Discovery of Oligosaccharide Antigens for Semi-Synthetic Glycoconjugate Vaccine Leads against *Streptococcus suis* Serotypes 2, 3, 9 and 14**

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Abstract: *Streptococcus suis* bacteria are one of the most serious health problems for pigs and an emerging zoonotic agent in humans working in the swine industry. *S. suis* bacteria express capsular polysaccharides (CPS) a major bacterial virulence factor that define the serotypes. Oligosaccharides resembling the CPS of *S. suis* serotypes 2, 3, 9, and 14 have been synthesized, glycans related to serotypes 2 and 9 were placed on glycan arrays to screen blood from infected pigs. Lead antigens for the development of semi-synthetic *S. suis* serotypes 2 and 9 glycoconjugate veterinary vaccines were identified in this way.

Introduction

*Streptococcus suis* causes bacterial infections in farm pigs globally,[1] but it is also a commensal bacterium that commonly inhabits the upper respiratory, digestive and reproductive systems of pigs.[2,3] Virulent strains can infect the bloodstream and eventually result in septic shock and meningitis in pigs, but can also cause septicemia and meningitis in humans.

*S. suis* is surrounded by a layer of polysaccharides forming the bacterial capsules that play a fundamental role for pathogen survival,[4] protect the bacterium and are important virulence factors. CPSs are able to trigger an adaptive immune response resulting in the production of specific antibodies rendering polysaccharides attractive targets for antibacterial vaccine development.[5] *S. suis* serotypes are distinguished based on the chemical composition of the capsules.[6–7] Serotypes 1/2, 2, 3, 7, 9 are most frequently isolated from infected animals and differ in geographical prevalence. Serotype 2 is particularly frequent in Europe and Asia, while serotypes 3 and 9 were mostly found in North America.

Veterinary vaccines are an effective strategy to limit disease in farm animals and reduce the spread of pathogens between animals and transmission to humans. Vaccinations help to reduce antibiotic consumption and slow the development of antimicrobial resistance.[8] All currently used anti-bacterial veterinary vaccines are prepared from live attenuated or inactivated bacteria that suffer shortcomings in terms of safety, stability and in some cases limited immunogenicity.[8–10] While glycoconjugate vaccines in humans are very successful, veterinary glycoconjugate vaccines remain a largely unexplored opportunity.[11]

The CPS structures of four major *S. suis* serotypes (2, 3, 9, and 14) have been elucidated (Figure 1).[12–15] The CPSs include rare sugars, a variety of glycosidic linkages, anionic charges and modifications such as acetyl esters and phospho- diesters. Serotypes 1, 2, and 14 are structurally very similar.

*S. suis* vaccine development using isolated polysaccharides has focused exclusively on serotype 2.[16] To date, no single antigen has been shown to be more efficacious than a suspension of killed or weakened bacteria,[16–18] or to be protective against *S. suis* serotype 9. Exact carbohydrate epitopes responsible for inducing protective antibodies are still unknown but are the basis for establishing structure–immunogenicity relationships for the design of carbohydrate antigens for vaccine studies. Investigations with isolated native CPSs produced inconclusive results.[19–23] Synthetic oligosaccharides related to CPS can help to determine antibody epitopes.

Here, we describe the first synthesis of well-defined oligosaccharides resembling the *S. suis* serotypes 2, 3, 9 and 14 CPSs. Glycans related to serotypes 2 and 9 were employed on the surface of glycan arrays to identify lead structures for the development of semi-synthetic glycoconjugate vaccines against *S. suis*. 
**Results and Discussion**

**Synthesis of Oligosaccharides Related to S. suis Serotype 2 CPS**

The *S. suis* serotype 2 CPS consists of a branched heptasaccharide repeating unit ([1→4](α-d-Neu5Ac[2→6]-β-d-Gal(1→4)-β-d-GlcNAc(1→3)-β-d-Gal(1→4)-α-L-Rha(1→4)-β-d-Glc(1→3)]-β-L-Rha(1→4)-β-d-Glc(1→3)) (Figure 2). The CPS from *S. suis* serotype 2 is essential for its virulence as it prevents phagocytosis when the bacterium infiltrates the bloodstream.\[^{[22]}\]

The CPS is the most promising antigen as it can induce protective IgM antibodies\[^{[23,24]}\] despite being poorly immunogenic—low levels of anti-CPS antibodies were seen in pigs after infection or immunization.\[^{[23]}\] An anti-serotype 2 glycoconjugate vaccine made from capsular polysaccharides isolated from fermented bacteria was evaluated in immunization experiments in animal models.\[^{[16]}\] The poor immunogenicity of CPS can be overcome and protection against *S. suis* can be achieved by active immunization with a glycoconjugate.

Five oligosaccharides (1–5) resembling the repeating unit of *S. suis* serotype 2 CPS were designed to obtain detailed structural information of antigenic epitopes of antibodies from *S. suis*-infected pigs (Figure 2). Three shorter fragments were included: trisaccharide 1 resembles the backbone while 2 and 3 represent the side-chain. Pentasaccharide 4 and heptasaccharide 5 were synthesized to cover almost the entire length of a repeating unit as well as branched sequences, and to understand whether the terminal N-acetyl neuraminic acid is directly engaged in antibody binding. All synthetic oligosaccharides contain an aminopentanol spacer at the reducing end sugar for creating microarrays and protein conjugates.

Seven orthogonally protected monosaccharide building blocks 6–12 were identified to create 1,2-cis glycosidic bonds, a branching point on L-rhamnose and the α-sialyl linkage (Figure 2). The synthesis of 1 via a linear approach used three monosaccharide building blocks (6–8, Scheme 1) and started with the introduction of the spacer at the reducing end monosaccharide with a glycosylation between N-protected aminopentanol 13 and glucose thioglycoside 6, followed by cleavage of the fluorenylmethoxycarbonyl (Fmoc) protective group to obtain 14. The reducing end glucose was then glycosylated with rhamnose thioglycoside 7. To assist the formation of the β-rhamnoside linkage and prepare for the subsequent introduction of the α-galactose, the C-3 hydroxy group was masked by a 2-pyridinecarbonyl ester (picoloyl ester—Pico) that ensures an H-bond mediated stereodirecting effect and is orthogonal to the benzyl ethers. The non-reducing end galactose was introduced with α configuration using known galactosyl thioglycoside 8, equipped with C-4 and C-6 acetyl esters to assist in the formation of the 1,2-cis glycosidic bond. The glycosylation of disaccharide 15 with thioglycoside 8 was carried out in a DCM/Et\(_2\)O mixture to increase the α selectivity through solvent effects. Only the α-linked product 16 was detected on TLC and isolated. Finally, protected trisaccharide 16 was fully deprotected by ester hydrolysis using sodium methoxide in methanol followed by catalytic hydrogenation, obtaining trisaccharide 1.

The linear synthesis of trisaccharide 2 used commercially available galactose building block 9 as starting point for both monosaccharides 17 and 20 (Supporting Information). Spacer 13 was regioselectively glycosylated with 17, before the resulting galactose was glycosylated with commercially available glucosamine 10, furnishing disaccharide 19 after Fmoc removal (Scheme 1). The disaccharide was finally glycosylated with galactose 20 to obtain fully protected trisaccharide 21. Decylation with sodium methoxide in methanol at 35°C was accompanied by partial hydrolysis of the trichloroacetamide due to the large excess of base such that N-acetylation became necessary. Finally, catalytic hydrogenation removed all ethers and afforded deprotected trisaccharide 2.
A convergent 2+2 glycosylation strategy was followed to assemble tetrasaccharide 3 by first coupling acceptor 19a and disaccharide 23 that contains a preinstalled α sialyl glycosydic bond. Disaccharide 23 was obtained by glycosylating galactose acceptor 22 with known sialyl glycosyl imidate 12 at −60 °C in a DCM/CH$_2$CN mixture. Isolation of the pure diastereoisomers by careful silica column chromatography gave pure α-sialylated galactoside 23 and the corresponding β-isomer in a 6:1 α/β ratio. The configuration was unequivocally determined by measuring the long-range $J_{\text{C-1,H-3ax}}$ (Supporting Information).

Disaccharide 23 was employed to glycosylate acceptor 19a, promoted by NIS and triflic acid. These conditions proved ineffective for tetrasaccharide formation. To improve the reactivity of the glycosylating agent, the thioglycoside was converted to the more reactive glycosyl phosphate 24. The glycosylation of acceptor 19a using disaccharide 24 proceeded poorly as no product was isolated from a complex mixture.
Likely, insufficient acceptor nucleophilicity was responsible for the failure of this reaction as both glycosylating agents 23 and 24 hydrolyzed but acceptor 22 was recovered. The C-4 hydroxy group in ester-protected glucosamine acceptors is a poor nucleophile and variations in the protecting group pattern can lead to improved couplings. When disaccharide 19b, containing an ether group instead of an ester at C-3 of the glucosamine unit, was glycosylated using disaccharide phosphate 24, the desired tetrasaccharide 25 was obtained in 67% yield. Removal of all protecting groups by ester hydrolysis under basic conditions followed by catalytic hydrogenation gave unprotected tetrasaccharide 3.

Oligosaccharides 4 and 5 were assembled using convergent syntheses from common trisaccharide acceptor 29 (Scheme 2). Trisaccharide 29 contains a challenging β-rhamnosidic linkage on a doubly substituted terminal rhamnose residue. Building block 7 was not suitable for the synthesis and fully orthogonal rhamnose building block 26 was prepared instead in seven steps from commercially available rhamnose (Supporting Information, Scheme S2). A glycosylation of the aminopentanol linker using rhamnose thioglycoside 26 and NIS/TIOH as activator resulted in low stereoselectivity (2.4:1, β/α) as judged by NMR. Instead, bromine activation of thioglycoside 26 afforded spacer-linked rhamnose in a slow reaction with higher stereoselectivity (10:1, β/α). Rhamnose 27 was then glycosylated with galactose 8 in a DCM/Et2O mixture and no appreciable amounts of β-linked galactose were isolated. To perform a second glycosylation and install a second galactose residue, the silyl ether was removed by TBAF to afford disaccharide acceptor 28 that was glycosylated with 9 to obtain desired trisaccharide 29 after Fmoc removal. The C-4 hydroxy group of rhamnose acted as a good nucleophile, despite its proximity to the α galactose unit.

With trisaccharide acceptor 29 in hand, the assembly of 4 continued with the preparation of disaccharide imidate 30 (Scheme 2 and Supporting Information, Scheme S3). Disaccharide 30 and acceptor 29 were coupled to obtain the protected pentasaccharide in 67% yield. Removal of all protective groups yielded pure pentasaccharide 3.

Sialylated hexasaccharide 5 required the preparation of trisaccharide glycosyl imidate 32 (Scheme 2 and Supporting Information, Scheme S4).
Information, Scheme S4). A 3+3 glycosylation of trisaccharide imidate 32 and trisaccharide 29 did not proceed below \(-20^\circ C\) as 32 degraded. Increasing amounts of acid activator (up to 0.5 equiv) and higher temperatures still resulted in highly complex mixtures as only 11% of hexasaccharide 31 were obtained. An identical acceptor was successfully employed in the synthesis of oligosaccharide 4. Therefore, likely trisaccharide glycosylating agent 32 is responsible for the low yields although the reasons remained unclear. Subsequently, ester cleavage and catalytic hydrogenation produced hexasaccharide 5.

**S. suis Serotype 2 Pentasaccharide is a Potential Vaccine Candidate**

Glycan arrays enable the screening of multiple serum samples to identify protective glycotopes. Synthetic oligosaccharides 1–5, the isolated native CPS, and other structurally related glycans were immobilized on glass slides to identify antibody binding patterns in serum samples from infected pigs and from rabbits immunized with native CPS (Figure 3). In addition, human reference serum 007sp was screened as control.

IgG antibodies from pig sera bound specifically to oligosaccharide 1 and, to a lesser extent, to 4. A five-fold stronger binding to oligosaccharides 4 and 5 was observed with anti-serotype 2 rabbit serum (Figure 3). Co-infections

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**Scheme 2. Synthesis of branched oligosaccharides 4 and 5.**

**Figure 3.** Glycan array analysis of *S. suis* serotype 2 oligosaccharides and native CPS. A) Printing pattern of microarray and binding pattern of rabbit serum to immobilized glycans. B) IgG antibody binding to glycans. A serum dilution of 1:100 was used. MFI, mean fluorescence in intensity (mean ± standard deviation); PB, printing buffer; CWPS, cell wall polysaccharide; CPS and synthetic glycans, see Figure S1.
with other *S. suis* serotypes or bacteria in pigs may be responsible for these differences as higher antibody levels are elicited following immunizations compared to a natural infection.\[^{34}\]

Trisaccharide 1 was recognized not only by pig sera, but also by *S. pneumoniae*-vaccinated human serum (007sp). This finding is likely the result of immune cross-reaction due to structural similarities in the CPS from different streptococcal species.\[^{35,36}\]

Pentasaccharide 4, a structure that covers most of the native repeating unit, appears to be the minimum glycoepitope useful to elicit an immune response, as both pig and rabbit antibodies recognized it strongly. It is worth noting that almost identical fluorescence signals were measured for compounds 4 and 5. The terminal sialic acid unit on hexasaccharide 5 neither increased nor impaired binding to these antibodies. Therefore, sialic acid is not a fundamental part of the minimal epitope of rabbit antibodies.

Additionally, class switch from IgM to IgG antibodies occurred as IgG titres were found to be much higher than the IgM response (Supporting Information, Figure S2), likely as a result of B cell differentiation to eliminate the pathogen following immunization or infection.\[^{16}\] Immunization resulted in higher antibody titres than natural infections,\[^{34}\] suggesting that vaccination with glycoconjugates is an promising means to elicit an even stronger immune response.

In conclusion, pentasaccharide 4 is an attractive lead for the development of a glycoconjugate vaccine to protect from *S. suis* serotype 2.

### Synthesis of Oligosaccharides Related to *S. suis* Serotype 3 CPS

The *S. suis* serotype 3 CPS disaccharide repeating unit [--→4]–β-D-GlcpA-(1→3)–β-D-QuipNAc4NAc–(1→] contains the rare diamino sugar di-N-acetyl-d-bacillosamine (QuipNAc4NAc) and D-glucuronic acid (GlcpA).\[^{37–39}\] QuipNAc4NAc is found in various bacterial capsular polysaccharides, including N-linked glycoproteins on *Campylobacter jejuni*, O-linked glycoproteins on *Neisseria gonorrhoeae* and O-antigens from many strains of Gram-negative bacteria.\[^{37–39}\] The *S. suis* disaccharide repeating unit is unique and we report the first synthesis of a series of oligosaccharides related to the native *S. suis* serotype 3 CPS as basis for immunological studies.

The main synthetic challenges are access to the rare diamino bacillosamine building block and complex functional interconversions to be performed at the tri- and tetrasaccharide level. Formation of glycosidic bonds to construct the oligosaccharides was challenging as several electron-withdrawing groups reduce the nucleophilicity of glycosyl acceptors.\[^{40,41}\] Since electron-withdrawing carboxy groups at C-6 may further reduce the reactivity of the glucose donors,\[^{42}\] they were introduced after the glycosylation. Finally, the densely nitrogen-functionalized target molecules necessitated additional deprotection steps. Stereoselective β-glycosidic bond formations were ensured by neighboring group participation with benzoyl groups (Bz) on the glucose and a trichoroacetamid (TCA) group on bacillosamine.

![Figure 4. Structure of *S. suis* serotype 3 CPS and synthetic oligosaccharide antigens resembling the *S. suis* serotype 3 CPS derived from building blocks 13, 41 and 42.](image-url)
Synthesis of the bacillosamine-containing reducing end commenced with the stereoselective glycosylation between selenoglycoside \(13\) (Supporting Information, Scheme S5) and protected amino linker \(13\) using TMSOTf and NIS as activators to yield exclusively the β-linked product \(43\) in 91% yield. Conversion of the azide group to the acetamide and contemporary reduction of the TCA group were carried out using zinc powder in a THF/\(\text{Ac}_2\text{O}/\text{AcOH}\) mixture. Removal of the remaining benzyl ethers and the benzoyloxy carbamate group was achieved by hydrogenolysis using Pd/C in EtOAc/\(\text{BuOH}/\text{H}_2\text{O}\) and gave linker-equipped bacillosamine \(40\) in 49% yield over two steps.

Scheme 3. Synthesis of oligosaccharides 34–40 related to \(S.\) suis serotype 3 CPS.
Cleavage of the 2-naphthylmethyl (Nap) protecting group on 43 with DDQ afforded alcohol 44 in 97% yield and subsequent union of 44 and 41[^1] yielded 67% β-linked disaccharide 45. Even when excess donor 41 (2.0 equiv) and prolonged reaction times were employed, unreacted acceptor 44 was always observed. Acid hydrolysis of the benzylidene acetal on 45, followed by selective oxidation of the C-6 hydroxy group with TEMPO/BAIB and protection of the carboxy moiety as benzyl ester gave disaccharide 47 in 77% yield over two steps. The C-4 hydroxy group was glycosylated with another bacillosamine unit to furnish β-linked trisaccharide 48 in 65% yield. Azide and TCA groups present in 47 and 48 were converted into acetamides by employing the same conditions used for compound 43. Subsequent hydrolysis under basic conditions and hydrogenolysis smoothly afforded oligosaccharides 38 and 36 in acceptable yield.

Linker-equipped glucuronic acid was prepared by first coupling protected glucose building block 41 and 13 under NIS/TMSOTf promotion to furnish β-linked product 49 in 71% yield. The benzylidene acetal on 49 was then hydrolyzed, before regioselective oxidation with TEMPO/BAIB and esterification furnished glucuronic acid benzyl ester 51 in

![Repeating unit of S. suis serotype 9 CPS](image)

**Figure 5.** Structure of S. suis serotype 9 CPS and related oligosaccharides 58–65 to be prepared from building blocks 66–73.
72% yield over two steps. Ester groups on monosaccharide 51 were hydrolyzed under basic conditions, and hydrogenolysis afforded deprotected 39 in 70% yield over two steps. Further elaboration of 51 continued with a glycosylation using building block 42 to furnish protected disaccharide 52 in 93% yield. The Nap ether was removed as preparation for another glycosylation with building block 41 to yield protected trisaccharide 54. Removal of the benzylidene acetal on 54 proved challenging. Treatment with (+)-camphor-10-sulfonic acid as a catalyst and ethanethiol as acetal exchange reagent[44,45] gave trisaccharide diol 55 in low yield. Instead, aqueous acetic acid (80%) at 60°C smoothly delivered 55 in 87% yield (Supporting Information). The primary C-6 alcohol of diol 55 was then oxidized and converted into the corresponding benzyl ester 56. Coupling of bacillosamine donor 42 with acceptor 56 produced tetrascaride 57 in 65% yield. Finally, oligosaccharides 52, 56 and 57 were globally deprotected via Zn-mediated reduction, basic ester hydrolysis and hydrogenolysis, to obtain oligosaccharides 37 (51% over three steps), 35 (50% over three steps) and 34 (40% over three steps), respectively (Scheme 3).

**Synthesis of Oligosaccharides Related to S. suis serotype 9 CPS**

The *S. suis* serotype 9 CPS repeating unit consists of a branched tetrasaccharide with a phosphorylated d-glucitol residue (Figure 5).[13] The presence of the labile C-4-keto sugar and the phosphodiester group pose considerable synthetic challenges. Synthetic C-4-keto sugars are difficult to handle during total synthesis but the reduced form is more stable, while maintaining the ability to induce an immune response against the CPS.[46] This isosteric chemical modification facilitates subsequent scale-up. We focused on the synthesis of the C-4-axial reduced form of *S. suis* serotype 9 CPS repeating unit and its analogs (Figure 5).

Five differentially protected monosaccharide building blocks (66–70) and three *H*-phosphonates (71, 72, 73) are needed to assemble the target oligosaccharides (Figure 5). Benzyl ethers serve as permanent protecting groups while TCA and levulinoyl ester ensure anchimeric assistance as C-2 protective groups. The phosphorylated d-glucitol will be introduced after assembly of the tetrasaccharide core.

Assembly of tetrasaccharide 79 commenced with the union of L-rhamnose 66 and D-fucosamine 67 in the presence of NIS/TfOH as promoter to afford disaccharide 74 in good stereoselectivity (α/β = 1:6, Scheme 4). Cleavage of the Nap ether of 74 in refluxing DCM gave diol 75 in 61% yield. NIS/TfOHemediated glycosylation of 75 with acceptor 67 under similar conditions gave disaccharide 78 in 92% yield, which was deprotected under basic conditions to yield diol 77 in 82% yield. Cleavage of the Nap ether of 77 with DDQ and β-pinene gave diol 76 in 63% yield. Phosphorylated d-glucitol was introduced after assembly of the tetrasaccharide core (Scheme 4).

**Scheme 4.** Synthesis of oligosaccharide fragments of *S. suis* serotype 9 CPS 58–65.
ether with DDQ and β-pinene gave disaccharide 75 (61 % yield) ready for glycosylation with 68 to produce trisaccharide 76 with complete β-selectivity. Cleavage of the levulinoyl ester with hydrazine gave trisaccharide 77 in 91 % yield. Control over stereoselectivity via solvent effects yielded consistent results. The NIS/TfOH-mediated glycosylation between thioglycoside 70 and 77 in a mixture of DCM and EtO produced 82 % tetrasaccharide 78. Acetyl ester cleavage with sodium methoxide furnished 79. Global deprotection of oligosaccharides 74, 76 and 79 gave the corresponding non-phosphorylated compounds 65, 62 and 59. H-phosphonates 71, 72 and 73 were coupled with the hydroxy group on 79 in the presence of pivaloyl chloride to give intermediate H-phosphate diesters.\(^{[48]}\) Subsequent oxidation with iodine and water in the same pot furnished the phosphates 80, 81 and 82 as triethylammonium salts in 69–86 % yield over two steps. Global deprotection of the phosphodiesters by Pd/C-catalyzed hydrogenolysis, followed by ion exchange column chromatography furnished 58, 60 and 61 as sodium salts (Supporting Information).

For the synthesis of trisaccharide phosphates, glycosylation between 75 and 69 yielded trisaccharide 83 (73 %) with complete β selectivity (Scheme 4). Removal of Nap provided 84 with a free C-3 hydroxy group to be coupled with H-phosphonates 72 and 71. The levulinoyl esters were cleaved to afford phosphate diesters 85 (73 % yield) and 86 (66 % yield). Target phosphates 63 and 64 were obtained after global deprotection.

The antibody-binding activity of a synthetic oligosaccharide antigen depends on the structure and configuration of the antigen.\(^{[49]}\) In order to investigate the importance of the β-linkage in the S. suis serotype 9 CPS, oligosaccharides 87–93 bearing an α-glycosidic linkage in place of the native β-glycosidic bond between d-fucosamine and l-rhamnose based on the S. suis serotype 9 CPS were prepared (Figure 6).

To install the α linkage, building block 94 was prepared (Supporting Information). Following the assembly sequence used for the β-linked oligosaccharides, union of 66 and 94 at 0°C produced disaccharide 95 with high α selectivity (Scheme 5). Conversion of the benzoyl ester to the benzyl ether over two steps followed by the cleavage of the Nap ether afforded disaccharide acceptor 98. Coupling of thioglycoside 68 with 98 produced exclusively β product 99 in 87 % yield. After levulinoyl ester cleavage, NIS/TfOH-mediated glycosylation of 100 with 70 produced tetrasaccharide 101. The azide moiety of 101 was reduced by zinc and the resulting amine was acetylated to give 102 in 76 % yield. Next, tetrasaccharide 103 with a free hydroxy group was obtained after saponification of the acetyl ester and following hydrogenolysis gave 88. Tetrasaccharide 103 was smoothly coupled with three different H-phosphonates 71, 72 and 73 to produce corresponding phosphates 104, 105 and 106. Subsequent hydrogenolysis and sodium ion exchange chromatography produced pure 87, 89 and 90.

The presence of an acetyl ester in 100 rendered benzyla-
tion under basic conditions not feasible. Treatment of 100 with freshly prepared silver oxide and benzyl bromide\(^{[50]}\) produced 107 in 47 % yield. The azide was converted to the corresponding acetamide using zinc and acetic anhydride to yield 108 (69 %). The synthesis of 91 was achieved after removal of the acetyl group and hydrogenolysis of the benzyl ethers on 108 in 46 % yield over two steps. To set the stage for phosphorylation, deacetylation of 108 yielded the requisite free hydroxy group that was coupled with 72 and 71 to furnish phosphates 109 and 110 as the triethylammonium salts.

Figure 6. α Oligosaccharide antigens resembling S. suis serotype 9 CPS.

\[\text{Figure 6. α Oligosaccharide antigens resembling S. suis serotype 9 CPS.}\]
Global deprotection of the phosphates afforded the corresponding final glycosides \( \text{92} \) and \( \text{93} \).

**Glycan Array Screening Identifies a Trisaccharide as *S. suis* Serotype 9 Glycoconjugate Vaccine Lead**

Sera from pigs infected with *S. suis* serotype 9, from immunized rabbits and human reference serum 007sp\([\text{51}]\) were screened for antibodies binding to synthetic oligosaccharides and isolated *S. suis* serotype 9 CPS using glycan microarrays (Figure 7). IgG antibodies present in pig sera bound weakly to oligosaccharides revealing a complex binding pattern. Rabbit sera on the other hand showed a clearer picture. Rabbit IgGs recognized strongly phosphorylated trisaccharides \( \text{63} \) and \( \text{64} \), disaccharide \( \text{65} \), and similarly the native CPS. Trisaccharide \( \text{63} \) includes both the sugar sequence of \( \text{65} \) and a terminal phosphate moiety. This functional group appears to be essential since the absence on phosphorylation on \( \text{62} \) strongly reduced binding. On the other hand, the glucitol chain on \( \text{64} \) did not show a significant effect. Branched oligosaccharides \( \text{58} - \text{61} \) were not bound specifically. These observations suggest that the minimal glycootope contains \( \text{l}-\text{rhamnose}, \text{d}-\text{fucosamine} \) and a phosphate moiety, indicating that trisaccharide \( \text{63} \) is the minimum glycootope useful to elicit an immune response.

Antibodies from 007sp serum bound to the longer oligosaccharides due to cross-reactivity with CPS *S. pneumoniae*, possibly because of the branched \( \text{a}-\text{galactose}. \)\([\text{35,52}]\) It had been shown previously that other *S. suis* serotypes cross-react with some serotypes of *S. pneumoniae*.\([\text{35,36}]\) The IgG response was higher than that of IgM, as IgM antibodies showed low or no binding for most cases (Supporting Information, Figure S4) indicating that isotype switching after infection in pigs or immunization in rabbits and humans was induced.

Oligosaccharides with an \( \text{a} \) in place of a \( \text{b} \) linkage between \( \text{d}-\text{fucosamine} \) and \( \text{l}-\text{rhamnose} (\text{87} - \text{93}) \) were found to be bound much weaker (Supporting Information, Figure S5). This finding suggests that the \( \text{b} \) linkage, present in the native CPS,\([\text{15}]\) has an important role for the recognition by antibodies.\([\text{49}]\)
In conclusion, trisaccharide 63 is an attractive lead for the development of a glycoconjugate vaccine against *S. suis* serotype 9.

**Synthesis of Oligosaccharides Related to *S. suis* Serotype 14 CPS**

*S. suis* serotype 14 is responsible for pig and human infections mainly in Asia and has been less studied than the more prevalent serotypes. Expression of the CPS is fundamental to inhibit phagocytosis in vitro and non-encapsulated bacteria are significantly less virulent in mouse models. The serotype 14 CPS (Figure 8) consists of a hexasaccharide repeating unit ([α-Neu5Ac(2→6)-β-D-Gal(1→4)]-β-D-GlcNAc(1→3)-β-D-Gal(1→3)-β-D-Gal-(1→4)-β-D-Glc(1→1)) composed of a trisaccharide backbone and a sialylated lactosamine side chain. Compared to the *S. suis* serotype 2 the β-rhamnose in the backbone is missing and the linkage between the glucose and galactose units is β-(1→6), instead of β-(1→4).

Recent studies aimed at elucidating glycotopes responsible for the production of protective antibodies but more detailed information on the structure of carbohydrate epitopes is needed. Moreover, the antigenic properties of the CPS, either alone or as part of a glycoconjugate, have not been evaluated.

Several substructures related to the repeating unit of serotype 14 CPS were designed (Figure 8), including three oligosaccharides carrying an aminopentyl spacer at the reducing end. To identify whether antibody binding involves mostly the backbone residues, hexasaccharide 111 was...
synthesized. Pentasaccharide 112 was prepared to evaluate whether antibody epitopes include the entire repeating unit. Hexasaccharide 113, representing its sialylated analogue, was included to address the role of sialylation in *S. suis* serotype 14 CPS.

Automated glycan assembly (AGA) was used to assemble all oligosaccharides from four building blocks and Merrifield resin functionalized with a photolabile linker (Scheme 6). Considering the challenges encountered during sialylation of *S. suis* serotype 2 oligosaccharides, a chemoenzymatic approach was adopted to obtain hexasaccharide 113 via one single step from pentasaccharide 112 using asialyltransferase.

Hexasaccharide 111 consists of two repetitions of a trisaccharide. Glycosyl phosphates 114, 115 and 116 were employed in AGA to obtain protected hexasaccharide 120, using glycosylation conditions previously optimized for glycosyl phosphates. The target compound was obtained after resin cleavage and HPLC purification in 20% overall yield. Deprotection by hydrogenolysis and basic ester hydrolysis produced compound 111 in 52% yield over two steps.

Pentasaccharide 112 contains the trisaccharide repeating unit in 111, plus a glucosamine and a galactose. Just three building blocks are needed: one glucose, one galactose and galactosamine 117. Employing the conditions used previously to synthesize the hexasaccharide, AGA proceeded smoothly and protected pentasaccharide 121 was obtained after resin cleavage and HPLC purification in 56% overall yield. Deprotection was carried out by hydrogenolysis followed by deacylation with sodium methoxide, to afford fully deprotected 112 in 53% yield.

Enzymatic sialylation of pentasaccharide 112 using a sialyltransferase from the marine bacterium *Photobacterium damselae* [55] (Pd2,6ST) produced the α(2→6) linkage in hexasaccharide 113. To limit double sialylation on the oligosaccharide chain observed during initial experiments, optimal reaction conditions (1.5 equiv of CMP-Neu5Ac and 7 h reaction time) were used to produce sialylated hexasaccharide 113 in 42% yield after purification.

**Conclusion**

We describe the synthesis of a collection of 30 novel oligosaccharides resembling the capsular polysaccharides related to four major serotypes (2, 3, 9, 14) of the bacterium *Streptococcus suis*. The syntheses tackled challenges associated with complex glycan targets, such as sialylation, the introduction of amino-rich sugars and labile phosphodiesters. The synthetic, conjugation-ready glycans were printed onto an array surface to give rise to glycan microarrays. The glycan microarrays were used to screen the sera of pigs infected with different *S. suis* serotypes as well as sera from immunized rabbits. With the help of glycan array studies, the glycan epitopes of lead antigens for the development of semi-synthetic glycoconjugate vaccines to protect from *S. suis* serotypes 2 and 9 were identified. Currently, vaccination of pigs for challenge studies is being prepared in an effort to develop efficacious vaccines to protect pigs as well as people working in the swine industry while reducing the use of antibiotics.

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**Conflict of interest**

The authors declare no conflict of interest.

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