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Chemical Synthesis Elucidates the Immunological Importance of a Pyruvate Modification in the Capsular Polysaccharide of *Streptococcus pneumoniae* Serotype 4

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Abstract: *Carbohydrate modifications are believed to strongly affect the immunogenicity of glycans. Capsular polysaccharides (CPS) from bacterial pathogens are frequently equipped with a pyruvate that can be placed across the 4,6-, 3,4-, or 2,3-positions. A trans-2,3-linked pyruvate is present on the CPS of the Gram-positive bacterium Streptococcus pneumoniae serotype 4 (ST4), a pathogen responsible for pneumococcal infections. To assess the immunological importance of this modification within the CPS repeating unit, the first total synthesis of the glycan was carried out. Glycan microarrays containing a series of synthetic antigens demonstrated how antibodies raised against natural ST4 CPS specifically recognize the pyruvate within the context of the tetrasaccharide repeating unit. The pyruvate modification is a key motif for designing minimal synthetic carbohydrate vaccines for ST4.*

The lives of hundreds of thousands of children have been saved by carbohydrate conjugate vaccines that prevent *Streptococcus pneumoniae* infections.[1] These vaccines have enjoyed immense commercial success. Nevertheless, the fight against *S. pneumoniae* continues, since more than 90 serotypes (STs) can be distinguished by the nature of their capsular polysaccharide (CPS).[2] While current pneumococcal conjugate vaccines (PCV) cover 10 and 13 serotypes, non-vaccine serotypes are gaining prevalence following the introduction of PCV, and antimicrobial resistance is becoming a serious health concern.[3] Some polysaccharide components in the current commercial vaccines exhibit limited efficacy.[4] Since *S. pneumoniae* serotype distribution varies by age, geography, and time, new vaccines will have to cover an increasing number of serotypes.[5] Glycoconjugate vaccines based on synthetic antigens may provide an attractive alternative.

A medicinal chemistry approach to carbohydrate vaccine design will require a fundamental understanding of minimal carbohydrate epitopes that are sufficient to induce a robust and specific immune response. Relatively short oligosaccharide fragments from different pneumococcal capsular polysaccharides (CPS) have been synthesized and induce an immune response against the pathogen that is equivalent to that induced by the polysaccharide.[6]

Identification of key epitopes that generate a robust antibody response against native CPS is a major step in the design of synthetic carbohydrate conjugate vaccines. The characteristics of epitopes in terms of length, sequence, terminal sugars, frame shifts, and substituents dictate the outcome of the immune response and reactivity to native CPS. Conjugation chemistry, hapten loading density, and the

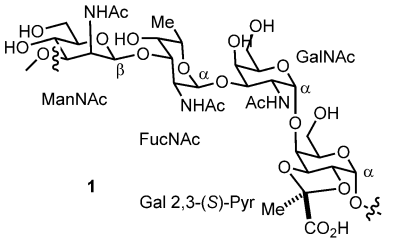

Figure 1. Repeating unit of the *S. pneumoniae* serotype 4 CPS.

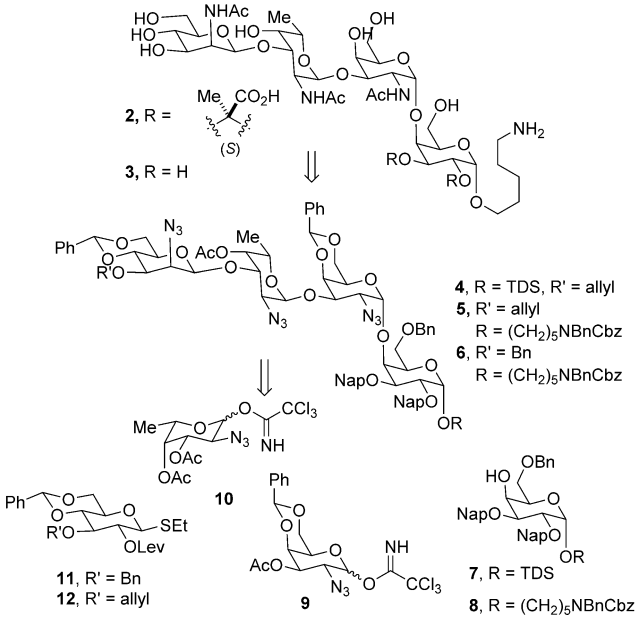

Figure 2. Retrosynthetic analysis of the repeating unit of ST4. Ac = acetyl; Bn = benzyl; Cbz = benzyloxycarbonyl; Lev = levulinyl, Nap = 2-naphthylmethyl; Ph = phenyl; TDS = thexyldimethylsilane.

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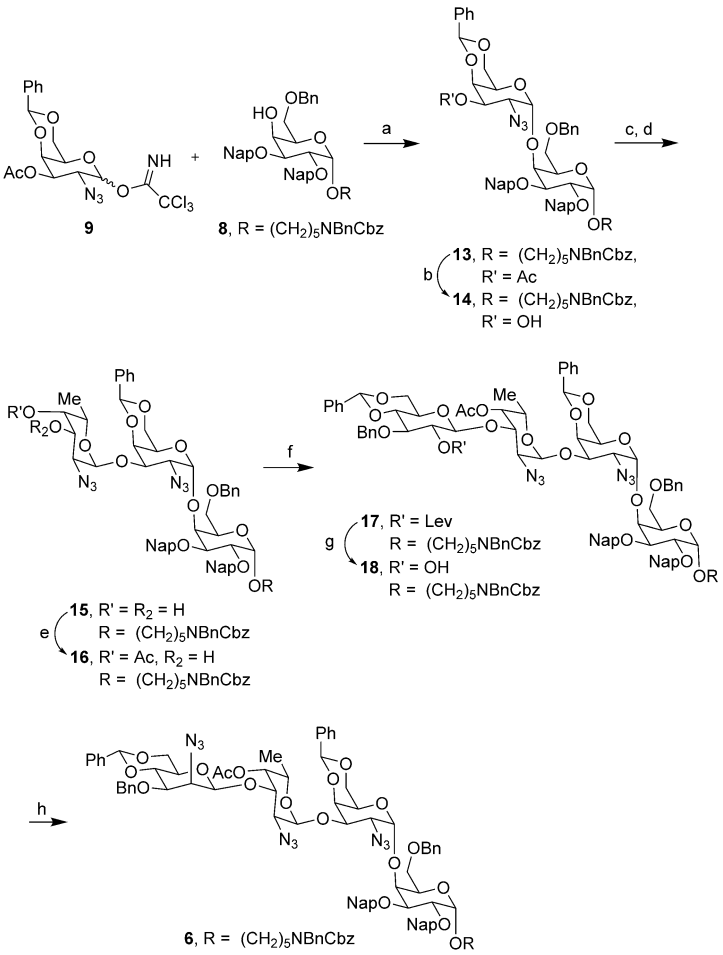
type of carrier also influence the immune response.[7] Glycan modifications such as pyruvate and acetate groups are potentially important immunological determinants.

S. pneumoniae serotype 4 (ST4) CPS is included in the commercial blockbuster vaccine Prevnar 13. While ST4 CPS was discovered in 1931, the structure of its repeating unit was assigned only in 1988.[8] The ST4 polysaccharide consists of a tetrasaccharide repeating unit made up of [3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p* 2, 3-(*S*)-Pyr-(1→] (Figure 1). The presence of N-acetyl sugars, an acid labile *trans*-2,3-(*S*)-pyruvate, the role of which has not been studied in detail, and the challenging β-mannoside and α-glycosidic linkages make this molecule a challenging synthetic and immunological target. To date, only the trisaccharide β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc, which lacks the pyruvalated galactose, has been synthesised.[9]

The key epitope within the ST4 repeat unit remains unknown and so far no detailed studies have been carried out to elucidate the impact of pyruvate modification on immunogenicity. To address these questions, we report herein the first chemical synthesis of the ST4 tetrasaccharide repeating unit (RU), its depyruvalated derivative, and deletion sequences as a basis for detailed immunological studies.

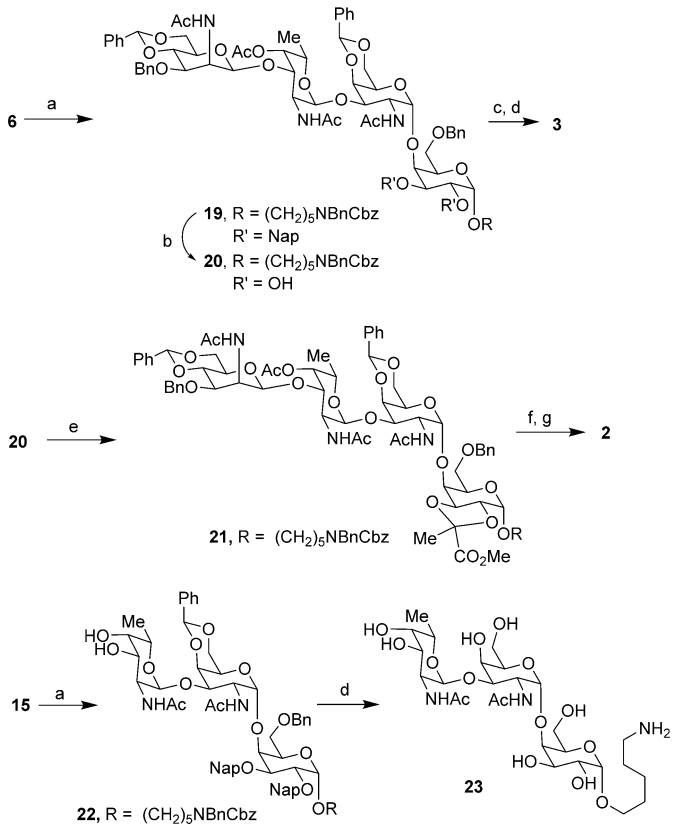
Flexibility to gain access to different target oligosaccharides and their fragments was a key guide for the retrosynthetic analysis of RU **2** of ST4. The synthesis should allow installation of more than one RU, attachment of an orthogonal linker at the reducing end for conjugation to a carrier protein, and introduction of the acid-labile *trans*-2, 3-(*S*)-pyruvate. Access to deletion sequences and RU frame shifts will facilitate in-depth immunological evaluation. Installation of the pyruvate at the final stage of the synthesis ensures access to both pyruvalated or depyruvalated ST4 RU from fully protected tetrasaccharide **6** (Figure 2). Tetrasaccharides **4** and **5**, which are required for chain elongation, will be obtained through a linear glycosylation approach from building blocks **7** or **8**, **9**, **10**, and **12**. Linear combination of building blocks **8**, **9**, **10**, and **11** will yield tetrasaccharide **6**.



Scheme 1. Synthesis of tetrasaccharide **6**. Reaction conditions: a) TMSOTf, CH_2Cl_2/ether (1:1), 90% (α/β = 7:1); b) NaOMe, MeOH, 98%; c) **10**, TMSOTf, CH_2Cl_2, −20°C, d) NaOMe, MeOH, 72% (α/β = 6:1) over two steps; e) 1. trimethyl orthoacetate, CSA, DMF; 2. 80% aq. AcOH, 92% over two steps; f) **11**, NIS, TfOH, CH_2Cl_2, 66%, β-only; g) N_2H_2·HOAc, EtOH/toluene/CH_2Cl_2, 70%; h) Tf_2O in CH_2Cl_2, pyridine, 30 min, then NaN_3, DMF, 80°C for 1.5 h, 61%. CSA = camphorsulphonic acid; DMF = dimethylformamide; Et = ethyl; Me = methyl, NIS = *N*-iodosuccinimide, TfOH = trifluoromethanesulfonic acid.

Synthesis of the reducing-end galactose **8** proceeded in four steps from a known thioglycoside, while a synthesis of fucosamine building block **10** from L-fucose was developed (see the Supporting Information). Galactosamine **9** and glucose **11** were synthesized following literature precedence.[10] Linking galactose **8** and galactosamine **9** provided disaccharide **13**, which was deacetylated to afford acceptor **14** (Scheme 1). Glycosylation of disaccharide **14** with fucosamine **10** followed by deacetylation, orthoester formation, and selective orthoester opening generated trisaccharide acceptor **16**.[11] Placement of the final β-mannosamine linkage employed an established strategy, albeit on a complex substrate: Glucosylation proceeded exclusively in β-fashion when building block **11** was activated by NIS and TfOH to furnish tetrasaccharide **17**. Inversion and amination at C2 to establish the desired stereocenter required cleavage of the 2-*O*-levulinoyl ester followed by conversion of the 2-*O*-hydroxy group into the triflate and its subsequent replacement with an azide nucleophile in the axial position to generate compound **6**, the fully protected core of the ST4 CPS repeating unit.[12] The coupling constants of the anomeric proton before (J = 7.2 Hz) and after inversion (J = 1.2 Hz) and the $J_{C,H}$ coupling of 160 Hz clearly confirm the formation of the desired β-mannosidic linkage.

With the protected intermediate **6** in hand, the stage was set to access the fully functionalized repeating unit as well as simpler fragments for immunological structure–function studies. The depyruvalated ST4 repeating unit **3** was completed via a three step deprotection sequence. Converting the azides to the *N*-acetyl derivative **19** by using thioacetic acid and pyridine was followed by removal of the 4-*O*-acetyl group



Scheme 2. Synthesis of the ST4 CPS tetrasaccharide repeating unit **2**. Reaction conditions: a) thioacetic acid, pyridine, 96 h, 81% for **19**, 72% for **22**; b) DDQ, CH_2Cl_2/H_2O, 3 h, 71%; c) NaOMe in MeOH, 2.5 h, 94%; d) 1st cycle $Pd(OH)_2/C$, H_2, EtOAc/MeOH/H_2O/AcOH, 24 h then 2nd cycle Pd/C, H_2, EtOAc/MeOH/H_2O/AcOH, 79% for **3**, 88% for **23**; e) methyl 2,2-bis(ethylthio)propanoate, DMTST, TTBP, 4 Å MS, CH_2Cl_2, 0°C, 2 h, 47%; f) aq. NaOH, MeOH, 12 h, quant.; g) $Pd(OH)_2/C$, H_2, EtOAc/MeOH/H_2O, 48 h, 33% for the *S* isomer. DMTST = dimethyl (methylthio) sulfonium trifluoromethanesulfonate; TTBP: 2,4,6-tri-tert-butylpyridine; DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

on FucNAc (Scheme 2).[13] Global deprotection of the tetrasaccharide afforded the final depyruvalated **3**, which has an aminopentyl linker at the reducing end.[10a] Substructures of depyruvalated tetrasaccharide **3** such as **23** were synthesized as basis for epitope mapping studies (Scheme 2 and the Supporting Information). Synthesis of tetrasaccharide **2**, which contains the *trans*-2,3-(*S*)-pyruvate, began with the removal of the 2-naphthylmethyl (Nap) protecting groups from **19** by using DDQ to obtain diol **20** (Scheme 2). Installation of the *trans*-2,3-pyruvate proved cumbersome since the only reported procedure for the formation of a *trans*-2,3-pyruvate and the many reported protocols for the formation of 4,6-pyruvates[14] lead to cleavage of the esters and acetals. The *trans*-2,3-pyruvate was finally incorporated into tetrasaccharide **20** by using methyl 2, 2-bis(ethylthio) propionate[15] in the presence of DMTST as an activator to obtain **21** as a mixture of *R* and *S* isomers. The reaction was not completely selective and the stereochemistry could not be assigned at this stage. A two-step removal of the esters and the permanent protecting groups gave **2** after HPLC purification. The *S* stereochemistry of the pyruvate in **2** was confirmed by 2D NMR spectroscopy (see the Supporting Information).

To assess the antigenicity of ST4 RU, synthetic structures were printed on microarray slides by using the reducing end aminopentyl linker for selective immobilization through coupling to activated carboxylic groups (Supporting Information).[16] Isolated polysaccharides such as ST4 CPS and pneumococcal cell-wall polysaccharide (CWPS) were printed as controls. The resulting glycan array was probed with rabbit anti-ST4 typing serum, a polyclonal serum that is specifically raised by immunizing animals with isolated ST4 CPS. As expected, high antibody levels were seen for native ST4 CPS but not for the other serotypes or CWPS (Figure 3). For the synthetic oligosaccharides, very specific binding was exclusively observed for pyruvalated tetrasaccharide **2** but not for any of the depyruvalated deletion sequences, including tetrasaccharide **3**. These observations highlight the important role of the pyruvate group in generating an immune response against the native polysaccharide. This carbohydrate modifi-

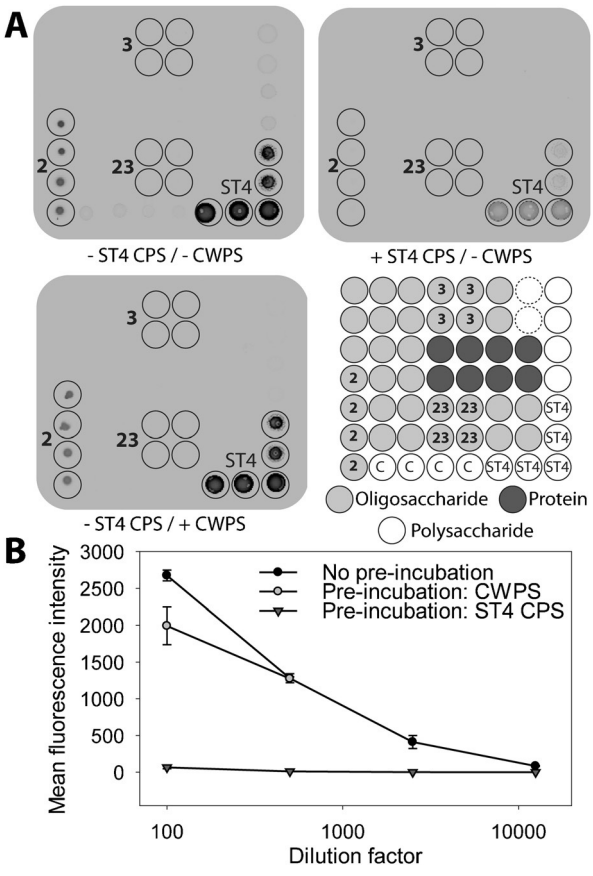


Figure 3. Glycan microarrays printed with synthetic oligosaccharides based on ST4 CPS and different native pneumococcal polysaccharides were probed with serum from rabbits immunized with isolated ST4 CPS. For inhibition studies, serum dilutions were preincubated with isolated polysaccharide. A) Images of representative wells: Bottom right: Schematic printing pattern outlining the positions of discussed structures (C = native *S. pneumoniae* CWPS; ST4 = native ST4 CPS; detailed printing pattern in the Supporting Information). B) Intensity comparison between spots printed with 50 μM of **2** in wells with and without inhibitor.

cation is key to designing novel vaccines, as hypothesized in the 1970s.[17] The binding specificity of the antibody was confirmed by inhibiting the interaction with isolated ST4 CPS. CWPS, an inevitable contaminant of pneumococcal CPS preparations, was also tested as an inhibitor. CWPS had no effect on the signal for the binding of **2**, whereas this was completely abrogated upon the addition of ST4 CPS. These inhibition studies confirm the specificity and cross-reactivity of synthetic tetrasaccharide **2** for binding to antibodies against ST4 CPS and the importance of the pyruvate for an immune response.

In summary, the synthesis of *trans*-2,3-(*S*)-pyruvalated tetrasaccharide ST4 CPS repeating unit **2** and the non-pyruvalated variant **3** formed the basis for demonstrating the importance of the carbohydrate modification for an immune response that recognizes *Streptococcus pneumoniae* serotype 4. This crucial insight is key for further refinement en route to a synthetic carbohydrate conjugate vaccine against ST4.

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Keywords: antigens · oligosaccharides · pyruvate acetal · *Streptococcus pneumoniae* · synthetic vaccines

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