide followed by di-*N*-hydroxysuccinimidyl adipate (12 equiv.). The reaction was stirred for 3 h, then the product was precipitated at 0 °C by adding ethyl acetate (9 volumes). The solid was washed 10 times with ethyl acetate (5 volumes each) and lyophilized. The activated sugar was conjugated to CRM197 or HSA in sodium phosphate 100 mM at a protein concentration of 20 mg/mL, using the saccharide/protein molar ratio of 75:1. After incubating overnight, the glycoconjugate was purified by dialysis against 10 mM

sodium phosphate buffer pH 7.2 (30 washings) in 30 kDa Vivaspın Turbo (Sartorius) centrifugal concentrators and reconstituted in the same buffer. Protein content in the purified glycoconjugates was determined by micro-BCA (Thermo-scientific). The saccharide content was estimated by CE-MS analysis.

Formulation Procedure

Glycoconjugates CRM-1, CRM-2, and CRM-3 were formulated with alum hydroxide (concentration 15.4 mg/mL, salt concentration of 2.3 mg/mL) as adjuvants using HyPureTMWFI QUALITY WATER (GE Lifescience), Histidine 100 mM and NaCl 2 M as buffer and excipients. The final formulates were composed of 2 mg/mL aluminum hydroxide, 10 mM histidine, and 150 mM NaCl and were characterized for the following quality attributes: pH (target 6.5 ± 0.5), osmolarity (target 300 mOsm/kg), formation of visible precipitates, antigen's identity and adsorption, endotoxin content, and bioburden detection. All quality attributes were in compliance with the development target range. SDS-Page confirmed that antigens maintained their integrity when formulated with AIOH and were completely adsorbed onto adjuvant.

Immunization

Animal studies were authorized by the Italian Ministry of Health and were undertaken in accordance with the regulations of the Directive 2010/63/EU.

Two groups of 10 female BALB/c mice were immunized by intraperitoneal injection of 0.5 μg in saccharide content of each produced glycoconjugate using alum hydroxide as an adjuvant. CRM-PSIII was used as the control. Mice received the vaccines at days 1, 21, and 35. Sera were bled at days 1, 35, and 49.

ELISA Protocol Using the Oligosaccharides Conjugated to HSA as Coating Reagent (IgG)

Microtiter plates (96 wells, NUNC, Maxisorp) were coated with 100 μL of 1 μg/mL (saccharide concentration) of HSA-oligosaccharides conjugates. Plates were incubated overnight at 2–8 °C, washed three times with PBST (0.05% Tween-20 in PBS pH 7.4) and saturated with 250 μL/well of PBST-B (2% Bovine Serum Albumin-BSA in PBST) for 90 min at 37 °C. Twofold serial dilutions of the sera in PBST-B were added to each well and tested in duplicate. Plates were then incubated at 37 °C for 2 h, washed with PBST, and then incubated for 1 h at 37 °C with antimouse IgG-alkaline phosphatase (Sigma) diluted 1:2000. After washing, the plates were developed with a 1 mg/mL solution of *p*-Nitrophenyl Phosphate (*p*NPP) in 1 M diethanolamine (DEA) pH 9.8, at room temperature for 30 min. After blocking with 7% EDTA, the absorbance was measured using a SPECTRAMax plate reader with the wavelength set at 405 nm. IgG concentrations were expressed as the reciprocal serum dilution giving OD 1.0.

OPKA

Functional activity of anti-GBS antibodies was estimated by OPKA using differentiated HL-60 cells and strains H36B-Ib, CJB111-V, DK21-II, COH1-III, and 515-Ia. The percent of killing was calculated as (mean Colony Forming Units at T0 – mean CFU at T60)/(mean CFU at T0). OPK titers were expressed as the reciprocal serum dilution mediating 50% bacterial killing, estimated through piecewise linear interpolation of the dilution-killing OPK data. The lower limit of detection was 1:30 and the assay coefficient of variation was approximately 30%.

Microscopy

GBS strains were used directly from frozen stocks as already described in the OPKA section. Briefly, 2 stocks of each strain were thawed, centrifuged, and resuspended in 1 mL of PBS. An aliquot of this suspension was diluted 1:1 with 4% formaldehyde and 150 μL spotted on polysinated slides for 30 min at room temperature to allow fixation and adhesion to the slide. Primary antisera and relative preimmune sera were diluted 1:50 in PBS and 150 μL added to fixed bacteria for 1 h at room temperature. After washing, 150 μL of 1:1000 diluted goat antimouse AlexaFluor488 were added to bacteria for 30 min. Slides were finally washed and mounted with ProLong Gold antifade with

DAPI. Images were acquired by using a 100x oil objective mounted on a Zeiss LSM microscope with Airyscan.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacsau.2c00302>.

Detailed description of all experimental details and full characterization of all new compounds, including ¹H and ¹³C spectra, and preparation and immunological evaluation of the glycoconjugates (PDF)

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Notes

The authors declare the following competing financial interest(s): E.B., A.P., G.B., M.F., M.R.R., L.D.B., and R.A. are employees of GSK group of companies.

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