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Defined neoglycoproteins as candidate vaccines against *Streptococcus pneumoniae* type 3

D.J. Lefeber

The research described in this thesis was financially supported by the European Union program VACNET (grant ERB BIO 4CT960158).

Defined neoglycoproteins as candidate vaccines against *Streptococcus pneumoniae* type 3

Gedefinieerde neoglycoproteïnen als kandidaatvaccin tegen
Streptococcus pneumoniae type 3

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op woensdag 3 oktober 2001 des middags te 14.30 uur

door

Dirk Jaap Lefeber

Geboren op 3 maart 1974, te Elst

Promotores: **Prof. Dr. J.F.G. Vliegenthart**
Prof. Dr. J.P. Kamerling

verbonden aan de sectie Bio-Organische Chemie
van het Bijvoet Centrum van de Faculteit Scheikunde
van de Universiteit Utrecht

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Lefeber, Dirk Jaap

Defined neoglycoproteins as candidate vaccines against *Streptococcus pneumoniae* type 3 / Dirk Jaap Lefeber. -Utrecht: Universiteit Utrecht, Faculteit Scheikunde.

Thesis Utrecht University. -With ref. -With Summary in Dutch.

ISBN: 90-393-2819-6

Subject headings: conjugate vaccines/ oligosaccharide synthesis/ *Streptococcus pneumoniae*

List of abbreviations

APT	attached proton test
CPS	capsular polysaccharide
CRM_{197}	cross-reacting material of diphtheria toxin
DABCO	diazabicyclo[2.2.2]octane
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
diethyl squarate	3,4-diethoxy-3-cyclobutene-1,2-dione
DIPEA	*N*,*N*-diisopropylethylamine
ELISA	enzyme-linked immunosorbent assay
HPAEC-PAD	high-pH anion-exchange chromatography with pulsed amperometric detection
KLH	keyhole limpet hemocyanin
MALDI-TOF	matrix-assisted laser desorption ionisation time-of-flight
NIS	*N*-iodosuccinimide
PBS	phosphate-buffered saline
ROESY	rotating frame nuclear Overhauser enhancement spectroscopy
SPR	surface plasmon resonance
TEA	triethylamine
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TOCSY	total correlation spectroscopy
TT	tetanus toxoid

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1

Introduction

1 General

Streptococcus pneumoniae

Since the first isolation of the Gram-positive bacterium *Streptococcus pneumoniae* in 1881 [1,2], this microorganism has been extensively studied [3-5]. The importance of studying this pathogenic bacterium lies in its heavy load on society, especially on children. This is indicated by the total number of deaths caused by pneumococcal diseases in children less than five years of age, which is estimated to be at least one million each year [6,7]. Furthermore, the mortality rate due to pneumococcal diseases is increasing [8] and severe illnesses cause many people to suffer. Finally, the economic costs for hospitalisation and treatment of these patients are enormous.

Many healthy adults carry *S. pneumoniae* in the respiratory tract. Rarely, pneumococcal diseases like pneumonia, meningitis or otitis media (infection of the lungs, meninges or middle ear) are caused by primary infection of the lungs. However, several factors predispose to infection such as damage to the respiratory tract, fatigue, malfunctioning of the immune system or viral diseases [9]. Groups at

high-risk that are especially vulnerable to pneumococcal infection include elderly persons, infants, especially under the age of two, and patients with chronic medical conditions such as heart, lung and kidney diseases, diabetes, alcoholism or immunosuppressive illnesses [10]. Multiplication of pneumococci in the lungs, middle ear or meninges will result in pneumococcal lysis and the release of bacterial products such as the toxin pneumolysin. The release of pneumolysin in the respiratory tract may perturb host defences, allowing bacterial proliferation and spread. Pneumococcal lysis will trigger the inflammatory process by activating phagocytes and complement. Inflammation is hypothesised to be responsible for the morbidity and mortality of pneumococcal infection [11]. Once the microorganism has breached the mucocutaneous barrier, opsonisation by type-specific antibodies and complement is required for clearance of the bacterium from tissue and blood.

S. pneumoniae belongs to the class of encapsulated bacteria that are enveloped by a carbohydrate coat. This serves as a cover against external influences and renders the bacterium more or less resistant to non-specific host defence. Antibodies raised against this capsular polysaccharide (CPS) were found to be essential for removal of the bacterium from the body [12]. *S. pneumoniae* is divided into different serotypes according to the chemical composition and structure of the CPSs as discovered by Avery, Heidelberger and Goebel [13-15]. A recent survey gives an update of the known structures of pneumococcal CPSs [16]. The total number of serotypes known today is 90 [17] that are numbered according to an American or Danish system [18]. The last is being commonly used and places serotypes with a similar CPS structure in one group such as types 6A and 6B that differ only in their rhamnose-ribitol linkage. The distribution of serotypes differs in time, geographical area [19-21], and with age [22,23] and is important for the composition of carbohydrate-based vaccines that are to be used in a specific area or age-group.

Vaccination

Antibiotics have long been an adequate way for treatment of bacterial diseases, including pneumococcal. Despite its use, death rates are still high and even increasing. The development of resistance against antibiotics in the last decades is a serious threat for successful treatment of infection [24-27]. For example, during a surveillance in the US from 1993-1996 the number of isolates nonsusceptible to penicillin or ceftriaxone doubled throughout the 3-year period [28,29]. An alternative approach is to prevent pneumococcal diseases by vaccination with CPSs isolated from bacterial cultures, which was shown to afford protection as early as 1923 [13].

As stated above, the capsular polysaccharide of the bacterium is the most immunogenic surface structure that can induce highly protective anti-CPS antibodies [30,31]. Thus, the CPS has been the main target for vaccine development. Vaccines containing isolated polysaccharides of 23 serotypes are commercially available as Pneumovax 23 (Merck) or Pnu-Immune 23 (Wyeth-Lederle). These vaccines cover over 90% of the infections caused by *Streptococcus pneumoniae* in the US [32].

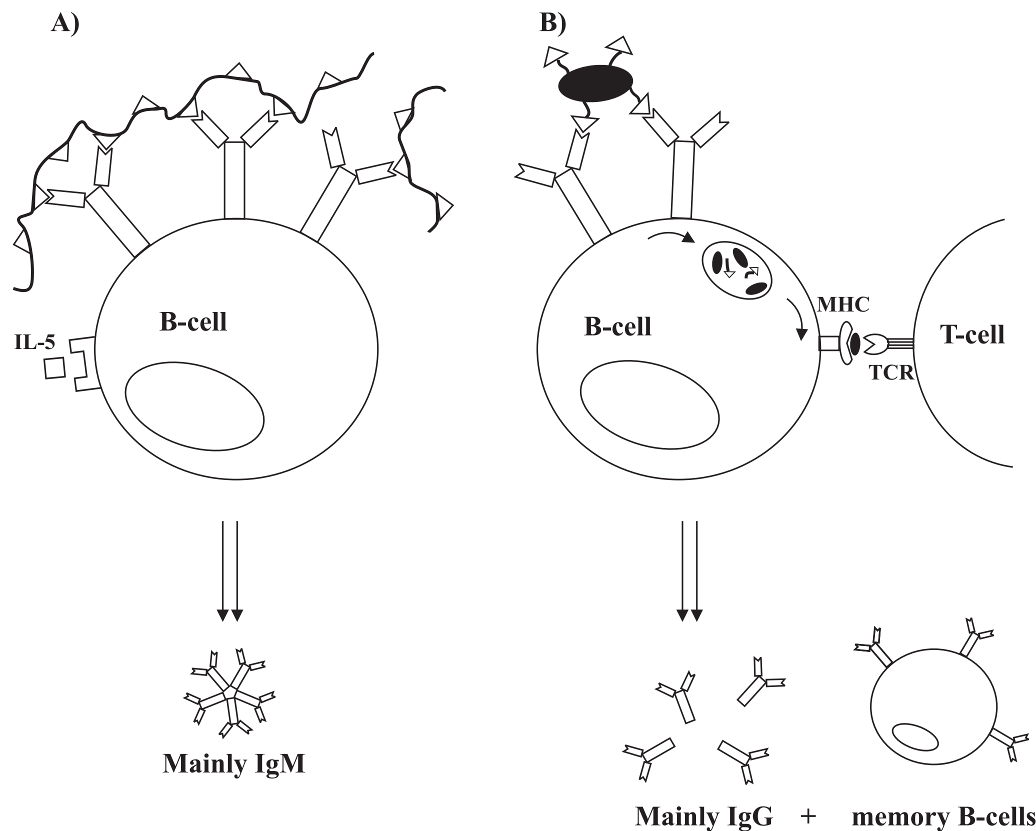

Figure 1. Simplified view on the humoral immune response to A) T-cell independent (TI) CPS antigens and B) T-cell dependent (TD) glycoprotein antigens. TCR: T-cell receptor; MHC: Major Histocompatibility Complex class II; IL-5: Interleukins.

Carbohydrates belong to the class of T-cell independent (TI) antigens. This type of antigens stimulates B-cells to produce antibodies without the involvement of T-cells. An immune response is elicited when surface-bound immunoglobulins are cross-linked (Figure 1A) and specific receptors interact with interleukins (IL-5). As a result of the absence of T-helper cells in this process, the antibodies formed are mainly of the IgM isotype and no memory B-cells are produced. Due to the immature immune system of children, they respond poorly to T-cell independent

carbohydrate antigens. As a consequence, the use of capsular polysaccharides (CPS) for vaccination is convenient in the short-term protection of healthy adults [30,33,34], and can give some protection in the elderly [35]. However, the results of vaccination of the high-risk groups with CPSs are not satisfactory, as described for example for children [36,37] and immunocompromised persons such as patients that are splenectomised or have a renal failure [38].

In contrast to carbohydrates, glycoproteins belong to the class of T-cell dependent (TD) antigens (Figure 1B). After recognition of a glycoprotein by surface bound immunoglobulins, proteolysis of the protein part in the B-cell generates peptide fragments, so-called T-cell epitopes, that interact with MHC class II molecules. The peptide-MHC complex is recognised by the T-cell receptor (TCR), thereby initiating the formation of both antibody-producing and memory B-cells and a class switch of the produced antibody from IgM to IgG. Thus, by immunisation with non-natural glycoproteins, so-called neoglycoproteins, containing carbohydrates from the bacterial capsule coupled to an immunogenic protein, long lasting immunity to encapsulated bacteria can be achieved.
Proteins that are used as carrier in human vaccines are tetanus [39,40] and diphtheria toxoid [41-43] (TT and DT), cross-reacting material of diphtheria toxoid (CRM_{197}) [44], and the outer membrane protein complex of *Neisseria meningitidis* (OMPC) [45]. Polysaccharide-protein conjugates have been described to give long-lasting protection in adults and persons of high-risk groups, especially in young children, as shown for *S. pneumoniae* types 6A and 23F [46] and *H. influenzae* type b conjugates [47]. Besides isolated polysaccharides, oligosaccharides derived from the CPS can be used for the preparation of conjugate vaccines. Both polysaccharide- and oligosaccharide-protein conjugates are being used as commercial vaccines against several encapsulated bacteria. A *Haemophilus influenzae* type b polysaccharide-protein vaccine is being routinely used in children vaccination programs in several countries [48], and a *Neisseria meningitidis* type C oligosaccharide-protein vaccine has been introduced in the UK in November 1999 [49,50]. In both cases death rates declined dramatically. A commercial conjugate vaccine against *S. pneumoniae* has become available recently, known as Prevnar in the US and as Prevenar in the EU [51-54]. So far, this vaccine has not yet been implemented in routine vaccination programs. The vaccine is safe and efficient in infants as shown in several reports [55] and effective in certain high-risk groups such as persons with sickle cell anaemia [56]. It consists of polysaccharides from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, covering about 80% of invasive pneumococcal diseases in children less than six years of age in the US [23]. Serotypes that will be introduced later are types 1 and 5 for a 9-valent, and types 3

and 7F for an 11-valent vaccine [57]. Due to differences in type distribution the composition of this vaccine should be adjusted to age and geographical area. One of the problems concerning the use of the 7-valent conjugate vaccine is the possible increase of infections by non-vaccine types of *S. pneumoniae*. Therefore, active epidemiological surveillance will remain important.

Alternative immunisation strategies

The combination of conjugates containing different serotypes into a single immunisation may result in competition among the constituents. As this feature may adversely affect the immunogenicity of the individual conjugates, it can lead to side-reactions and immune suppression upon immunisation [58,59]. Furthermore, the use of single component vaccines facilitates the manufacturing process and analysis of the products. For these reasons, other surface components of *S. pneumoniae* are investigated as vaccine candidates. Pneumolysoid, a non-toxic mutant of the highly conserved protein pneumolysin, gives some protection in animal models [60]. Both phosphocholine and pneumococcal surface protein A (PspA) can elicit antibodies and protect mice from experimental *S. pneumoniae* [61,62]. However, several different PspAs have been identified and the protective ability of the antibodies was consistently shown to be less than that of anti-CPS antibodies [63,64]. Immunisation with other cell-wall components failed to induce protective antibodies. Nevertheless, several authors have described the need to study alternatives to neoglycoprotein vaccines such as pneumococcal surface proteins [65-67].

Another strategy comprises the use of pneumococcal proteins as carrier in conjugate vaccines. For this purpose, the meningococcal outer membrane protein complex (OMPC) [68] and pneumolysoid, a non-toxic pneumolysin derivative [69,70] are most often used. In addition to the protection by type-specific carbohydrates, this approach affords aspecific protection, irrespective of the serotypes used.

An alternative approach is to use only the minimal structural elements required to elicit a protective immune response. Examples in this category are oligosaccharide-peptide conjugates in which the peptide contains a T-cell epitope [71-74] and the use of multiple antigenic glycopeptide (GMAP) constructs [75,76]. In this way, defined products are obtained, and the amount of carrier protein is reduced, which could help to solve the problems described above.

2. Well-defined neoglycoproteins

Guidelines for the preparation of defined neoglycoproteins

The currently used conjugate vaccines are heterogeneous products, which is caused by the preparation process. Conjugation of polysaccharides to a protein by random activation results in lattice-type complexes with an undefined structure. In oligosaccharide-protein conjugates the carbohydrates are usually present as mixtures with respect to chain length and presented epitope. This heterogeneity has a few disadvantages. The presence of lattice-type structures or oligosaccharide mixtures complicates the analysis of the products, which is increasingly important for product control. This is especially true for a pneumococcal conjugate vaccine, wherein many serotypes have to be included. Furthermore, carbohydrate material isolated from bacterial culture can be contaminated, which complicates the results obtained from immunological assays [77]. Another disadvantage is that these conjugates can not be used to study the influence of structural parameters in the immune response of conjugate vaccines. This can only be done when well-defined structures are available with a variation in only one parameter. Subtle differences in the nature of the carbohydrate part (*i.e.* chain length, presented epitope, and conformation), the protein part, the carbohydrate-protein ratio, the linker and the coupling chemistry can influence the immunogenicity of the vaccine.
In many studies it has been tried to correlate the length of the carbohydrate to the immunogenicity of the neoglycoproteins. With respect to polysaccharide-protein conjugates, in two cases polysaccharide conjugates were found to be more immunogenic than oligosaccharide formulations. However, the conjugates were prepared by random activation of the poly- and oligosaccharides with cyanogen bromide [78] or with sodium periodate [79], which resulted in both cases in undefined structures and therefore no general rules could be deduced.
As stated by Jennings [80], coupling methods are preferred, in which saccharides are coupled to a protein via their reducing end, since the resulting products are better defined. In the case of a Group B Streptococcus type III TT-conjugate the optimal length of oligosaccharides was found to be 14 repeating units (70 monosaccharides), while fragments of 8 and 19 repeating units showed decreased immunogenicity [81]. In a similar report on several pneumococcal oligosaccharide conjugates, no variation in antibody titres was found with increasing chain length [82]. For type 3, fragments of 8, 16, and 27 repeating units were tested. In the two examples described above, oligosaccharide mixtures were used with a wide range of chain lengths coupled to protein in varying oligosaccharide loadings. Long oligosaccharides (> 10 repeating units) were said to express the same conformational epitope as the polysaccharide [83] and should therefore be used.

Short oligosaccharides (1-3 repeating units) were said to be insufficient to generate an efficient immune response.

The use of synthetic oligosaccharides

In contrast to the findings described in 2.1, several studies have revealed the potency of oligosaccharide-protein conjugates containing the minimum antigenic determinant needed to induce CPS-specific antibodies. Synthetic tri- and tetrameric fragments of *Haemophilus influenzae* type b could induce anti-CPS antibodies in mice and monkeys [84], and a pneumococcal type 23F conjugate containing one synthetic repeating unit could induce type-specific antibodies in rabbits, although it was shown that human IgG recognised a larger epitope [85]. Recent studies with *Streptococcus pneumoniae* type 6B neoglycoproteins showed that a synthetic tetrasaccharide fragment, *i.e.* one repeating unit of the CPS, coupled to KLH (keyhole limpet hemocyanin) was sufficient to generate a protective antibody response in mice [86]. In a study on *Shigella dysenteriae* type 1 [87], synthetic oligosaccharides (1-4 tetrasaccharide repeating units) were coupled to HSA (human serum albumin) in varying oligosaccharide loadings ranging from 4 to 23. The conjugate containing the largest carbohydrate with an intermediate loading of 9 induced the highest IgG levels in mice. Conjugates with higher loadings showed a decreased level of IgG. This effect was explained by the inaccessibility of the T-cell epitopes to proteolytic enzymes due to covering of the protein by the carbohydrates. Clearly, neoglycoconjugates containing a unique carbohydrate structure as described in this last example, are useful to unravel the structural parameters that are important for their immunogenicity. In a recent publication [88], an overview is given of the immunological results obtained with this type of well-defined conjugates.
As described above, synthetic oligosaccharide-protein conjugates can be useful in establishing the chain length and number of incorporated carbohydrate residues that are required for optimal immunological activity. Furthermore, they can overcome the problems of product heterogeneity and biological contamination associated with the use of native carbohydrate material. Finally, they can help to elucidate the structural and antigenic characteristics of different parts of a CPS by synthesising overlapping repeating units [89], analogs [90] or mimics [91]. A review about *Streptococcus pneumoniae* summarises the synthetic oligosaccharides related to several serotypes [16].

Conjugation procedures

Besides the use of defined carbohydrates, another prerequisite to obtain defined neoglycoconjugates is the establishment of a specific linkage between carbohydrate and protein. As stated above, single point attachment via the reducing end of the oligosaccharide is the preferred mode of coupling. Activation of the protein carrier like bromoacetylation [84,92] introduces additional immunogenic determinants. This could lead to unwanted side-reactions that should be avoided. Instead, methods based on the coupling of saccharides to more defined positions on the protein such as cysteine [93] or lysine residues are preferred. Different methods are in use for linking carbohydrates to lysine, like the conjugation of oligosaccharides that contain an amino spacer with thiophosgene [94,95], diethyl squarate [96,97] or *N*-hydroxysuccinimide active diesters [98]. Furthermore, reductive amination of oligosaccharides with an aldehyde-containing spacer [99,100] can be used, and introduction of an amide bond via the carboxylic acid containing Lemieux-spacer [101,102]. In several reviews on neoglycoconjugate vaccines [103,104,16], other less defined coupling procedures have been summarised.

Analysis of neoglycoprotein antigens

Detailed analysis of neoglycoproteins is of equal importance as the preparation of defined antigens. First, analysis of the structural parameters of a neoglycoprotein, *i.e.* its integrity, size, carbohydrate structure and heterogeneity, is important for product control and batch-to-batch consistency in the manufacturing process. Furthermore, knowing the structure of the conjugate into more detail, for example the attachment sites of the oligosaccharide with respect to the T-cell epitopes, will help to establish a more thorough understanding of the immune response at a molecular level.

A growing number of studies report on the use of modern analytical techniques for characterisation of the conjugates. Several methods are used to analyse the carbohydrate part. For example, high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [105,106] can be used to analyse the carbohydrate composition, and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy [107,108] are in use for the analysis of the structure and identity of the saccharides. For establishment of the polysaccharide size, high-performance size-exclusion chromatography (HPSEC) with multi-angle laser light scattering (MALLS) and refractive index (RI) detection is the most commonly used technique [109].

Investigation of the structure of neoglycoproteins as a whole is complicated by their size and heterogeneous nature. Some approaches, however, can give valuable information. The conformational difference between uncoupled protein and

conjugate was studied with optical spectroscopy [110], and the integrity of two Meningococcal C-CRM$_{197}$ conjugates was compared by using size exclusion chromatography, HPAEC-PAD and fluorescence spectroscopy [111]. Mass spectrometry in combination with enzymatic digestion of CRM$_{197}$-conjugates was applied to determine the carbohydrate attachment sites [112]. Among the 39 lysine residues, at least five have been shown to be highly reactive, and one histidine residue was found to be reactive to iodoacetylation [113]. A look at the amino acid sequence of CRM$_{197}$ [114] shows that conjugation of saccharides to lysine will not occur within close vicinity of an assumed T-cell epitope [74]. This could lead to an easier processing of the T-cell epitope that can interact with MHC-II molecules to generate a T-cell dependent immune response. More analytical investigations are needed to get a more thorough understanding of the immune response of neoglycoprotein antigens.

3. *Streptococcus pneumoniae* type 3

Structural characteristics

The first isolation of a type specific polysaccharide was performed by Heidelberger and Avery in 1923 and was shown to be of *Streptococcus pneumoniae* type 3 [13]. The capsular polysaccharide (CPS) of this type can be isolated from bacterial cultures and it was the first of which the structure was elucidated. The CPS of *S. pneumoniae* type 3 consists of [→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→] repeating units [115,116] (Figure 2) and is cross-reactive with serotype 8 [117]. Its size is estimated to be around 37 repeating units, and its average molecular mass 14 kDa [82]. X-ray crystallographic data of purified CPS and conformational analysis [118] revealed that the CPS crystallises with a slowly winding extended helix.

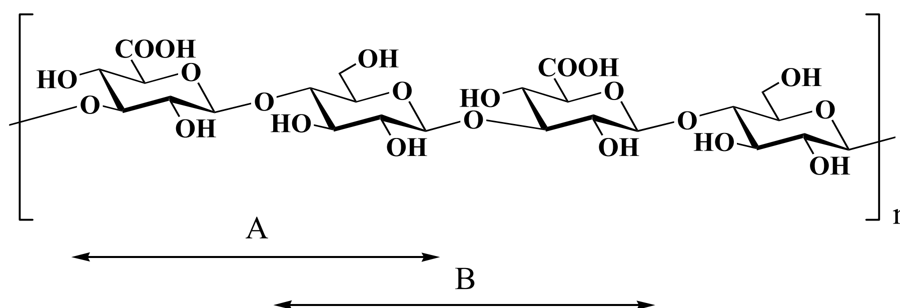

Figure 2. Two repeating units of the CPS of *Streptococcus pneumoniae* type 3. Epitope A can be obtained by acid hydrolysis, epitope B by degradation with an enzyme from *Bacillus palustris*.

Oligosaccharide fragments from *Streptococcus pneumoniae* type 3

Acid hydrolysis of the polysaccharide has been described [119-121] to generate oligosaccharide fragments with glucuronic acid at the nonreducing end, *i.e*. epitope A (Figure 2). Enzymatic digestion of the polysaccharide using a depolymerase from *Bacillus palustris* [119,120,122-124] generates the alternative epitope (B). Isolation of individual fragments has been described [125,126], however mostly mixtures of oligosaccharides were used for conjugation. Synthetic oligosaccharide fragments have been prepared by Chernyak et al. [127,128] as copolymers of acrylamide. The glycopolymer consisting of a disaccharide with sequence A (Figure 2) showed a slightly weaker interaction with *S. pneumoniae* type 3 antiserum than the native CPS, but much stronger than the glycopolymer with disaccharide B.
The oligosaccharides obtained by any of these methods can be used to study antibody characteristics with well-established methods like the precipitin reaction [121,129,130], ELISA inhibition assays [126] or for coupling to a protein carrier. These studies are helpful in finding the minimal and optimal antigenic determinant using oligosaccharides of different chain length and repeating unit (Figure 2).

Experimental *Streptococcus pneumoniae* type 3 vaccines

Immunisation experiments using carbohydrate material from *S. pneumoniae* type 3 have been carried out with purified capsular polysaccharide (CPS) [131,132], polysaccharide-protein conjugates [15,132,133] and oligosaccharide-protein conjugates [82,125]. In most cases, the obtained oligosaccharides were conjugated to a carrier protein as mixtures with an average chain length [82] or via multiple point attachment [132]. The CPS vaccines elicited mainly IgM antibodies and no memory B-cells were produced, whereas the protein-containing conjugates elicited both IgM and IgG antibodies and induced memory. A conjugate of a hexasaccharide of type 3 incorporated into a liposome [134] induced mainly IgM antibodies and no memory, thus showing the characteristics of a TI-antigen.

4. Aim of the thesis

As outlined in the introduction, well-defined neoglycoconjugates are valuable tools for studying the influence of the structural parameters on the immune response of conjugate vaccines. Furthermore, the use of this type of structures facilitates product analysis and will eventually lead to the development of more efficient vaccines. The goal of the research presented in this thesis was to develop methods for the preparation of well-defined oligosaccharides related to the capsular polysaccharide of *Streptococcus pneumoniae* type 3. Furthermore, these structures

were aimed to be used in the preparation of neoglycoproteins with a defined carbohydrate part for studying the influence of the carbohydrate structure on the immune response of conjugate vaccines.
For the preparation of defined oligosaccharides, two methods were investigated. Chemical synthesis (Chapters 2 and 4) was used to prepare spacer-containing oligosaccharide fragments of 1-3 repeating units and to prepare glycoclusters of a synthetic disaccharide repeating unit (Chapter 6). Partial acid hydrolysis (Chapter 5) of the capsular polysaccharide was studied to obtain larger fragments of 1-7 repeating units. To study the effect of the structural parameters oligosaccharide chain length and carbohydrate-protein ratio on the immune response of conjugate vaccines, the synthetic oligosaccharide fragments were coupled to a protein carrier (Chapter 2). The immune response of the saccharide-CRM_{197} conjugates was investigated by immunisation experiments in mice (Chapter 3).

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2

Synthesis of *Streptococcus pneumoniae* type 3 neoglycoproteins varying in oligosaccharide chain length, loading and carrier protein

Dirk J. Lefeber, Johannis P. Kamerling, and Johannes F.G. Vliegenthart

Abstract

The preparation is described of a range of neoglycoproteins containing synthesised fragments of the capsular polysaccharide of *Streptococcus pneumoniae* type 3, *i.e.* β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$ (**1**), β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$ (**2**), and β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$ (**3**). A blockwise approach was developed for the synthesis of the protected carbohydrate chains, in which the carboxylic groups were introduced prior to deprotection by selective oxidation of HO-6 in the presence of HO-4 by using TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy). After deprotection, the 3-aminopropyl spacer of the fragments was elongated with diethyl squarate (3,4-diethoxy-3-cyclobutene-1,2-dione) and the elongated oligosaccharides were conjugated to CRM_{197} (cross-reacting material of diphtheria toxin), KLH (keyhole limpet hemocyanin) or TT (tetanus toxoid). The resulting neoglycoconjugates varied in oligosaccharide chain length, oligosaccharide loading and protein carrier. These well-defined conjugates are ideal probes for evaluating the influence of the different structural parameters in immunological tests.

Introduction

The Gram-positive bacterium *Streptococcus pneumoniae* is one of the most prevalent infectious pathogens that causes life-threatening diseases like meningitis, pneumonia and otitis media. In the past, these infections could be efficiently treated with antibiotics. However, death rates have not declined and furthermore, resistance to pneumococcal strains is still growing [1]. Immunocompetent people can be protected efficiently by vaccination with the available 23-valent capsular polysaccharide (CPS) vaccines [2]. The highest incidence of pneumococcal infections is, however, in young children, the elderly and immunocompromised patients. People in these groups do not benefit from the mentioned vaccines, since they do not respond adequately to the T-cell independent polysaccharides [3]. However, conjugation of carbohydrate antigens to a protein results in a T-cell dependent neoglycoconjugate antigen that can give an efficient immune response in the population at high risk [4].

Currently, neoglycoconjugate vaccines are in use against *Haemophilus influenzae* type b [5], *Neisseria meningitidis* type C [6], and *Streptococcus pneumoniae* serotypes [7]. These neoglycoproteins are prepared by conjugation of isolated capsular polysaccharides or a mixture of polysaccharide-derived oligosaccharides to a protein carrier. The polysaccharide-protein conjugates have a very complex and undefined structure, whereas the oligosaccharide-protein conjugates contain the carbohydrate as mixtures with respect to chain length and presented epitope. Especially in the case of a pneumococcal conjugate vaccine, wherein many serotypes have to be included, the presence of mixtures complicates the analysis of the products, which is increasingly important for product control. The use of synthetic polysaccharide-related oligosaccharide fragments with a unique structure could help to solve these problems. Furthermore, it creates the opportunity for the preparation of tailor-made neoglycoproteins that can be used for vaccination, for ELISA tests not hindered by bacterial contamination [8], and for studying the immune response at a molecular level. Although synthetic neoglycopeptides contain a defined carbohydrate and a defined peptide part, the use of neoglycoproteins with a defined carbohydrate part allows a comparison with the currently used vaccines.

Recent studies with *Streptococcus pneumoniae* type 6B neoglycoproteins showed that a synthetic tetrasaccharide fragment, *i.e.* one repeating unit of the 6B CPS, coupled to KLH was sufficient to generate a protective antibody response in mice [9]. An overview of the synthetic oligosaccharides from several serotypes of *S. pneumoniae* has been given by Kamerling [10], and an overview of well-defined neoglycoconjugates related to several encapsulated bacteria by Pozsgay [11].

To obtain well-defined products for the simplification of product control, and to

investigate the influence of oligosaccharide chain length, oligosaccharide loading and carrier protein on the immunogenicity of the neoglycoproteins, here we present the development of a versatile synthetic route for the preparation of oligosaccharides related to the CPS of *S. pneumoniae* type 3 and their conjugation to a protein in varying ratios. For the conjugation, the defined coupling chemistry of diethyl squarate (3,4-diethoxy-3-cyclobutene-1,2-dione) was applied, and as carrier proteins were used CRM_{197} (cross-reacting material of diphtheria toxin) and TT (tetanus toxoid), which are relevant for human vaccination, and KLH (keyhole limpet hemocyanin), which is suitable for animal studies.

Results and discussion

1. Retrosynthetic strategy

The CPS of *S. pneumoniae* type 3 consists of β(1→3)-linked cellobiuronic acid repeating units. A route for the synthesis of small fragments has been reported by Chernyak et al. [12], based on 3,6-lactonisation of glucuronic acid containing structures to prepare acceptors with a free HO-3 group for the introduction of the (1→3)-linkage. However, the yields for lactonisation and opening of the lactone were moderate in the synthesis of the disaccharide acceptor and can be expected to be even lower in the preparation of larger fragments. During the progress of our work, the synthesis of a protected disaccharide was described by Garegg et al. [13]. In contrast to these earlier reports, in our approach the carboxylic functions are introduced in a late stage of the synthetic route by regioselective oxidation using TEMPO. This approach avoids elaborate protecting group manipulations and possibly low yielding coupling steps. Furthermore, the blockwise strategy employing disaccharide building blocks paves the way for the versatile preparation of larger fragments.

For the synthesis of the target oligosaccharide fragments **1**, **2** and **3** (Figure 1), three monosaccharide building blocks were designed (Scheme 1).

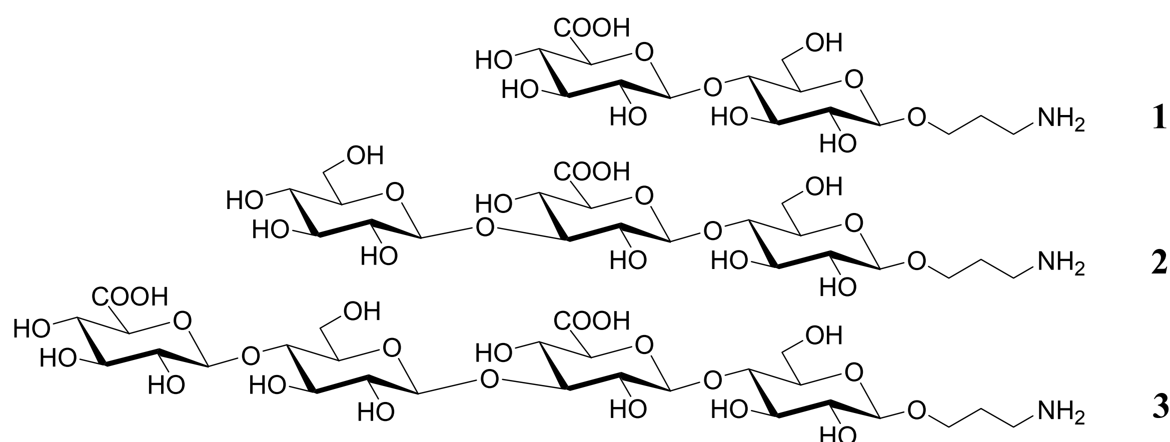
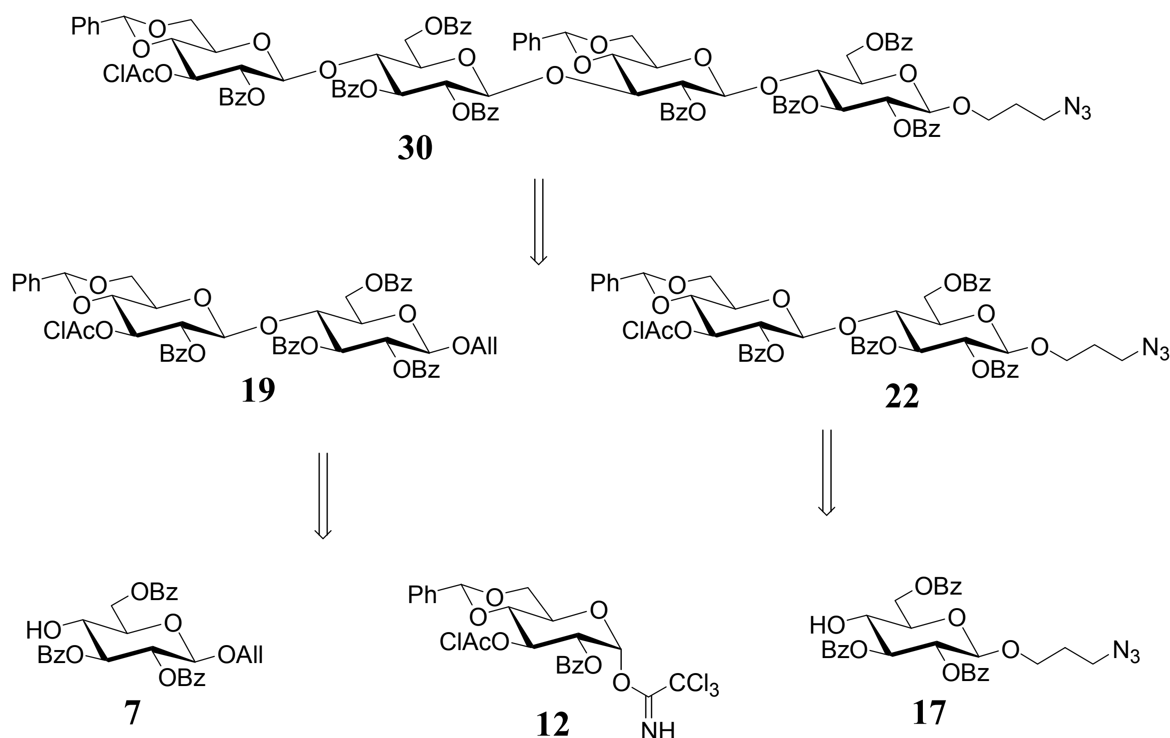


Figure 1. Synthesised oligosaccharide fragments to be coupled to a carrier protein. Coupling of imidate donor **12** with acceptor **7** or **17** results in the formation of disaccharide **19** or **22**, respectively. Dechloroacetylation of **22** results in an acceptor for the synthesis of the tri- and tetrasaccharide fragments. Disaccharide **19** can be deallylated and imidated to obtain a disaccharide donor, which can be coupled with dechloroacetylated **22** to give **30**. Introduction of the carboxylic groups is achieved by selective 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) mediated oxidation of the primary hydroxyl groups after liberation of the benzylidene protecting groups. This selective oxidation eliminates the need for protection of HO-4. After debenzoylation, the azide can be hydrogenated and the resulting aminopropyl spacer coupled via diethyl squarate to lysine residues of CRM_{197}, KLH or TT. The tetrasaccharide acceptor can be elongated to obtain longer fragments by coupling with the mentioned disaccharide or other donors.



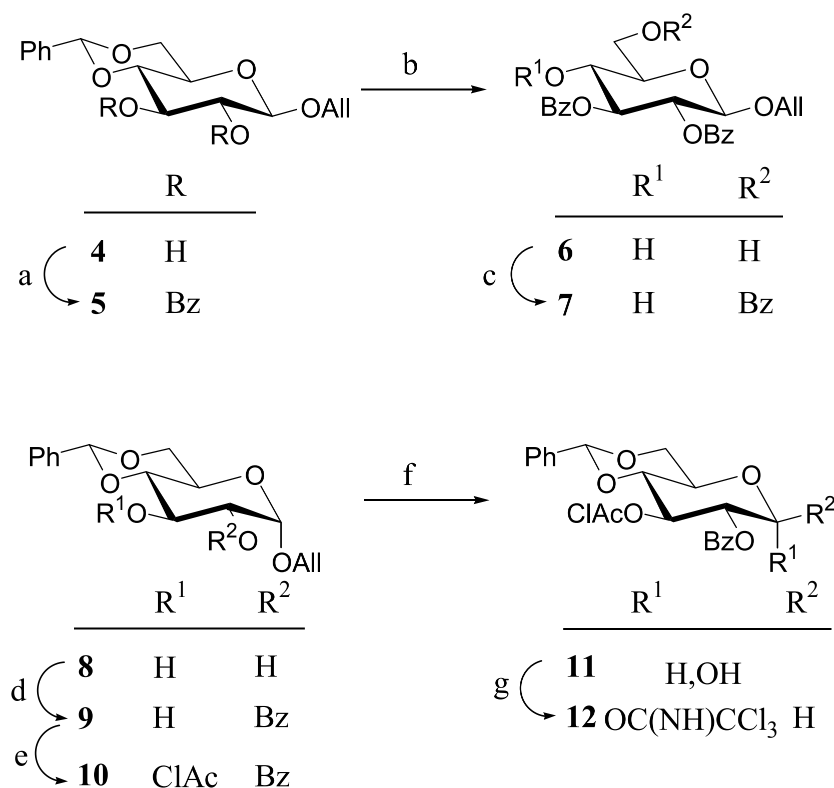
Scheme 1. Retrosynthetic strategy.

2. Synthesis of the saccharide fragments

Monosaccharide acceptor allyl 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (**7**) was prepared as depicted in Scheme 2. Benzoylation of allyl 4,6-*O*-benzylidene-β-D-glucopyranoside (**4**, [14]) by using benzoyl chloride (→**5**, 92%), and subsequent debenzylidenation with trifluoroacetic acid afforded **6** in a yield of 92%. Selective benzoylation of the primary hydroxyl function with benzoyl imidazole [15] gave

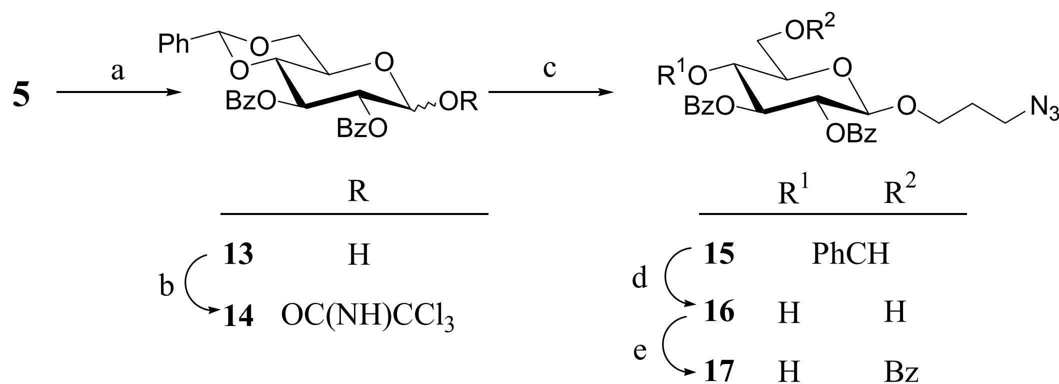
monosaccharide acceptor **7** in 95% yield.

For the synthesis of imidate donor **12** (Scheme 2), selective protection of allyl 4,6-*O*-benzylidene-β-D-glucopyranoside (**4**) was attempted. However, several methods, for example, using stannylene acetals (Bu_2SnO) [16], benzoyl chloride at -50 °C, benzoyl imidazole, heterogeneous catalysis with tetrabutylammonium iodide and potassium carbonate [17], or silylation [18] failed to give satisfactory isolated yields of compounds with a protecting group on HO-2 or HO-3. In the related α-glucoside of **4** the acidity of HO-2 is increased by H-bond formation with the anomeric oxygen [19], thereby increasing the difference in reactivity between HO-2 and HO-3. For this reason, selective benzoylation of allyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**8**, [20]) was performed with benzoyl imidazole, which resulted in the formation of **9** in a good yield of 74%. Subsequent chloroacetylation [21] (→**10**, 96%), deallylation by isomerisation of the double bond with Wilkinson's catalyst [22] and removal of the 1-propenyl group with *N*-iodosuccinimide and water [23] (→**11**, 77%), and trichloroacetimidation [24] afforded monosaccharide donor **12** (93%).



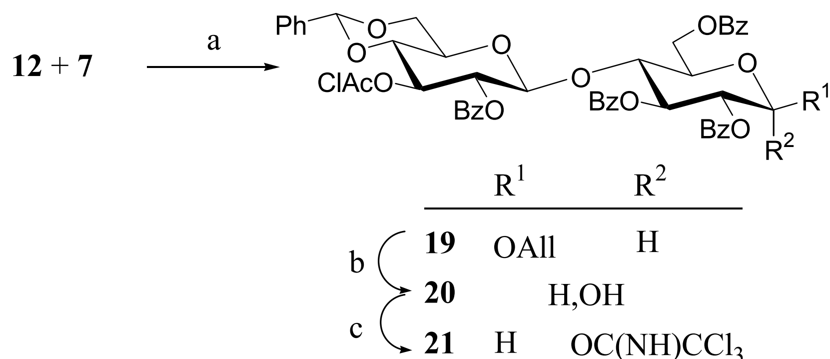
Scheme 2. Synthesis of the monosaccharide building blocks **7** and **12**. Reagents and conditions: a) PhCOCl, C_5H_5N, CH_2Cl_2, 92%; b) CF_3COOH, H_2O, CH_2Cl_2, 92%; c) PhCOCl, imidazole, CH_2Cl_2, 95%; d) PhCOCl, imidazole, CH_2Cl_2, 74%; e) ClAcCl, C_5H_5N, CH_2Cl_2, 96%; f) *i* $(PPh_3)_3Rh(I)Cl$, $CH_3C_6H_5$/EtOH (9:4), *ii* NIS, H_2O, THF, 77%; g) Cl_3CCN, DBU, CH_2Cl_2, 93%.

Monosaccharide acceptor **17**, containing a 3-azidopropyl spacer, was prepared from **5** (Scheme 3). Deallylation of **5** by using Wilkinson's catalyst and *N*-iodosuccinimide (→**13**, 75%) and subsequent trichloroacetimidation afforded monosaccharide donor **14** (82%). Coupling of **14** with 3-azido-1-propanol (**18**), prepared in 74% from 3-bromo-1-propanol by treatment with sodium azide, was performed in dichloromethane using 8% TMSOTf as a promoter (→**15**, 65%). Debenzylidenation of **15** by using trifluoroacetic acid (→**16**, 63%) and selective benzoylation of HO-6 with benzoyl imidazole afforded acceptor **17** in 96% yield.



Scheme 3. Synthesis of the monosaccharide building block **17**. Reagents and conditions: a) *i* $(PPh_3)_3Rh(I)Cl$, $CH_3C_6H_5$/EtOH (5:2), *ii* NIS, H_2O, THF, 75%; b) Cl_3CCN, DBU, CH_2Cl_2, 82%; c) **18**, 8% TMSOTf, CH_2Cl_2, 65%; d) CF_3COOH, H_2O, CH_2Cl_2, 63%; e) PhCOCl, imidazole, CH_2Cl_2, 96%.

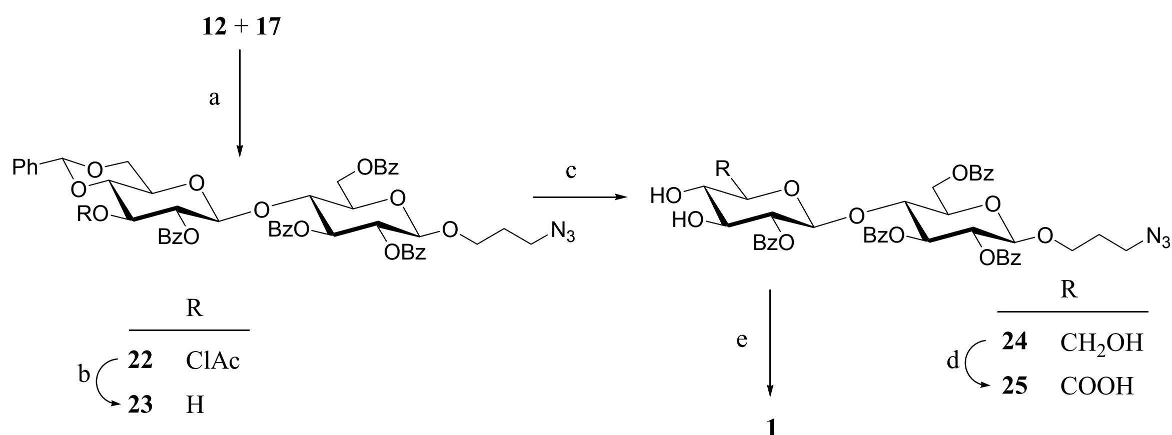
The preparation of disaccharide donor **21** is presented in Scheme 4. Coupling of donor **12** with acceptor **7** using 5% TMSOTf as a promoter afforded disaccharide **19** in an excellent yield of 96%. Deallylation with tris(triphenylphosphine)rhodium(I) chloride and *N*-iodosuccinimide (→**20**, 70%) and subsequent trichloroacetimidation gave disaccharide donor **21** (88%).



Scheme 4. Synthesis of disaccharide donor **21**. Reagents and conditions: a) 5% TMSOTf, CH_2Cl_2, 96%; b) *i* $(PPh_3)_3Rh(I)Cl$, DABCO, $CH_2Cl_2/CH_3C_6H_5$/EtOH

(0.6:9:4), *ii* NIS, H_2O, THF, 70%; c) Cl_3CCN, DBU, CH_2Cl_2, 88%.

In Scheme 5, the strategies for the preparation of the disaccharide acceptor (**23**) and target disaccharide (**1**) are shown. Test-oxidations with TEMPO [25] of monosaccharide **6** showed that the allyl group was incompatible with the reaction conditions. Therefore, the 3-azidopropyl spacer was introduced prior to oxidation. Coupling of disaccharide imidate **21** to 3-azido-1-propanol **18** to afford **22** was very troublesome and gave irreproducible yields of around 50% due to orthoester formation ($J_{1,2}$ = 5.1 Hz). Coupling via the glycosyl bromide, prepared from **20** by reaction with Vilsmeier reagent, [26] gave similar problems. Alternatively, the synthesis of disaccharide **22** was achieved by coupling of donor **12** and the spacer-containing monosaccharide acceptor **17** using 8% TMSOTf as a promoter in a yield of 75%. Subsequent dechloroacetylation with diazabicyclo[2.2.2]octane (DABCO) [27] gave disaccharide acceptor **23** (98%).

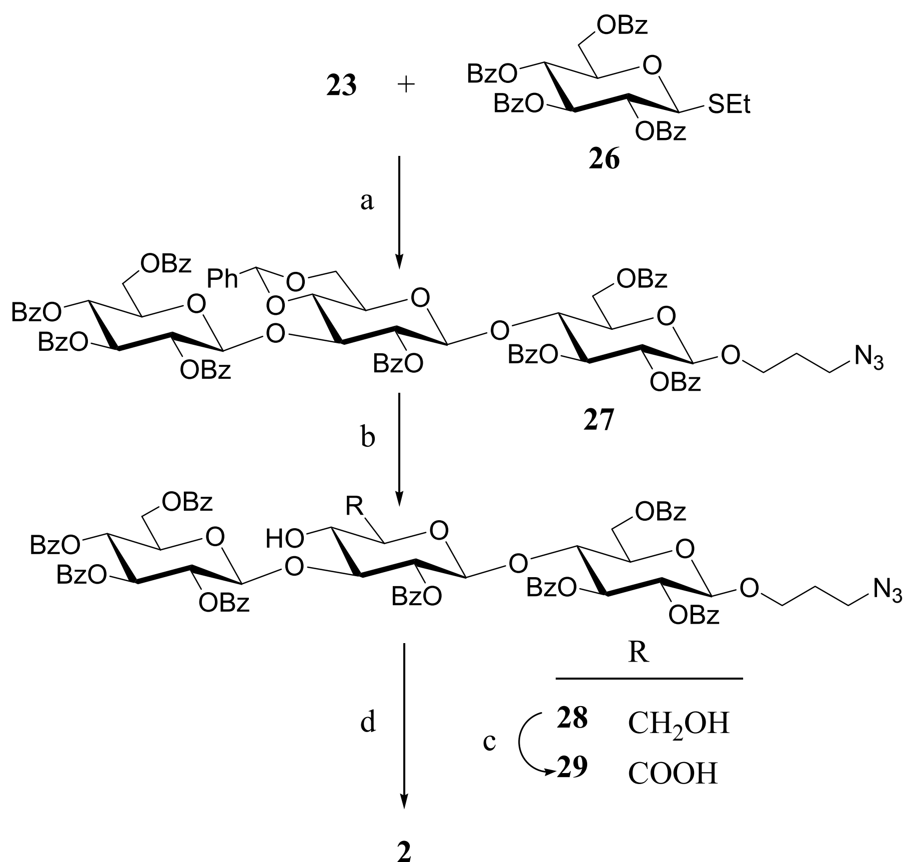


Scheme 5. Synthesis of disaccharide acceptor **23** and target disaccharide **1**. Reagents and conditions: a) 8% TMSOTf, CH_2Cl_2, 78%; b) 15 eq DABCO, $CH_3C_6H_5$/EtOH (1:1), 55 °C, 98%; c) CF_3COOH, H_2O, CH_2Cl_2, 90%; d) TEMPO, aqueous NaOCl, KBr, Bu_4NBr, aqueous NaCl, aqueous $NaHCO_3$, CH_2Cl_2, 85%; e) *i* NaOMe, MeOH (pH 11), *ii* $NaBH_4$, 10% Pd/C, H_2O, 90%.

For the synthesis of the target disaccharide fragment **1**, first **23** was debenzylidenated using trifluoroacetic acid (→**24**, 90%). Then, selective oxidation by using TEMPO and aqueous sodium hypochlorite afforded the protected cellobiuronic acid derivative **25** (85%). ^{1}H NMR analysis after methylation by using diazomethane proved the identity of the compound (δ = 3.37, COOCH_3). Debenzoylation of **25** using sodium methoxide in methanol at pH 11, followed by hydrogenation of the azide should give **1**. However, using the conventional hydrogenolysis method with 10% Pd/C and hydrogen, a side-product in about 20% was generated. ^{1}H NMR analysis showed the removal of some of the spacer

signals, but the full identity of the compound could not be disclosed. When using dithiothreitol and DBU [28], the same side-product was formed. Reduction with sodium borohydride and 10% Pd/C in H_2O afforded disaccharide fragment **1** in 90% yield over two steps with the formation of only small amounts of the mentioned side-product.

Focusing on target trisaccharide fragment **2**, the synthesis of protected trisaccharide **27** (Scheme 6) was quite troublesome.

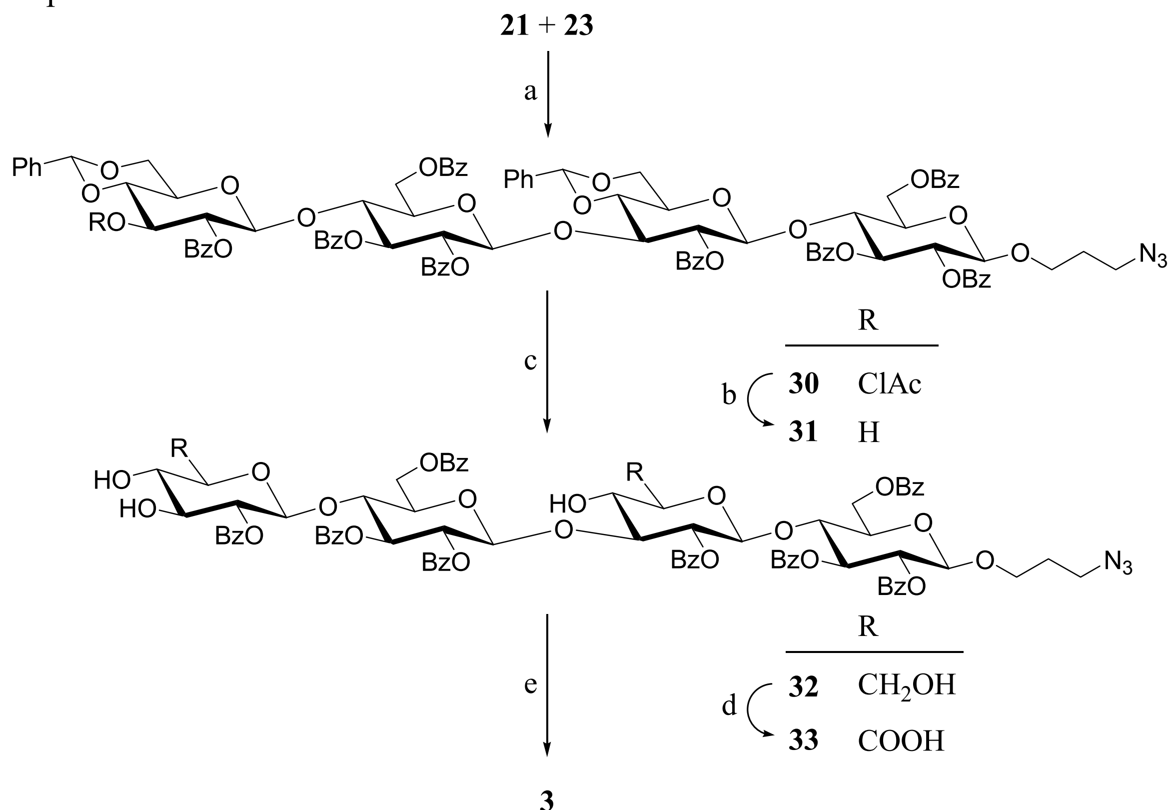


Scheme 6. Synthesis of target trisaccharide **2**. Reagents and conditions: a) NIS, TfOH, Et_2O/CH_2Cl_2 (1:2), 82%; b) CF_3COOH, H_2O, CH_2Cl_2, 70%; c) TEMPO, aqueous NaOCl, KBr, Bu_4NBr, aqueous NaCl, aqueous $NaHCO_3$, CH_2Cl_2, 76%; d) *i* NaOMe, MeOH (pH 11), *ii* $NaBH_4$, 10% Pd/C, H_2O, 89%.

Coupling of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate with **23** using 10% TMSOTf as a promoter gave a complex reaction mixture. The use of the corresponding glucosyl bromide or thioglucoside as a donor resulted in the same complex mixture with one of the main side-products being the orthoester ($J_{1'',2''}$ = 5.2 Hz). Coupling of 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate with **23** using 10% TMSOTf as a promoter gave trisaccharide **27** in 70% yield. The yield was, however, irreproducible due to the formation of

orthoester and some other side-products. Finally, the use of ethyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-glucopyranoside (**26**) as donor was found to result in an efficient glycosylation of **23**. Activation with triflic acid and *N*-iodosuccinimide gave trisaccharide **27** in a reproducible yield of 82%. Debenzylidenation using trifluoroacetic acid (→**28**, 70%) and subsequent oxidation of HO-6 using TEMPO and aqueous sodium hypochlorite gave the protected trisaccharide fragment **29** (76%) (^{1}H NMR: δ = 3.62, d, $J_{4',5'}$ = 9.3 Hz, 1 H; H-5'). Deprotection of **29** as described for **25** afforded trisaccharide fragment **2** in 89% yield over two steps.

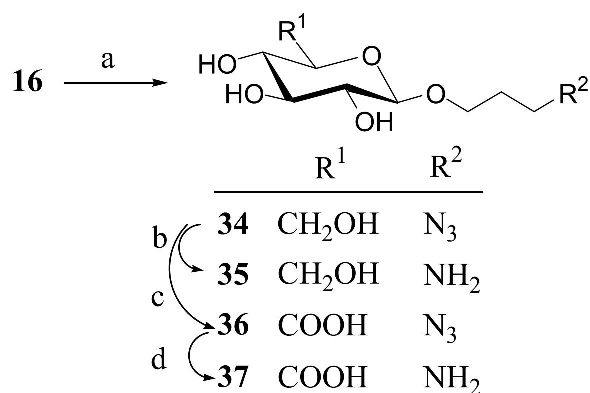
In the synthesis of target tetrasaccharide fragment **3** (Scheme 7), as a first step disaccharide donor **21** was coupled with disaccharide acceptor **23** using 10% TMSOTf as a promoter, resulting in **30** (75%). Dechloroacetylation of **30** using thiourea (→**31**, 84%), followed by debenzylidenation using trifluoroacetic acid (→**32**, 70%), and selective oxidation using TEMPO and aqueous sodium hypochlorite gave **33** in a yield of 65%. ^{1}H NMR analysis after methylation with diazomethane showed two singlets at δ = 3.39 and 3.34 (COOCH_3). Deprotection of **33** as described for **25** gave tetrasaccharide fragment **3** in 60% yield over two steps.



Scheme 7. Synthesis of target tetrasaccharide **3**. Reagents and conditions: a) 10% TMSOTf, CH_2Cl_2, 75%; b) thiourea, EtOH/C_5H_5N (8:1), 90 °C, 84%; c) CF_3COOH, H_2O, CH_2Cl_2, 70%; d) TEMPO, aqueous NaOCl, KBr, Bu_4NBr, aqueous NaCl, aqueous $NaHCO_3$, CH_2Cl_2, 65%; e) *i* NaOMe, MeOH (pH 11), *ii*

$NaBH_4$, 10% Pd/C, H_2O, 60%.

In order to make a comparison possible of the immunological studies of the aimed di-, tri- and tetrasaccharide-protein conjugates with relevant blanks, the glucose and glucuronic acid conjugates were also prepared. To this end, 3-aminopropyl glucopyranoside (**35**) and 3-aminopropyl glucopyranosiduronic acid (**37)** (Scheme 8) were prepared from **16**. Debenzoylation of **16** (→**34**), followed by reduction of the azide with sodium borohydride and 10% Pd/C in water gave **35** in 88% over two steps. Oxidation of **34** with TEMPO and aqueous sodium hypochlorite in water gave **36**, which was purified by conventional acetylation with acetic anhydride and pyridine, column chromatography, and deacetylation using sodium methoxide. Glucuronic acid derivative **37** was obtained after reduction of **36** as described for **34** (80% over three steps).



Scheme 8. Synthesis of 3-aminopropyl glucopyranoside **35** and 3-aminopropyl glucopyranosiduronic acid **37**. Reagents and conditions: a) NaOMe, MeOH (pH 10); b) $NaBH_4$, 10% Pd/C, H_2O, 88%; c) TEMPO, aqueous NaOCl, KBr, H_2O; d) $NaBH_4$, 10% Pd/C, H_2O, 80%.

The ^{1}H NMR data of the target saccharide fragments **1-3**, **35** and **37**, derived from 2D TOCSY and ROESY measurements, are presented in Table 1.

Table 1. 500 MHz ^{1}H NMR data of **1**, **2**, **3**, **35**, and **37** at 300K (in ppm)[a]. $J_{1,2}$ couplings are presented in parentheses.

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
35	4.49 (7.9)	3.29	3.50	3.38	3.48	3.93	3.73
37	4.48 (7.9)	3.33	3.51	3.51	3.72		
1	4.51 (7.7)	3.33	3.66	3.61	3.61	3.98	3.81
1'	4.51 (7.7)	3.36	3.52	3.52	3.75		
2	4.51 (7.7)	3.33	3.66	3.61	3.61	3.98	3.80
2'	4.54 (8.3)	3.57	3.79	n.d.[b]	n.d.		
2''	4.79 (7.7)	3.36	3.51	3.40	3.46	3.92	3.72
3	4.51 (8.2)	3.33	3.65	3.62	n.d.	3.98	3.81
3'	4.54 (7.7)	3.58	3.79	n.d.	n.d.		
3''	4.82 (8.2)	3.38	3.68	3.61	n.d.	3.98	3.82
3'''	4.51 (8.2)	3.36	3.52	3.52	3.76		

[a] The signals for the 3-aminopropyl spacer were similar for all compounds: δ = 4.03 and 3.84 ($OCH_2CH_2CH_2NH_2$), 3.15 ($OCH_2CH_2CH_2NH_2$), 2.00 ($OCH_2CH_2CH_2NH_2$). [b] n.d. = not determined.

3. Preparation of the protein conjugates

Studying neoglycoconjugates in which only one parameter is varied is important to understand the different factors that influence the immunogenicity of the conjugates. To this end, focusing on CPS-related oligosaccharides from *S. pneumoniae* type 3, neoglycoproteins with a different oligosaccharide chain length, carbohydrate-protein ratio, and protein carrier have been prepared.

The synthesised saccharide fragments were conjugated to CRM_{197}, TT or KLH, using diethyl squarate as linker (Figure 2) [29,30]. Elongation of the saccharides

with diethyl squarate was performed in ethanol/0.1 M sodium phosphate (pH 6.9). The reaction products were purified by solid phase extraction and coupled to protein in 0.1 M sodium borate buffer at pH 9.5.

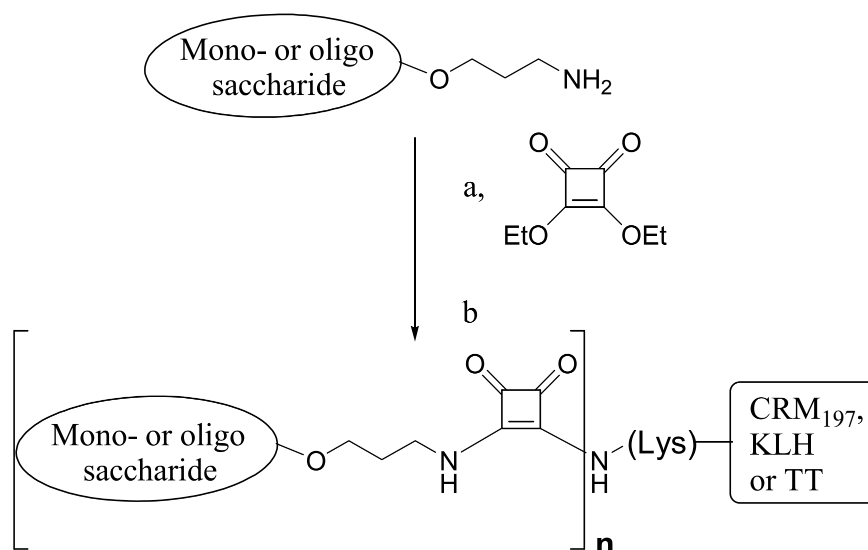


Figure 2. Coupling of saccharide fragments to a protein via diethyl squarate. a) EtOH/sodium phosphate buffer (0.1 M, pH 6.9); b) sodium borate buffer (0.1 M, pH 9.5), CRM_{197}, KLH or TT.

CRM_{197} is a non-toxic product of a single mutation in the diphtheria toxin gene [31] and exists as a pure and well-defined protein that is often used as a carrier in human neoglycoconjugate vaccines [32].

Table 2. Oligosaccharide loading and coupling efficiency for the conjugation of **1**, **2**, **3**, **35** and **37** to CRM_{197}.

Entry	Glycoconjugate	Oligosaccharide loading (mol/mol)	Coupling efficiency (%)
1	CRM_{197}-**35**	8.5	77
2	CRM_{197}-**37**	6.6	67
3	CRM_{197}-**1**	3.1	56
4	CRM_{197}-**1**	6.7	52
5	CRM_{197}-**2**	4.9	45
6	CRM_{197}-**2**	6.8	41
7	CRM_{197}-**2**	12.0	54
8	CRM_{197}-**3**	2.9	26
9	CRM_{197}-**3**	6.7	32
10	CRM_{197}-**3**	8.1	37

As can be seen from the coupling efficiencies in Table 2, the yield for the conjugation of the saccharides to CRM_{197} decreases when charged structures are coupled (entries 1 and 2), or when the oligosaccharides become larger (entries 1, 3, 5, and 8).
The amount of carbohydrate incorporation (Table 2) was measured by MALDI-TOF analysis (see Figure 3 for some representative spectra). It should be noted that analysis of the CRM_{197}-conjugates by MALDI-TOF was possible only when about 10 mM sodium borate buffer was still present in the sample. Attempts to measure the molecular mass in water failed.

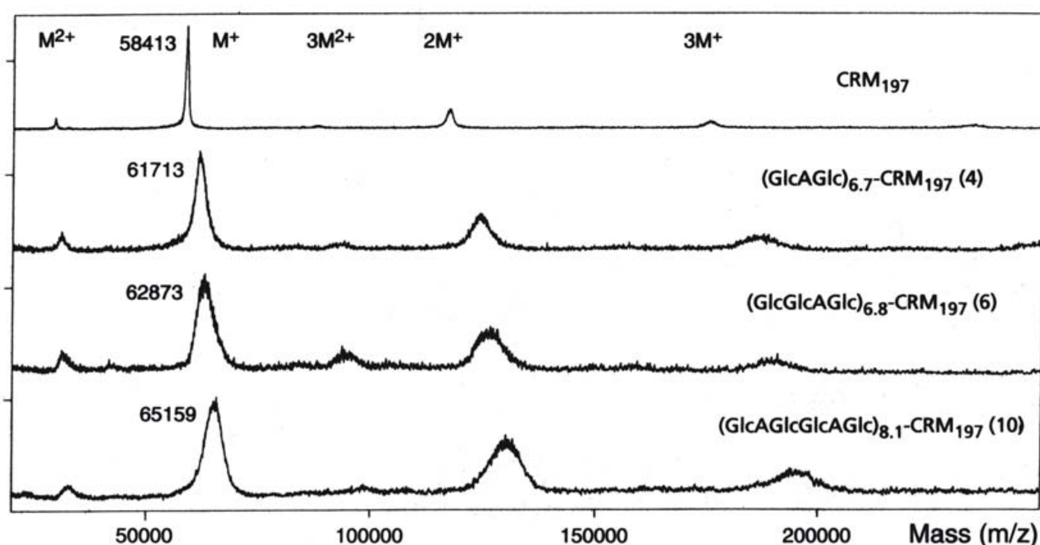


Figure 3. MALDI-TOF spectra of CRM_{197} and entries 4, 6 and 10 (Table 2).

To investigate the immunological effect of the protein carrier, two other proteins were coupled with the saccharide fragments. Tetanus toxoid (TT), prepared by inactivation of the crude toxin with formalin, is frequently used in human immunisation studies, and KLH is frequently used for immunisation studies in animals. The results for the coupling of the saccharide fragments to TT and KLH are shown in Table 3. Since TT and KLH are heterogeneous protein preparations, the carbohydrate loading of these conjugates could not be derived reliably by MALDI-TOF analysis. Therefore, the loading was determined by analysis of the carbohydrate (Dubois, [33]) and protein content (Pierce, [34]).

Table 3. Oligosaccharide loading and coupling efficiency for the conjugation of **1**, **2** and **3** to KLH and TT.

Entry	Glycoconjugate	Oligosaccharide loading (μg/mg)	Coupling efficiency (%)
1	KLH-**1**	14.1	9
2	KLH-**2**	13.3	15
3	KLH-**3**	24.6	9
4	TT-**1**	31.3	16
5	TT-**2**	23.6	11
6	TT-**3**	22.9	8

In conclusion, we have synthesised a range of neoglycoproteins related to *S. pneumoniae* type 3. The immunological properties of these well-defined neoglycoconjugates will be evaluated in order to gain insight in the structural factors influencing the immune response of this type of neoglycoconjugate vaccines. Preliminary results of the immunisation of mice with the CRM_{197}-conjugates show that they can induce a protective immune response [35].

Acknowledgements

This work was supported by the European Union program VACNET (grant ERB BIO 4CT960158). We thank Dr. P. Hoogerhout for the kind gift of tetanus toxoid.

Experimental section

General: All chemicals were of reagent grade, and were used without any further purification. A solution of tetanus toxoid (TT; 6.12 mg mL^{-1}) was obtained from the Dutch National Institute of Health (RIVM, Bilthoven, The Netherlands); a solution of keyhole limpet hemocyanin (KLH; 50 mg mL^{-1}) was obtained from the Eijkman-Winkler Institute for Microbiology (Utrecht University, Utrecht, The Netherlands); a solution of a non-toxic variant of diphtheria toxin (cross-reacting material, CRM_{197}; 61.15 mg mL^{-1}) was obtained from Chiron (Siena, Italy). Reactions were monitored by TLC on Silica gel 60 F_{254} (Merck); after examination under UV light, compounds were visualised by heating with 10% (v/v) ethanolic H_2SO_4, orcinol (2 mg mL^{-1}) in 20% (v/v) methanolic H_2SO_4, or ninhydrin (1.5 mg mL^{-1}) in BuOH/H_2O/HOAc (38:1.75:0.25). In the work-up procedures of reaction mixtures, organic solutions were washed with appropriate amounts of the indicated aqueous solutions, then dried ($MgSO_4$), and concentrated under reduced pressure at 20-40 °C on a water-bath. Column chromatography was performed on Silica gel 60 (Merck, 0.063-0.200 mm). Optical rotations were measured in $CHCl_3$, unless stated otherwise, with a Perkin-Elmer 241 polarimeter, using a 10-cm l-mL cell. ^1H NMR spectra in $CDCl_3$ were recorded at 27 °C with a Bruker AC 300 spectrometer; the δ_H values are given in ppm relative to the signal for internal Me_4Si ($\delta = 0$). ^{13}C (APT, 75 MHz) NMR

spectra in $CDCl_3$ were recorded at 27 °C with a Bruker AC 300 spectrometer; indicated ppm values for δ_C are relative to the signal of $CDCl_3$ (δ = 76.9). ^{1}H NMR spectra in D_2O were recorded at 27 °C with a Bruker AMX 500 spectrometer, and the δ_H values are given in ppm relative to the signal for internal acetone (δ = 2.225). Two-dimensional TOCSY and ROESY spectra in D_2O were recorded using a Bruker AMX 500 apparatus (500 MHz) at 27 °C to assign the spectra of compounds **1**, **2**, **3**, **35** and **37**. Fast-atom-bombardment mass spectrometry (FABMS) was performed on a JEOL JMS SX/SX 102A four-sector mass spectrometer. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) spectra were obtained on a Voyager-DE™ mass spectrometer using 2,4-dihydroxybenzoic acid (DHB) in H_2O as a matrix. Elemental analyses were carried out by H. Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany).

Allyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside (5): To a solution of allyl 4,6-*O*-benzylidene-β-D-glucopyranoside **4** [14] (10.5 g, 34.1 mmol) in CH_2Cl_2 (80 mL) and pyridine (10 mL) was added dropwise benzoyl chloride (10.3 mL, 88.7 mmol). After 3 h, the mixture was diluted with CH_2Cl_2, washed twice with 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (CH_2Cl_2) to obtain **5** as a white solid (16.2 g, 92%). R_f 0.81 (CH_2Cl_2/EtOAc 9:1); $[\alpha]_D^{20}$= +19 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 7.98-7.94 and 7.39-7.29 (2 m, 15 H; *Ph*CH, 2 *Ph*CO), 5.84-5.70 (m, 1 H; OCH_2CH=CH_2), 5.79 (t, $J_{2,3}$ = 9.5, $J_{3,4}$ = 9.5 Hz, 1 H; H-3), 5.54 (s, 1 H; PhC*H*), 5.51 (dd, $J_{1,2}$ = 7.8 Hz, 1 H; H-2), 5.27-5.10 (m, 2 H; OCH_2CH=CH_2), 4.85 (d, 1 H; H-1), 4.43 (dd, $J_{5,6a}$ = 4.9, $J_{6a,6b}$ = 10.3 Hz, 1 H; H-6a), 4.39-4.32 and 4.17-4.09 (2 m, each 1 H; OCH_2CH=CH_2), 3.94 (t, $J_{4,5}$ = 9.5 Hz, 1 H; H-4), 3.88 (t, $J_{5,6b}$ = 10.3 Hz, 1 H; H-6b), 3.69 (ddd, 1 H; H-5); ^{13}C NMR ($CDCl_3$): δ = 165.4 and 165.0 (2 Ph*C*O), 117.6 (OCH_2CH=CH_2), 101.3 (Ph*C*H), 100.3 (C-1), 78.7, 72.3, 72.0, and 66.5 (C-2,3,4,5), 70.1 (OCH_2CH=CH_2), 68.5 (C-6); elemental analysis calcd (%) for $C_{30}H_{28}O_8$ (516.6): C 69.76, H 5.46; found C 69.88, H 5.38.

Allyl 2,3-di-*O*-benzoyl-β-D-glucopyranoside (6): To a solution of **5** (1.50 g, 2.90 mmol) in CH_2Cl_2 (40 mL) were added CF_3COOH (1.64 mL) and H_2O (0.22 mL). The mixture was stirred for 3 h, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHCO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (CH_2Cl_2/MeOH 98:2) to obtain **6** (1.14 g, 92%). R_f 0.15 (CH_2Cl_2/acetone 9:1); $[\alpha]_D^{20}$= +90 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 7.97-7.94 and 7.51-7.26 (2 m, 10 H; 2 *Ph*CO), 5.85-5.72 (m, 1 H; OCH_2CH=CH_2), 5.27-5.10 (m, 2 H; OCH_2CH=CH_2), 4.78 (d, $J_{1,2}$ = 7.7 Hz, 1 H; H-1), 4.38-4.31 and 4.18-4.11 (2 m, each 1 H; OCH_2CH=CH_2), 3.58 (ddd, $J_{4,5}$ = 9.6, $J_{5,6a}$, $J_{5,6b}$ = 3.6, 4.5 Hz, 1 H; H-5); ^{13}C NMR ($CDCl_3$): δ = 165.2 (2 Ph*C*O), 117.6 (OCH_2CH=CH_2), 99.8 (C-1), 77.3, 75.7, 71.3, and 69.9 (C-2,3,4,5), 70.1 (OCH_2CH=CH_2), 62.1 (C-6); elemental analysis calcd (%) for $C_{23}H_{24}O_8$ (428.4): C 64.48, H 5.65; found C 64.32, H 5.64.

Allyl 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (7): To a solution of imidazole (0.15 g, 2.2 mmol) in CH_2Cl_2 (4 mL) was added dropwise benzoyl chloride (0.15 mL, 1.28 mmol). The suspension was filtered, and the filtrate was added dropwise to a solution of **6** (0.50 g, 1.17 mmol) in CH_2Cl_2 (4 mL). After stirring for 36 h, the mixture was diluted with CH_2Cl_2, washed with 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography (CH_2Cl_2/EtOAc 95:5) to obtain **7** (0.59 g, 95%). R_f 0.73 (CH_2Cl_2/EtOAc 9:1); $[\alpha]_D^{20}$= +63 (*c* = 1); ^1H NMR ($CDCl_3$): δ = 8.09-7.94 and 7.47-7.33 (2 m, 15 H; 3 *Ph*CO), 5.85-5.72 (m, 1 H; $OCH_2CH{=}CH_2$), 5.24-5.07 (m, 2 H; $OCH_2CH{=}CH_2$), 4.81 (d, $J_{1,2}$ = 7.6 Hz, 1 H; H-1), 4.75 (dd, $J_{5,6b}$ = 4.5, $J_{6a,6b}$ = 12.1 Hz, 1 H; H-6b), 4.68 (dd, $J_{5,6a}$ = 2.5 Hz, 1 H; H-6a), 4.38-4.31 and 4.18-4.10 (2 m, each 1 H; $OCH_2CH{=}CH_2$), 3.83 (ddd, $J_{4,5}$ = 9.7 Hz, 1 H; H-5); ^{13}C NMR ($CDCl_3$): δ = 167.1, 166.8, and 165.1 (3 Ph*C*O), 117.5 ($OCH_2CH{=}CH_2$), 99.6 (C-1), 76.5, 74.3, 71.4, and 69.5 (C-2,3,4,5), 69.9 ($OCH_2CH{=}CH_2$), 63.4 (C-6); elemental analysis calcd (%) for $C_{30}H_{28}O_9$ (532.5): C 67.66, H 5.29; found C 67.53, H 5.20.

Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranoside (9): To a solution of imidazole (0.80 g, 11.73 mmol) in CH_2Cl_2 (10 mL) was added dropwise benzoyl chloride (0.68 mL, 5.81 mmol). The suspension was filtered and the filtrate was added dropwise to a solution of allyl 4,6-*O*-benzylidene-α-D-glucopyranoside **8** [20] (1.50 g, 4.86 mmol) in CH_2Cl_2 (9.5 mL). After stirring at boiling under reflux for 36 h, the mixture was diluted with CH_2Cl_2, washed with 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography, whereby the 2,3-di-*O*-benzoylated compound was eluted with toluene/EtOAc (95:5), and **9** with toluene/EtOAc (9:1) (1.48 g, 74%). R_f 0.61 (toluene/EtOAc 7:3); $[\alpha]_D^{20}$= +99 (*c* = 1); ^1H NMR ($CDCl_3$): δ = 8.09-8.06 and 7.56-7.35 (2 m, 10 H; *Ph*CH, *Ph*CO), 5.87-5.74 (m, 1 H; $OCH_2CH{=}CH_2$), 5.54 (s, 1 H; Ph*CH*), 5.30-5.22 and 5.15-5.10 (2 m, each 1 H; $OCH_2CH{=}CH_2$), 5.20 (d, $J_{1,2}$ = 3.8 Hz, 1 H; H-1), 5.04 (dd, $J_{2,3}$ = 9.6 Hz, 1 H; H-2), 4.36 (bt, $J_{3,4}$ = 9.4 Hz, 1 H; H-3), 4.29 (dd, $J_{6a,6b}$ = 10.2, $J_{5,6a}$ = 4.8 Hz, 1 H; H-6a), 3.75 (t, $J_{5,6b}$ = 10.2 Hz, 1 H; H-6b), 3.59 (t, 1 H; H-4), 2.68 (s, 1 H; HO-2); ^{13}C NMR ($CDCl_3$): δ = 166.0 (Ph*C*O), 136.9 (*Ph*CH, quaternary C), 129.4 (*Ph*CO, quaternary C), 117.5 ($OCH_2CH{=}CH_2$), 101.8 (Ph*C*H), 95.8 (C-1), 81.3, 73.8, 68.7, and 62.2 (C-2,3,4,5), 68.7 and 68.6 (C-6, $OCH_2CH{=}CH_2$); elemental analysis calcd (%) for $C_{23}H_{24}O_7$ (412.4): C 66.98, H 5.86; found C 67.18, H 5.76.

Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-α-D-glucopyranoside (10): To a solution of **9** (0.35 g, 0.84 mmol) in CH_2Cl_2 (30 mL) and pyridine (1.5 mL) was added chloroacetyl chloride (70 μL, 0.88 mmol) at 5 °C. After stirring for 2 h, another portion of chloroacetyl chloride (35 μL, 0.44 mmol) was added, and stirring was continued for 1 h. Then, the mixture was co-concentrated with toluene, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated.

The residue was purified by column chromatography (toluene/EtOAc 95:5) to afford **10** (0.38 g, 96%). R_f 0.50 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +104 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 8.04-8.02 and 7.35-7.48 (2 m, 10 H; *Ph*CH, *Ph*CO), 5.88-5.75 (m, 1 H; $OCH_2CH{=}CH_2$), 5.87 (t, $J_{2,3}$ = 9.9, $J_{3,4}$ = 9.8 Hz, 1 H; H-3), 5.54 (s, 1 H; PhC*H*), 5.31-5.23 and 5.16-5.12 (2 m, each 1 H; $OCH_2CH{=}CH_2$), 5.28 (d, $J_{1,2}$ = 3.8 Hz, 1 H; H-1), 5.12 (dd, 1 H; H-2), 4.33 (dd, $J_{6a,6b}$ = 10.3, $J_{5,6a}$ = 4.8 Hz, 1 H; H-6a), 4.00 and 3.94 (2 d, J_{gem} = 14.8 Hz, each 1 H; $ClCH_2CO$), 3.81 (t, $J_{5,6b}$ = 10.3 Hz, 1 H; H-6b), 3.78 (t, $J_{4,5}$ = 9.6 Hz, 1 H; H-4); ^{13}C NMR ($CDCl_3$): δ = 166.3 and 165.7 (Ph*C*O, $ClCH_2CO$), 136.6 (*Ph*CH, quaternary C), 128.7 (*Ph*CO, quaternary C), 117.8 ($OCH_2CH{=}CH_2$), 101.5 (Ph*C*H), 95.7 (C-1), 78.8, 72.0, 70.8, and 62.5 (C-2,3,4,5), 68.7 and 68.6 (C-6, $OCH_2CH{=}CH_2$), 40.4 ($ClCH_2CO$); elemental analysis calcd (%) for $C_{25}H_{25}ClO_8$ (488.9): C 61.41, H 5.15; found C 61.22, H 5.03.

2-*O*-Benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-D-glucopyranose (11): To a solution of **10** (0.10 g, 0.21 mmol) in absolute EtOH (20 mL) and toluene (45 mL) was added tris(triphenylphosphine)rhodium(I) chloride (70 mg). After stirring at boiling under reflux for 2.5 h, TLC (toluene/EtOAc 9:1) showed the formation of a new spot (R_f 0.52), and the mixture was concentrated. The residue was dissolved in THF (18 mL), and water (2 mL) and NIS (0.13 g) were added. After 20 min, the mixture was concentrated, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHSO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography. Impurities were eluted with toluene/EtOAc (9:1), and **11** was obtained as a light brown syrup by elution with toluene/EtOAc (8:2) (71 mg, 77%; α/β = 4:1). R_f 0.29 (toluene/EtOAc 9:1); ^{1}H NMR ($CDCl_3$): δ = 8.04-8.02 and 7.46-7.34 (2 m, 10 H; *Ph*CH, *Ph*CO), 5.54 (s, 0.8 H; PhCH^{α}), 5.52 (s, 0.2 H; PhCH^{β}), 5.88 (t, $J_{2,3}$ = 9.9, $J_{3,4}$ = 9.8 Hz, 1 H; H-3), 5.57 (d, $J_{1,2}$ = 3.6 Hz, 0.8 H; H-1^{α}), 5.19 (dd, $J_{1,2}$ = 7.9 Hz, 0.2 H; H-2^{β}), 5.10 (dd, 0.8 H; H-2^{α}), 4.92 (d, 0.2 H; H-1^{β}), 4.01 and 3.95 (2 d, J_{gem} = 14.8 Hz, each 1 H; $ClCH_2CO$); ^{13}C NMR ($CDCl_3$): δ = 166.6 and 165.8 (Ph*C*O, $ClCH_2CO$), 136.6 (*Ph*CH, quaternary C), 101.5 (Ph*C*H), 95.8 (C-1^{α}), 90.7 (C-1^{β}), 68.8 (C-6^{α}), 68.3 (C-6^{β}), 40.4 ($ClCH_2CO^{\alpha}$), 40.3 ($ClCH_2CO^{\beta}$).

2-*O*-Benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-α-D-glucopyranosyl trichloroacetimidate (12): To a solution of **11** (1.06 g, 2.43 mmol) in dry CH_2Cl_2 (28 mL) were added Cl_3CCN (2.7 mL) and DBU (87 μL). After 2 h, the mixture was concentrated, and the residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **12** (1.31 g, 93%). R_f 0.49 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +67 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 8.60 (s, 1 H; N*H*), 8.01-7.98 and 7.48-7.36 (2 m, 10 H; *Ph*CH, *Ph*CO), 6.71 (d, $J_{1,2}$ = 3.8 Hz, 1 H; H-1), 5.94 (t, $J_{2,3}$ = 9.9, $J_{3,4}$ = 9.9 Hz, 1 H; H-3), 5.59 (s, 1 H; PhC*H*), 5.40 (dd, 1 H; H-2), 4.41 (dd, $J_{5,6a}$ 4.9, $J_{6a,6b}$ = 10.4 Hz, 1 H; H-6a), 4.21 (dt, $J_{4,5}$ = 9.9, $J_{5,6b}$ = 10.2 Hz, 1 H; H-5), 4.03 and 3.97 (2 d, J_{gem} = 14.8 Hz, each 1 H; $ClCH_2CO$), 3.92 (t, 1 H; H-4), 3.84 (t, 1 H; H-6b); ^{13}C NMR ($CDCl_3$): δ = 166.3 and 165.3 (Ph*C*O,

ClCH$_2$*C*O), 160.6 (O*C*(NH)CCl$_3$), 136.3 (*Ph*CH, quaternary C), 101.6 (Ph*C*H), 93.4 (C-1), 78.1, 70.8, 70.5, and 65.1 (C-2,3,4,5), 68.4 (C-6), 40.4 (Cl*C*H$_2$CO); elemental analysis calcd (%) for $C_{24}H_{21}Cl_4NO_8$ (593.1): C 48.59, H 3.56; found C 48.10, H 3.42.

2,3-Di-*O*-benzoyl-4,6-*O*-benzylidene-D-glucopyranose (13): To a solution of **5** (3.02 g, 5.84 mmol) in EtOH (100 mL) and toluene (250 mL) was added tris(triphenylphosphine)rhodium(I) chloride (2.1 g). After stirring at boiling under reflux for 3 h, TLC (toluene/EtOAc 8:2) showed the formation of a new spot (R_f 0.81), and the mixture was concentrated. The residue was dissolved in THF (250 mL), and water (30 mL) and NIS (2.0 g) were added. After 20 min, the mixture was concentrated, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHSO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography. Impurities were eluted with toluene/EtOAc (9:1), and **13** was obtained as a light brown syrup by elution with toluene/EtOAc (8:2) (2.09 g, 75%; α/β = 3:2). R_f 0.25 (toluene/EtOAc 8:2). ^{1}H NMR (CDCl$_3$): δ = 7.99-7.94 and 7.45-7.25 (2 m, 15 H, *Ph*CH, 2 *Ph*CO), 6.14 (t, $J_{2,3}$ = 9.8, $J_{3,4}$ = 9.8 Hz, 0.6 H; H-3^α), 5.83 (t, $J_{2,3}$ = 9.6, $J_{3,4}$ = 9.6 Hz, 0.4 H; H-3^β), 5.66 (d, $J_{1,2}$ = 3.7 Hz, 0.6 H; H-1^α), 5.54 (s, 0.6 H; PhC*H*^{α}), 5.49 (s, 0.4 H; PhC*H*^{β}), 5.37 (dd, $J_{1,2}$ = 7.9 Hz, 0.4 H; H-2^β), 5.30 (dd, 0.6 H; H-2^α), 4.99 (d, 0.4 H; H-1^β); ^{13}C NMR (CDCl$_3$): δ = 166.0 and 165.7 (2 Ph*C*O), 101.4 (Ph*C*H^α), 101.3 (Ph*C*H^β), 96.1 (C-1^α), 91.0 (C-1^β), 68.3 (C-6^α), 66.6 (C-6^β); elemental analysis calcd (%) for $C_{27}H_{24}O_8$ (476.4): C 68.06, H 5.08; found C 68.08, H 5.06.

2,3-Di-*O*-benzoyl-4,6-*O*-benzylidene-D-glucopyranosyl trichloroacetimidate (14): To a solution of **13** (1.82 g, 3.82 mmol) in dry CH_2Cl_2 (65 mL) at 0 °C were added Cl_3CCN (4.1 mL) and DBU (0.14 mL). After 3 h, the mixture was concentrated, and the residue was purified by column chromatography. Impurities were eluted with toluene/EtOAc (95:5), and **14** was obtained by elution with toluene/EtOAc (9:1) (1.88 g, 82%; α/β = 3:2). R_f 0.68 (toluene/EtOAc 8:2). ^{1}H NMR (CDCl$_3$): δ = 8.72 (s, 0.4 H; N*H*^{β}), 8.60 (s, 0.6 H; N*H*^{α}), 6.76 (d, $J_{1,2}$ = 3.8 Hz, 0.6 H; H-1^α), 6.23 (d, $J_{1,2}$ = 7.2 Hz, 0.4 H; H-1^β), 6.17 (t, $J_{2,3}$ = 9.8, $J_{3,4}$ = 9.9 Hz, 0.6 H; H-3^α), 5.84 (dd, $J_{2,3}$ = 8.2, $J_{3,4}$ = 9.1 Hz, 0.4 H; H-3^β), 5.75 (t, 0.4 H; H-2^β), 5.60 (s, 0.6 H; PhC*H*^{α}), 5.57 (s, 0.4 H; PhC*H*^{β}), 5.56 (dd, 0.6 H; H-2^α); ^{13}C NMR (CDCl$_3$): δ = 165.3 (2 Ph*C*O), 160.7 (O*C*(NH)CCl$_3$), 101.5 (Ph*C*H), 95.5 and 93.6 (C-1^α,1^β), 68.4 (C-6).

3-Azidopropyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside (15): A solution of 3-azido-1-propanol **18** (1.4 g, 14 mmol) and **14** (1.80 g, 2.90 mmol) in dry CH_2Cl_2 (35 mL), containing 4 Å molecular sieves, was stirred under Ar for 1 h. Then, TMSOTf (42 μL, 0.23 mmol) was introduced, and after 30 min, the mixture was neutralised with dry pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **15** (1.05

g, 65%). R_f 0.70 (toluene/EtOAc 8:2); $[\alpha]_D^{20}$= +19 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 7.98-7.94 and 7.52-7.25 (2 m, 15 H, *Ph*CH, 2 *Ph*CO), 5.78 (t, $J_{2,3}$ = 9.5, $J_{3,4}$ = 9.5 Hz, 1 H; H-3), 5.55 (s, 1 H; PhC*H*), 5.46 (dd, $J_{1,2}$ = 7.8 Hz, 1 H; H-2), 4.79 (d, 1 H; H-1), 4.44 (dd, $J_{6a,6b}$ = 10.3, $J_{5,6a}$ = 4.9 Hz, 1 H; H-6a), 3.93 (t, $J_{4,5}$ = 9.6 Hz, 1 H; H-4), 3.88 (t, $J_{5,6b}$ = 10.1 Hz, 1 H; H-6b), 3.70 (dt, 1 H; H-5), 3.98 (dt, 1 H; OC*H*HCH$_2$CH$_2$N$_3$), 3.61 (ddd, 1 H; OCH*H*CH$_2$CH$_2$N$_3$), 3.29-3.17 (m, 2 H; OCH$_2$CH$_2$C*H*$_2$N$_3$), 1.83-1.72 (m, 2 H; OCH$_2$C*H*$_2$CH$_2$N$_3$); ^{13}C NMR ($CDCl_3$): δ = 136.6 (*Ph*CH, quaternary C), 101.7 and 101.4 (Ph*C*H, C-1), 78.7, 72.4, 71.9, and 66.6 (C-2,3,4,5), 68.5 (C-6), 66.7 (O*C*H$_2$CH$_2$CH$_2$N$_3$), 47.7 (OCH$_2$CH$_2$*C*H$_2$N$_3$), 28.9 (OCH$_2$*C*H$_2$CH$_2$N$_3$); elemental analysis calcd (%) for $C_{30}H_{29}O_8N_3$ (559.5): C 64.39, H 5.22; found C 64.25, H 5.27.

3-azidopropyl 2,3-di-*O*-benzoyl-β-D-glucopyranoside (16): To a solution of **15** (1.24 g, 2.21 mmol) in CH_2Cl_2 (40 mL) were added CF_3COOH (1.0 mL) and H_2O (0.16 mL). The mixture was stirred for 3 h, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHCO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (CH_2Cl_2/EtOAc 9:1→CH_2Cl_2/acetone 6:4) to obtain **16** (0.66 g, 63%). R_f 0.07 (CH_2Cl_2/EtOAc 9:1); $[\alpha]_D^{20}$= +37 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.01-7.94 and 7.51-7.29 (2 m, 10 H, 2 *Ph*CO), 4.73 (d, $J_{1,2}$ = 7.9 Hz, 1 H; H-1), 3.59 (ddd, 1 H; OCH*H*CH$_2$CH$_2$N$_3$), 3.33-3.17 (m, 2 H; OCH$_2$CH$_2$C*H*$_2$N$_3$), 1.83-1.73 (m, 2 H; OCH$_2$C*H*$_2$CH$_2$N$_3$); ^{13}C NMR ($CDCl_3$): δ = 101.0 (C-1), 77.1, 75.8, 71.3, and 69.9 (C-2,3,4,5), 66.3 (O*C*H$_2$CH$_2$CH$_2$N$_3$), 62.1 (C-6), 47.7 (OCH$_2$CH$_2$*C*H$_2$N$_3$), 28.9 (OCH$_2$*C*H$_2$CH$_2$N$_3$); elemental analysis calcd (%) for $C_{23}H_{25}O_8N_3$ (471.4): C 58.59, H 5.34; found C 58.32, H 5.40.

3-Azidopropyl 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (17): To a solution of imidazole (0.14 g, 2.11 mmol) in CH_2Cl_2 (10 mL) was added dropwise benzoyl chloride (0.20 mL, 1.69 mmol). The suspension was filtered, and the filtrate was added dropwise to a solution of **16** (0.66 g, 1.41 mmol) in CH_2Cl_2 (10 mL). After stirring for 18 h, the mixture was diluted with CH_2Cl_2, washed with 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **17** (0.79 g, 96%). R_f 0.41 (toluene/EtOAc 8:2); $[\alpha]_D^{20}$= +58 (*c* = 0.8); ^{1}H NMR ($CDCl_3$): δ = 8.09-7.93 and 7.50-7.30 (2 m, 15 H, 3 *Ph*CO), 5.53 (dd, $J_{2,3}$ = 9.8, $J_{3,4}$ = 8.6 Hz, 1 H; H-3), 5.41 (dd, $J_{1,2}$ = 7.8 Hz, 1 H; H-2), 4.76 (d, 1 H; H-1), 3.61 (ddd, 1 H; OCH*H*CH$_2$CH$_2$N$_3$), 3.26-3.15 (m, 2 H; OCH$_2$CH$_2$C*H*$_2$N$_3$), 1.85-1.68 (m, 2 H; OCH$_2$C*H*$_2$CH$_2$N$_3$); ^{13}C NMR ($CDCl_3$): δ = 167.0, 166.9, and 165.2 (3 Ph*C*O), 101.0 (C-1), 76.1, 74.3, 71.4, and 69.4 (C-2,3,4,5), 66.4 (O*C*H$_2$CH$_2$CH$_2$N$_3$), 63.3 (C-6), 47.7 (OCH$_2$CH$_2$*C*H$_2$N$_3$), 28.8 (OCH$_2$*C*H$_2$CH$_2$N$_3$); elemental analysis calcd (%) for $C_{30}H_{29}O_9N_3$ (575.5): C 62.60, H 5.08; found C 62.68, H 5.14.

3-Azido-1-propanol (18): To a solution of 3-bromo-1-propanol (1.54 g, 11.1 mmol) in

DMF (9 mL) was added sodium azide (3.58 g, 55.0 mmol). After stirring at boiling under reflux for 36 h, the mixture was filtered, and co-concentrated with toluene. The residue was diluted with CH_2Cl_2 and washed twice with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. An analytically pure sample was obtained by distillation under reduced pressure (0.83 g, 74%). n_D (1.4521, 23 °C); lit [36] (1.4569, 28 °C); ^{1}H NMR ($CDCl_3$): δ = 3.73 (t, 2 H; HOC*H*$_2$CH$_2$CH$_2$N$_3$), 3.44 (t, 2 H; HOCH$_2$CH$_2$C*H*$_2$N$_3$), 1.87-1.79 (m, 2 H; HOCH$_2$C*H*$_2$CH$_2$N$_3$); IR (KBr; liquid film): ν = 2100 cm^{-1} (N_3).

Allyl (2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (19): A solution of **7** (0.42 g, 0.80 mmol) and **12** (0.85 g, 1.43 mmol) in dry CH_2Cl_2 (13 mL), containing 4 Å molecular sieves, was stirred under Ar for 1 h. Then, TMSOTf (15 μL, 84 μmol) was added. After 30 min, the mixture was neutralised with dry pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **19** (0.73 g, 96%). R_f 0.35 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +29 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.01-7.91 and 7.50-7.25 (2 m, 25 H; *Ph*CH, 4 *Ph*CO), 5.77-5.64 (m, 1 H; OCH$_2$C*H*=CH$_2$), 5.69 (t, $J_{2,3}$ = 9.7 Hz, 1 H; H-3), 5.39 (dd, $J_{1,2}$ = 7.8 Hz, 1 H; H-2), 5.38 (t, $J_{2',3'}$ = $J_{3',4'}$ = 9.4 Hz, 1 H; H-3'), 5.27 (dd, $J_{1',2'}$ = 7.6 Hz, 1 H; H-2'), 5.20 (s, 1 H; PhC*H*), 5.16-5.10 and 5.07-5.03 (2 m, each 1 H; OCH$_2$CH=C*H*$_2$), 4.77 and 4.71 (2 d, each 1 H; H-1,1'), 3.91 and 3.85 (2 d, J_{gem} = 14.8 Hz, each 1 H; ClC*H*$_2$CO); ^{13}C NMR ($CDCl_3$): δ = 166.3, 165.6, 165.1 (2 C), and 164.9 (4 Ph*C*O, ClCH$_2$*C*O), 136.2 (*Ph*CH, quaternary C), 117.6 (OCH$_2$CH=*C*H$_2$), 101.5, 101.1, and 99.2 (Ph*C*H, C-1,1'), 69.8 (O*C*H$_2$CH=CH$_2$), 67.3 (C-6'), 62.2 (C-6), 40.2 (Cl*C*H$_2$CO); elemental analysis calcd (%) for $C_{52}H_{47}O_{16}Cl$ (963.3): C 64.83, H 4.91; found C 64.59, H 4.97.

(2-*O*-Benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-D-glucopyranose (20): To a solution of **19** (0.74 g, 0.78 mmol) in absolute EtOH (20 mL), toluene (45 mL), and CH_2Cl_2 (3 mL) were added a catalytic amount of DABCO and tris(triphenylphosphine)rhodium(I) chloride (0.82 g). After stirring at boiling under reflux for 2.5 h, TLC (toluene/EtOAc 9:1) showed the formation of a new spot (R_f 0.38), and the mixture was concentrated. The residue was dissolved in THF (55 mL), and water (8 mL) and NIS (0.33 g) were added. After 20 min, the mixture was concentrated, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHSO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography. Impurities were eluted with toluene/EtOAc (9:1), and **20** was obtained as a light brown syrup by elution with toluene/EtOAc (8:2) (0.50 g, 70%; α/β = 4:1). R_f $0.29^{\alpha}/0.34^{\beta}$ (toluene/EtOAc 8:2); ^{1}H NMR ($CDCl_3$): δ = 8.06-7.93 and 7.50-7.31 (2 m, 25 H; *Ph*CH, 4 *Ph*CO), 6.04 (dd, $J_{2,3}$ = 10.2, $J_{3,4}$ = 9.2 Hz, 0.8 H; H-3$^{\alpha}$), 5.48 (d, $J_{1,2}$ = 3.5 Hz, 0.8 H; H-1$^{\alpha}$), 5.42 (t, $J_{2',3'}$ = $J_{3',4'}$ =

9.4 Hz, 1 H; H-3'), 5.30 (dd, $J_{1',2'}$ = 7.6 Hz, 1 H; H-2'), 5.24 (s, 0.8 H; PhCH^{α}), 5.22 (s, 0.2 H; PhCH^{β}), 5.14 (dd, 0.8 H; H-2$^{\alpha}$), 4.89 and 4.81 (2 d, $J_{1,2} \approx$ 7.8 Hz, each 0.2 H; H-1$^{\beta}$,1'$^{\beta}$), 4.85 (d, 0.8 H; H-1'$^{\alpha}$), 3.93 and 3.87 (2 d, J_{gem} = 14.9 Hz, each 1 H; ClCH_2CO); ^{13}C NMR ($CDCl_3$): δ = 166.7, 166.4, 165.8, and 165.0 (2 C) (4 Ph*C*O, ClCH$_2$*C*O), 136.3 (*Ph*CH, quaternary C), 101.6 and 101.2 (Ph*C*H, C-1'), 95.4 (C-1$^{\beta}$), 90.0 (C-1$^{\alpha}$), 67.4 (C-6'), 62.0 (C-6), 40.3 (Cl*C*H$_2$CO); elemental analysis calcd (%) for $C_{49}H_{43}O_{16}Cl$ (923.3): C 63.74, H 4.69; found C 63.44, H 4.73.

(2-*O*-Benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate (21): To a solution of **20** (0.40 g, 0.44 mmol) in dry CH_2Cl_2 (9 mL) were added Cl_3CCN (0.60 mL) and DBU (18 μL). After 2 h, the mixture was concentrated and the residue was purified by column chromatography (toluene/EtOAc 95:5) to yield **21** (0.41 g, 88%). R_f 0.32 (toluene/EtOAc 9:1); ^{1}H NMR ($CDCl_3$): δ = 8.54 (s, 1 H; N*H*), 8.05-7.90 and 7.49-7.25 (2 m, 25 H; 4 *Ph*CO, *Ph*CH), 6.67 (d, $J_{1,2}$ = 3.7 Hz, 1 H; H-1), 6.08 (dd, $J_{2,3}$ = 10.1, $J_{3,4}$ = 8.8 Hz, 1 H; H-3), 5.46 (dd, 1 H; H-2), 5.41 (t, $J_{2',3'}$ = 9.2 Hz, 1 H; H-3'), 5.23 (dd, $J_{1',2'}$ = 7.6 Hz, 1 H; H-2'), 5.28 (s, 1 H; PhC*H*), 4.87 (d, 1 H; H-1'), 3.91 and 3.85 (2 d, J_{gem} = 14.8 Hz, each 1 H; ClCH_2CO); ^{13}C NMR ($CDCl_3$): δ = 166.3, 165.4, 165.3, 164.9, and 164.8 (4 Ph*C*O, ClCH$_2$*C*O), 160.4 (O*C*(NH)CCl$_3$), 136.3 (*Ph*CH, quaternary C), 101.7 and 101.2 (Ph*C*H, C-1'), 92.8 (C-1), 67.5 (C-6'), 61.6 (C-6), 40.2 (Cl*C*H$_2$CO).

3-Azidopropyl (2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (22): A solution of **12** (0.50 g, 0.84 mmol) and **17** (0.28 g, 0.49 mmol) in dry CH_2Cl_2 (10 mL), containing 4 Å molecular sieves, was stirred under Ar for 1 h. Then, TMSOTf (11.5 μL, 63 μmol) was added. After 30 min, the mixture was neutralised with dry pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **22** (0.38 g, 78%). R_f 0.39 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +42 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.06-7.91 and 7.48-7.26 (2 m, 25 H; *Ph*CH, 4 *Ph*CO), 5.69 (t, $J \approx$ 9.3 Hz, 1 H; H-3), 5.38 (t, $J \approx$ 9.5 Hz, 1 H; H-3'), 5.35 and 5.27 (2 dd, $J_{1,2}$, $J_{1',2'}$ = 7.6, 7.8 Hz, each 1 H; H-2,2'), 5.20 (s, 1 H; PhC*H*), 4.78 and 4.65 (2 d, each 1 H; H-1,1'), 4.07 and 3.53 (2 t, $J \approx$ 9.4 Hz, each 1 H; H-4,4'), 3.91 and 3.86 (2 d, each 1 H; ClCH_2CO), 3.16 (dt, 1 H; OCH$_2$CH$_2$CH_2N$_3$), 1.77-1.61 (m, 2 H; OCH$_2$CH_2CH$_2$N$_3$); ^{13}C NMR ($CDCl_3$): δ = 166.3, 165.5, 165.1, 164.9, and 164.8 (4 Ph*C*O, ClCH$_2$*C*O), 136.3 (*Ph*CH, quaternary C), 101.5, 101.2, and 100.7 (Ph*C*H, C-1,1'), 67.4 (C-6'), 66.3 (O*C*H$_2$CH$_2$CH$_2$N$_3$), 62.1 (C-6), 47.7 (OCH$_2$CH$_2$*C*H$_2$N$_3$), 40.2 (Cl*C*H$_2$CO), 28.7 (OCH$_2$*C*H$_2$CH$_2$N$_3$); IR (KBr, liquid film): ν = 2096 cm^{-1} (N_3); elemental analysis calcd (%) for $C_{52}H_{48}O_{16}ClN_3$ (1006.4): C 62.05, H 4.80; found C 62.15, H 4.86.

3-Azidopropyl (2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-

***O*-benzoyl-β-D-glucopyranoside (23)**: To a solution of **22** (0.26 g, 0.26 mmol) in toluene (14 mL) and ethanol (14 mL) was added DABCO (0.43 g, 3.8 mmol). After 2.5 h at 55 °C, the mixture was diluted with CH_2Cl_2, washed with aqueous 0.05 M HCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 9:1) to afford **23** (0.23 g, 98%). R_f 0.25 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +31 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.08-7.91 and 7.50-7.29 (2 m, 25 H; *Ph*CH, 4 *Ph*CO), 5.68 (t, $J_{2,3}$ = 9.8 Hz, 1 H; H-3), 5.36 (dd, $J_{1,2}$ = 7.9 Hz, 1 H; H-2), 5.22 (s, 1 H; PhC*H*), 5.17 (dd, $J_{1',2'}$ = 8.0, $J_{2',3'}$ = 9.0 Hz, 1 H; H-2'), 4.67 and 4.62 (2 d, each 1 H; H-1,1'), 4.05 and 3.36 (2 t, $J \approx 9.3$ Hz, each 1 H; H-4,4'), 3.87 (t, 1 H; H-3'), 3.19-3.11 (m, 2 H; $OCH_2CH_2C\mathit{H}_2N_3$), 1.74-1.60 (m, 2 H; $OCH_2C\mathit{H}_2CH_2N_3$); ^{13}C NMR ($CDCl_3$): δ = 165.8, 165.3, 165.1, and 165.0 (4 Ph*C*O), 136.6 (*Ph*CH, quaternary C), 101.5, 101.4, and 100.7 (Ph*C*H, C-1,1'), 67.5 (C-6'), 66.3 ($O\mathit{C}H_2CH_2CH_2N_3$), 62.4 (C-6), 47.7 ($OCH_2CH_2\mathit{C}H_2N_3$), 28.7 ($OCH_2\mathit{C}H_2CH_2N_3$); elemental analysis calcd (%) for $C_{50}H_{47}O_{15}N_3$ (929.9): C 64.58, H 5.09; found C 64.65, H 5.20.

3-Azidopropyl (2-*O*-benzoyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (24): To a solution of **23** (80 mg, 87 μmol) in CH_2Cl_2 (5 mL) were added CF_3COOH (60 μL) and H_2O (8 μL). The mixture was stirred for 3 h, then diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHCO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 8:2→toluene/EtOAc 6:4) to obtain **24** (70 mg, 90%). R_f 0.04 (toluene/EtOAc 6:4); $[\alpha]_D^{20}$= +28 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.04-7.90 and 7.61-7.32 (2 m, 20 H; 4 *Ph*CO), 5.66 (t, $J_{2,3}$ = 9.7 Hz, 1 H; H-3), 5.38 (dd, $J_{1,2}$ = 7.8 Hz, 1 H; H-2), 4.97 (dd, $J_{1',2'}$ = 7.8, $J_{2',3'}$ = 9.5 Hz, 1 H; H-2'), 4.67 and 4.65 (2 d, each 1 H; H-1,1'), 3.52 (ddd, 1 H; $OC\mathit{H}HCH_2CH_2N_3$), 3.19-3.14 (m, 2 H; $OCH_2CH_2C\mathit{H}_2N_3$), 1.79-1.64 (m, 2 H; $OCH_2C\mathit{H}_2CH_2N_3$); ^{13}C NMR ($CDCl_3$): δ = 165.9 (3 C) and 165.2 (4 Ph*C*O), 100.7 (C-1,1'), 66.4 ($O\mathit{C}H_2CH_2CH_2N_3$), 62.6 and 61.3 (C-6,6'), 47.7 ($OCH_2CH_2\mathit{C}H_2N_3$), 28.8 ($OCH_2\mathit{C}H_2CH_2N_3$); elemental analysis calcd (%) for $C_{43}H_{43}O_{15}N_3$ (841.3): C 61.35, H 5.14; found C 61.59, H 5.22.

3-Azidopropyl (2-*O*-benzoyl-β-D-glucopyranosyluronic acid)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (25): To a solution of **24** (65 mg, 0.072 mmol) in CH_2Cl_2 (0.65 mL) were added a catalytic amount of TEMPO, and 0.14 mL of a solution of KBr (7.8 mg) and Bu_4NBr (10.4 mg) in saturated aqueous $NaHCO_3$ (1.4 mL). The mixture was stirred vigorously at 0 °C, when a solution of saturated aqueous NaCl (0.14 mL), saturated aqueous $NaHCO_3$ (78 μL), and aqueous NaOCl (13% Cl active; 0.18 mL) was added dropwise. After 45 min, the mixture was acidified with 4 M HCl (pH 4), diluted with CH_2Cl_2, and the organic layer was washed with 10% (w/v) aqueous NaCl, dried, filtered, and concentrated. The residue was purified by column chromatography. Impurities were eluted with CH_2Cl_2/acetone (8:2), and **25** (55 mg, 85%) was eluted with CH_2Cl_2/acetone/HOAc (8:2:0.5). R_f 0.30 (CH_2Cl_2/acetone/HOAc 8:2:1); ^{13}C NMR

($CDCl_3$): δ = 100.6 and 100.4 (C-1,1'), 66.3 (O*C*$H_2CH_2CH_2N_3$), 62.3 (C-6), 47.6 (OCH_2CH_2*C*H_2N_3), 28.7 (OCH_2*C*$H_2CH_2N_3$).

A small amount was methylated with diazomethane and acetylated with Ac_2O and pyridine for analysis. ^{1}H NMR ($CDCl_3$): δ = 7.94-7.89 and 7.50-7.26 (2 m, 20 H; 4 *Ph*CO), 4.87 and 4.65 (2 d, $J_{1,2}$, $J_{1',2'}$ = 7.4, 7.7 Hz, each 1 H; H-1,1'), 3.73 (d, $J_{4',5'}$ = 9.9 Hz, 1 H; H-5'), 3.37 (s, 3 H; COOC*H_3*), 3.16-3.13 (m, 2 H; OCH_2CH_2C*H_2*N_3), 1.93 and 1.84 (2 s, each 3 H; C*H_3*CO), 1.75-1.57 (m, 2 H; OCH_2C*H_2*CH_2N_3); elemental analysis calcd (%) for $C_{48}H_{47}O_{18}N_3$ (953.9): C 60.44, H 4.97; found C 60.65, H 5.03.

3-Aminopropyl (β-D-glucopyranosyluronic acid)-(1→4)-β-D-glucopyranoside (1): To a solution of **25** (90 mg, 0.11 mmol) in MeOH (9 mL) was added NaOMe until pH 11. After stirring for 1.5 h, TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of a new spot (R_f 0.37), and the mixture was neutralised with Dowex H^+, filtered, and concentrated. A solution of the residue in water was washed with CH_2Cl_2 (3x), and the aqueous layer was concentrated. MALDI-TOF analysis of the residue showed a peak at *m/z* 462 [*M*+Na]$^+$. Then, a solution of the residue in 0.1 M NaOH (1.1 mL) was added dropwise to a suspension of 10% Pd/C (2 mg) and $NaBH_4$ (8.7 mg) in bidistilled water (0.55 mL). After 45 min, when TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of a ninhydrin-positive spot on the baseline, the mixture was acidified with Dowex H^+ (pH 4), then loaded on a short column of Dowex 50 W × 2 (H^+, 200-400 mesh). After elution of contaminants with water, elution with 1% NH_4OH afforded **1** after concentration and co-concentration with water (2x) (39 mg, 90%). $[\alpha]_D^{20}$= -92 (*c* = 0.6, H_2O); MS (MALDI-TOF): *m/z* 414 [*M*+H]$^+$, 436 [*M*+Na]$^+$. For ^{1}H NMR data, see Table 1.

3-Azidopropyl (2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-(1→3)-(2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (27): A solution of **23** (53 mg, 57 μmol) and ethyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-glucopyranoside **26** (62 mg, 96 μmol) in dry CH_2Cl_2 (0.26 mL), containing 4 Å molecular sieves, was stirred under Ar for 30 min at 0 °C. Then, 0.48 mL of a solution of NIS (420 mg, 1.87 mmol) and TfOH (12 μL) in Et_2O/CH_2Cl_2 (1:1) (9.6 mL) was added. After 5 min, the mixture was neutralised with dry pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous $NaHSO_3$ (2x) and 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 9:1) to afford **27** (70 mg, 82%). R_f 0.41 (toluene/EtOAc 85:15); $[\alpha]_D^{20}$= +27 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 5.64 and 5.56 (2 t, $J_{2'',3''}$ = 9.3, $J_{3'',4''}$ = 9.4 Hz, each 1 H; H-3",4"), 5.62 (dd, $J_{2,3}$ = 9.8, $J_{3,4}$ = 9.0 Hz, 1 H; H-3), 5.39 and 5.31 (2 dd, $J_{1,2}$, $J_{1'',2''}$ = 7.8 Hz, each 1 H; H-2,2"), 5.29 (s, 1 H; PhC*H*), 5.27 (dd, $J_{1',2'}$ = 7.8, $J_{2',3'}$ = 9.0 Hz, 1 H; H-2'), 4.91 (d, 1 H; H-1"), 4.59 and 4.58 (2 d, each 1 H; H-1,1'), 3.47 (ddd, 1 H; OC*H*$HCH_2CH_2N_3$), 3.13 (dt, 2 H; OCH_2CH_2C*H_2*N_3), 1.72-1.58 (m, 2 H; OCH_2C*H_2*CH_2N_3); ^{13}C NMR ($CDCl_3$): δ = 165.9, 165.1, 164.9, 164.8 (2 C), 164.6, and 164.0 (2 C) (8 Ph*C*O), 136.6 (*Ph*CH, quaternary C), 101.5, 101.2, and 100.5 (2 C) (Ph*C*H,

C-1,1',1"), 67.5 (C-6'), 66.3 (O*C*$H_2CH_2CH_2N_3$), 62.8 (2 C) (C-6,6"), 47.7 (OCH_2CH_2*C*H_2N_3), 28.7 (OCH_2*C*$H_2CH_2N_3$); MS (MALDI-TOF): *m/z* 1530 $[M+Na]^+$.

3-Azidopropyl (2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-(1→3)-(2-*O*-benzoyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (28): To a solution of **27** (0.13 g, 82 μmol) in CH_2Cl_2 (5 mL) were added CF_3COOH (60 μL) and H_2O (8 μL). The mixture was stirred for 3 h, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHCO_3$ (2 x), and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 8:2→toluene/EtOAc 7:3) to obtain **28** (86 mg, 70%). R_f 0.37 (toluene/EtOAc 7:3); $[\alpha]_D^{20}$= +14 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 5.76, 5.54, and 5.53 (3 t, $J \approx 9.5$ Hz, each 1 H; H-3,3",4"), 5.46 (dd, $J_{1'',2''}$ = 7.9, $J_{2'',3''}$ = 9.8 Hz, 1 H; H-2"), 5.35 (dd, $J_{1,2}$ = 7.7, $J_{2,3}$ = 9.6 Hz, 1 H; H-2), 5.14 (dd, $J_{1',2'}$ = 8.0, $J_{2',3'}$ = 9.2 Hz, 1 H; H-2'), 4.81 (d, 1 H; H-1"), 4.56 (d, 1 H; H-1), 4.51 (d, 1 H; H-1'), 3.14 (dt, 2 H; OCH_2CH_2C*H_2*N_3), 1.75-1.62 (m, 2 H; OCH_2C*H_2*CH_2N_3); ^{13}C NMR ($CDCl_3$): δ = 165.9, 165.5, 165.1 (2 C), 164.9, 164.7, 163.8, and 160.2 (8 Ph*C*O), 101.5 and 100.8 (2 C) (C-1,1',1"), 66.3 (O*C*$H_2CH_2CH_2N_3$), 62.5 and 62.2 (2 C) (C-6,6',6"), 47.7 (OCH_2CH_2*C*H_2N_3), 28.8 (OCH_2*C*$H_2CH_2N_3$); MS (MALDI-TOF): *m/z* 1442 $[M+Na]^+$.

3-Azidopropyl (2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-(1→3)-(2-*O*-benzoyl-β-D-glucopyranosyluronic acid)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (29): To a solution of **28** (50 mg, 34 μmol) in CH_2Cl_2 (0.6 mL) were added TEMPO (catalytic amount), and 70 μL of a solution of KBr (3.6 mg) and Bu_4NBr (4.9 mg) in saturated aqueous $NaHCO_3$ (0.7 mL). The mixture was stirred vigorously at 0 °C, when a solution of saturated aqueous NaCl (70 μL), saturated aqueous $NaHCO_3$ (33 μL), and aqueous NaOCl (13% Cl active; 90 μL) was added dropwise. After 45 min, the mixture was acidified with 4 M HCl (pH 4), diluted with CH_2Cl_2, and the organic layer was washed with 10% (w/v) aqueous NaCl, dried, filtered, and concentrated. The residue was purified by column chromatography. Impurities were eluted with CH_2Cl_2/acetone (8:2), and **29** (38 mg, 76%) was eluted with CH_2Cl_2/acetone/HOAc (8:2:0.5). R_f 0.65 (CH_2Cl_2/acetone/HOAc 8:2:1); $[\alpha]_D^{20}$= +25 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 5.76, 5.64, and 5.53 (3 t, $J_{2,3}$ = 9.6, $J_{2'',3''}$ = 9.8, $J_{3'',4''}$ = 9.3 Hz, each 1 H; H-3,3",4"), 5.45 (dd, $J_{1'',2''}$ = 7.9 Hz, 1 H; H-2"), 5.27 (dd, $J_{1,2}$ = 7.7 Hz, 1 H; H-2), 5.18 (dd, $J_{1',2'}$ = 7.9 Hz, 1 H; H-2'), 4.86 (d, 1 H; H-1"), 4.68 (d, 1 H; H-1'), 4.58 (d, 1 H; H-1), 3.62 (d, $J_{4',5'}$ = 9.3 Hz, 1 H; H-5'), 3.12 (dt, 2 H; OCH_2CH_2C*H_2*N_3), 1.71-1.59 (m, 2 H; OCH_2C*H_2*CH_2N_3); ^{13}C NMR ($CDCl_3$): δ = 169.1 (C-6'), 166.0, 165.7, 165.5, 165.3, 165.1, 164.9, 164.7, and 163.8 (8 Ph*C*O), 101.4, 100.7, and 100.6 (C-1,1',1"), 66.3 (O*C*$H_2CH_2CH_2N_3$), 62.5 and 62.1 (C-6,6"), 47.7 (OCH_2CH_2*C*H_2N_3), 28.8 (OCH_2*C*$H_2CH_2N_3$); MS (MALDI-TOF): *m/z* 1456 $[M+Na]^+$.

3-Aminopropyl (β-D-glucopyranosyl)-(1→3)-(β-D-glucopyranosyluronic acid)-(1→4)-β-D-glucopyranoside (2): To a solution of **29** (50 mg, 33 μmol) in MeOH (3 mL) was

added NaOMe until pH 11. After stirring for 16 h, TLC (EtOAc/MeOH/H_2O 12:5:2) showed the formation of a new spot (R_f 0.27), and the mixture was neutralised with Dowex H^+, filtered, and concentrated. A solution of the residue in water was washed with CH_2Cl_2 (3x), and the aqueous layer was concentrated. MALDI-TOF analysis of the residue showed a peak at *m/z* 624 $[M+Na]^+$. Then, a solution of the residue in 0.1 M NaOH (0.33 mL) was added dropwise to a suspension of 10% Pd/C (0.6 mg) and $NaBH_4$ (2.7 mg) in bidistilled water (0.17 mL). After 45 min, when TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of a ninhydrin-positive spot on the baseline, the mixture was acidified with Dowex H^+ (pH 4), then loaded on a short column of Dowex 50 W × 2 (H^+, 200-400 mesh). After elution of contaminants with water, elution with 1% NH_4OH afforded **2**, after concentration and co-concentration with water (2x) (17 mg, 89%). $[\alpha]_D^{20}$= -18 (*c* = 0.8, H_2O); MS (MALDI-TOF): *m/z* 575 $[M+H]^+$, 597 $[M+Na]^+$. For ^1H NMR data, see Table 1.

3-Azidopropyl (2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-chloroacetyl-β-D-glucopyranosyl) - (1→4) - (2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl) - (1→4) - 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (30): A solution of **21** (0.50 g, 0.47 mmol) and **23** (0.27 g, 0.29 mmol) in dry CH_2Cl_2 (4.4 mL), containing 4 Å molecular sieves, was stirred under Ar for 1 h. Then, TMSOTf (8.5 μL, 47 μmol) was added. After 15 min, the mixture was neutralised with dry pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **30** (0.39 g, 75%). R_f 0.36 (toluene/EtOAc 85:15); $[\alpha]_D^{20}$= +34 (*c* = 1); ^1H NMR ($CDCl_3$): δ = 5.58, 5.40, and 5.25 (3 t, $J \approx 9.5$ Hz, each 1 H; H-3,3'',3'''), 5.17 and 5.11 (2 s, each 1 H; 2 PhC*H*), 4.72, 4.55, 4.53, and 4.53 (4 d, $J \approx 7.7$ Hz, each 1 H; H-1,1',1'',1'''), 3.87 and 3.81 (2 d, J_{gem} = 14.9 Hz, each 1 H; ClC*H*$_2$CO), 1.79-1.65 (m, 2 H; $OCH_2CH_2CH_2N_3$); ^{13}C NMR ($CDCl_3$): δ = 136.4 and 136.2 (2 *Ph*CH, quaternary C), 101.4 (2 C), 101.1 (2 C), 100.6, and 99.8 (2 Ph*C*H, C-1,1',1'',1'''), 67.5 and 67.3 (C-6',6'''), 66.3 ($OCH_2CH_2CH_2N_3$), 62.0 and 61.9 (C-6,6''), 47.7 ($OCH_2CH_2CH_2N_3$), 40.2 (ClCH$_2$CO), 28.7 ($OCH_2CH_2CH_2N_3$); MS (FAB): *m/z* 1834 $[M+H]^+$, 1856 $[M+Na]^+$.

3-Azidopropyl (2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl) - (1→4) - (2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl) - (1→4) - 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (31): To a solution of **30** (0.39 g, 0.22 mmol) in ethanol (5 mL) and pyridine (0.66 mL) was added thiourea (50 mg). After stirring for 3 h at 90 °C, the mixture was cooled to room temperature, co-concentrated with toluene, and the residue was purified by column chromatography (toluene/EtOAc 95:5→toluene/EtOAc 9:1) to afford **31** (0.32 g, 84%). R_f 0.25 (toluene/EtOAc 8:2); $[\alpha]_D^{20}$= +52 (*c* = 1); ^1H NMR ($CDCl_3$): δ = 5.58 and 5.39 (2 t, $J \approx$ 9.5 Hz, each 1 H; H-3,3''), 5.17 and 5.13 (2 s, each 1 H; 2 PhC*H*), 4.73, 4.54 (2 H), and

4.49 (3 d, $J \approx 7.8$ Hz, 4 H; H-1,1',1'',1'''), 3.11 (bt, 2 H; $OCH_2CH_2CH_2N_3$), 1.70-1.59 (m, 2 H; $OCH_2CH_2CH_2N_3$); ^{13}C NMR ($CDCl_3$): δ = 165.9, 165.6, 165.2, 165.1, 164.8 (2 C), 164.7, and 163.9 (8 Ph*C*O), 136.4 (2 *Ph*CH, quaternary C), 101.4 (3 C), 101.1, 100.6, and 100.0 (2 Ph*C*H, C-1,1',1'',1'''), 67.4 (2 C) (C-6',6'''), 66.3 ($OCH_2CH_2CH_2N_3$), 62.1 (2 C) (C-6,6''), 47.7 ($OCH_2CH_2CH_2N_3$), 28.7 ($OCH_2CH_2CH_2N_3$); MS (FAB): *m/z* 1758 $[M+H]^+$, 1780 $[M+Na]^+$.

3-Azidopropyl (2-*O*-benzoyl-β-D-glucopyranosyl)-(1→4)-(2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl)-(1→3)-(2-*O*-benzoyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (32): To a solution of **31** (0.20 g, 0.11 mmol) in CH_2Cl_2 (6.5 mL) were added CF_3COOH (0.12 mL) and H_2O (10 μL). The mixture was stirred for 2.5 h, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHCO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 1:1→toluene/EtOAc 3:7) to obtain **32** (0.12 g, 70%). R_f 0.14 (toluene/EtOAc 1:1); $[\alpha]_D^{20}$= +36 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 5.52 (bt, $J \approx 9.3$ Hz, 2 H; H-3,3''), 5.31, 5.29, 5.05, and 4.91 (4 dd, each 1 H; H-2,2',2'',2'''), 4.63, 4.61, 4.54, and 4.45 (4 d, $J \approx 7.5$ Hz, each 1 H; H-1,1',1'',1'''), 1.68-1.56 (m, 2 H; $OCH_2CH_2CH_2N_3$); ^{13}C NMR ($CDCl_3$): δ = 165.9, 165.8, 165.7, 165.1 (2 C), 165.0, 164.9, and 163.9 (8 Ph*C*O), 101.2, 100.7 (2 C), and 100.6 (C-1,1',1'',1'''), 66.3 ($OCH_2CH_2CH_2N_3$), 62.3 (2 C), 62.0, and 61.3 (C-6,6',6'',6'''), 47.7 ($OCH_2CH_2CH_2N_3$), 28.8 ($OCH_2CH_2CH_2N_3$). MS (FAB): *m/z* 1582 $[M+H]^+$, 1604 $[M+Na]^+$.

3-Azidopropyl (2-*O*-benzoyl-β-D-glucopyranosyluronic acid)-(1→4)-(2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl)-(1→3)-(2-*O*-benzoyl-β-D-glucopyranosyluronic acid)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (33): To a solution of **32** (95 mg, 57 μmol) in CH_2Cl_2 (0.6 mL) were added TEMPO (catalytic amount), and 0.12 mL of a solution of KBr (6.6 mg) and Bu_4NBr (8.7 mg) in saturated aqueous $NaHCO_3$ (1.2 mL). The mixture was stirred vigorously at 0 °C, then a mixture of saturated aqueous NaCl (0.12 mL), saturated aqueous $NaHCO_3$ (0.06 mL), and aqueous NaOCl (13% Cl active; 0.15 mL) was added dropwise. After 45 min, the mixture was acidified with 4 M HCl (pH 4), diluted with CH_2Cl_2, and the organic layer was washed with 10% (w/v) aqueous NaCl, dried, filtered, and concentrated. The residue was purified by column chromatography. Impurities were eluted with CH_2Cl_2/acetone (8:2), and **33** (64 mg, 65%) was eluted with CH_2Cl_2/acetone/HOAc (8:2:0.5). R_f 0.41 (CH_2Cl_2/acetone/HOAc 8:2:1).
A small sample was methylated with diazomethane for analysis. ^{1}H NMR ($CDCl_3$): δ = 5.56 (bt, $J \approx 9.3$ Hz, 2 H; H-3,3''), 5.36, 5.28, 5.22, and 5.13 (4 dd, each 1 H; H-2,2',2'',2'''), 4.84, 4.66, 4.58, and 4.54 (4 d, $J \approx 7.8$ Hz, each 1 H; H-1,1',1'',1'''), 3.39 and 3.34 (2 s, each 3 H; 2 $COOCH_3$), 3.11 (dt, 2 H; $OCH_2CH_2CH_2N_3$), 1.68-1.56 (m, 2 H; $OCH_2CH_2CH_2N_3$). MS (MALDI-TOF): *m/z* 1610 $[M+H]^+$, 1632 $[M+Na]^+$.

3-Aminopropyl (β-D-glucopyranosyluronic acid)-(1→4)-(β-D-glucopyranosyl)-(1→3)-

(β-D-glucopyranosyluronic acid)-(1→4)-β-D-glucopyranoside (3): To a solution of **33** (75 mg, 43 μmol) in MeOH (3 mL) was added NaOMe until pH 11. After stirring for 16 h, TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of a new spot (R_f 0.30), and the mixture was neutralised with Dowex H^+, filtered, and concentrated. A solution of the residue in water was washed with CH_2Cl_2 (3x), and the aqueous layer was concentrated. MALDI-TOF analysis of the residue showed a peak at *m/z* 800 $[M+Na]^+$. Then, a solution of the residue in 0.1 M NaOH (0.43 mL) was added dropwise to a suspension of 10% Pd/C (0.8 mg) and $NaBH_4$ (3.5 mg) in bidistilled water (0.22 mL). After 45 min, when TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of a ninhydrin-positive spot on the baseline, the mixture was acidified with Dowex H^+ (pH 4), then loaded on a short column of Dowex 50 W × 2 (H^+, 200-400 mesh). After elution of contaminants with water, elution with 1% NH_4OH afforded **3** after concentration and co-concentration with water (2x) (19 mg, 60%). $[\alpha]_D^{20}$= +124 (*c* = 0.7, H_2O); MS (MALDI-TOF): *m/z* 752 $[M+H]^+$, 774 $[M+Na]^+$. For ^1H NMR data, see Table 1.

3-Aminopropyl β-D-glucopyranoside (35): To a solution of **16** (81 mg, 0.12 mmol) in MeOH (3 mL) was added NaOMe until pH 10. After stirring for 2 h, when TLC (EtOAc/MeOH/H_2O 6:3:1) showed the formation of **34** (R_f 0.63), the mixture was neutralised with Dowex H^+, filtered, and concentrated. A solution of the residue in water was washed with CH_2Cl_2 (3x), then the aqueous layer was concentrated, and a solution of the residue (crude **34**) in 0.1 M NaOH (1.1 mL) was added dropwise to a suspension of 10% Pd/C (2 mg) and $NaBH_4$ (9.7 mg) in bidistilled water (0.5 mL). After 45 min, when TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of **35** as a ninhydrin-positive spot on the baseline, the mixture was acidified with Dowex H^+ (pH 4) and loaded on a short column of Dowex 50 W × 2 (H^+, 200-400 mesh). Contaminants were eluted with water, and after elution with 1% NH_4OH, **35** was obtained after concentration and co-concentration with water (2x) (25 mg, 88%). $[\alpha]_D^{20}$= +6 (*c* = 0.2, H_2O); MS (MALDI-TOF): *m/z* 238 $[M+H]^+$, 260 $[M+Na]^+$. For ^1H NMR data, see Table 1.

3-Aminopropyl β-D-glucopyranosiduronic acid (37): To a cooled solution (5 °C) of **34** (30 mg, 0.11 mmol) in H_2O (3 mL) were added TEMPO (0.12 mg) and KBr (5.6 mg). Aqueous NaOCl (13% Cl active; 0.37 mL) was brought to pH 10 by addition of 4 M HCl and cooled to 5 °C. The two solutions were combined and pH 10 was maintained by addition of 0.5 M NaOH. After stirring for 3 h at 5 °C, the mixture was neutralised with 4 M HCl and concentrated. Desalting of the mixture could not be achieved by size-exclusion chromatography. Therefore, the crude residue was acetylated by stirring with Ac_2O (1 mL) and pyridine (1 mL) for 4 h, and after concentration, the residue was purified by column chromatography (toluene/EtOAc 7:3). To a solution of the residue in MeOH (1 mL) and H_2O (0.5 mL) was added 0.5 M NaOH until pH 10 was reached. After stirring overnight, Dowex H^+ was added until pH 6, and the mixture was filtered and concentrated to obtain **36**. A solution of the residue in 0.1 M NaOH (1 mL) was added dropwise to a

suspension of 10% Pd/C (2 mg) and $NaBH_4$ (8.1 mg) in H_2O (0.5 mL). After 30 min, when TLC (EtOAc/MeOH/H_2O 12:5:2) showed the formation of **37** as a ninhydrin-positive spot on the baseline, the mixture was acidified with Dowex H^+ (pH 4) and loaded on a short column of Dowex 50 W × 2 (H^+, 200-400 mesh). Contaminants were eluted with water, and after elution with 1% NH_4OH, **37** was obtained after concentration and co-concentration with water (2x) (22 mg, 80%). $[\alpha]_D^{20}$= -25 (*c* = 1, H_2O); MS (MALDI-TOF): *m/z* 252 $[M+H]^+$, 274 $[M+Na]^+$. For ^1H NMR data, see Table 1.

General procedure for the elongation of 1, 2, 3, 35 and 37 with diethyl squarate: To a solution of 3-aminopropyl glycoside (1 μmol) in 0.1 M sodium phosphate (40 μL for **35** and **37**, 75 μL for **1**-**3**; pH 6.9) was added a solution of diethyl squarate (1 μmol) in EtOH (76.2 μL, stock solution of 24.9 μL diethyl squarate in 12.8 mL EtOH). After stirring for 16 h, EtOH was evaporated by flushing with N_2. To the water layer was added H_2O (**35**, 4 mL) or aqueous HOAc pH 4 (**1**-**3**, **37**, 6 mL), and the mixture was loaded on a C18 Bakerbond spe™ column (500 mg). Rinsing with H_2O (10 mL) eluted the starting material, and elution with MeOH (4 mL) and evaporation of the solvent by flushing with N_2, afforded the pure elongated fragment that was used for the conjugation reaction.

General procedure for the conjugation of the elongated saccharides to a protein carrier:

CRM_{197}-conjugates: For a targeted oligosaccharide incorporation of about 11 mol mol^{-1}, the elongated saccharide fragment (~1 μmol) was dissolved in 0.1 M sodium borate buffer (400 μL; pH 9.5), and a solution of CRM_{197} (61.15 mg mL^{-1}; 86 μL, 0.09 μmol) was added. After stirring for two to three days, the mixture was diluted with H_2O to a protein concentration of 1 mg mL^{-1}, samples were taken for MALDI-TOF analysis, and the mixture was dialysed against 50 mM sodium phosphate buffer (pH 7.2). To obtain different oligosaccharide loadings, the amount of elongated saccharide was varied. For MALDI-TOF analysis, samples were mixed on the target plate in a ratio of 1:1 (v/v) with the matrix 3,5-dimethoxy-4-hydroxycinnamic acid in 30% aqueous CH_3CN containing 0.3% CF_3COOH.

KLH-conjugates: The elongated saccharide fragment (~1 μmol) was dissolved in 0.1 M sodium borate buffer (400 μL; pH 9.5), and a solution of KLH (50 mg mL^{-1}; 75 μL for **1**, 60 μL for **2**, and 40 μL for **3**) was added. After stirring for two to three days, the mixture was diluted with 50 mM sodium phosphate buffer (pH 7.2) and dialysed against the same buffer.

TT-conjugates: The elongated saccharide fragment (~1 μmol) was dissolved in 0.1 M sodium borate buffer (300 μL; pH 9.5), and a solution of TT (6.12 mg mL^{-1}; 0.61 mL for **1**, 0.49 mL for **2**, and 0.33 mL for **3**) was added. The pH was adjusted to 9.5 by addition of 0.1 M NaOH. After stirring for two to three days, the reaction mixture was diluted with 50 mM sodium phosphate buffer (pH 7.2) and dialysed against the same buffer.

The protein concentrations of all conjugates were determined by the Pierce assay [34] and

the carbohydrate content by MALDI-TOF (CRM_{197}) or Dubois (KLH and TT) analysis [33]. Coupling efficiencies (Tables 2 and 3) were expressed as percentage of the targeted incorporation.

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3

Synthetic polysaccharide type 3 related di-, tri- and tetrasaccharide-CRM_{197} conjugates induce protection against *Streptococcus pneumoniae* type 3 in mice

Barry Benaissa-Trouw[1], Dirk J. Lefeber[2], Johannis P. Kamerling[2], Johannes F.G. Vliegenthart[2], Kees Kraaijeveld,[1] and Harm Snippe[1]

[1]*Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation, Vaccines Section, Utrecht Medical Center;* [2]*Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, The Netherlands*

Abstract

Di-, tri- and tetrasaccharides, synthesized according to the chemical structure of pneumococcal polysaccharide type 3, were coupled to the cross-reacting material (CRM_{197}) of modified diphtheria toxin in different molar carbohydrate-protein ratios using the squarate coupling method. To study protective immunity, female BALB/c mice were immunized twice subcutaneously with a 3-week interval using the amount of conjugates corresponding to 2.5 μg oligosaccharide per mouse. The conjugates evoked polysaccharide type 3 (PS3) binding IgG antibodies that lasted for at least 7 weeks after the booster. Immunogenicity was not influenced by the carbohydrate-protein ratio. All mice with PS3 specific antibodies survived the intraperitoneal challenge with *S. pneumoniae* type 3. Therefore, synthetic oligosaccharide-protein conjugates might have potential as vaccines.

Introduction

The encapsulated bacterium *Streptococcus pneumoniae* is still a major cause of upper and lower respiratory tract infections. Of the 90 serotypes known, about 23 are causing the majority (~90 %) of pneumococcal infections such as otitis media, pneumonia and meningitis [4]. *S. pneumoniae* type 3 strains are related to invasive pneumococcal infection in adults [1,4] and are often used in experimental meningitis [9] and otitis media models [7] in rabbits and rats. Furthermore, because of the high virulence in mice, *S. pneumoniae* type 3 offers a good model to study protective immunogenic properties of candidate vaccines [8,14,15].

Protection against encapsulated bacteria is primarily mediated by anticapsular antibodies. However, capsular polysaccharides are thymus-independent type 2 antigens and thus induce low affinity antibodies that display a limited subclass distribution. These antigens evoke no B-cell memory either. Vaccines consisting of polysaccharides coupled to a protein carrier can circumvent these disadvantages with an increased antibody response to capsular polysaccharides [3,11,16].

Neoglycoprotein preparations containing polysaccharides or oligosaccharide fragments obtained by degradation of the polysaccharides are sometimes contaminated with other pneumococcal components. They also have an ill-defined structure due to multiple coupling sites or the use of oligosaccharide pools of different chain lengths and they lose their reducing end upon conjugation to a carrier. The use of small chemically synthesized oligosaccharides (OS) results in conjugates with a precisely defined carbohydrate part. This offers a possibility to evaluate the immunogenic properties of a conjugate vaccine by varying its specific structural parameters, for example the length of the saccharide fragment and the carbohydrate-protein ratio.

Materials and Methods

Oligosaccharide-protein conjugates were prepared as follows: the synthetic spacer-containing monosaccharides β-D-Glc*p*-(1→$O(CH_2)_3NH_2$ and β-D-Glc*p*A-(1→$O(CH_2)_3NH_2$, disaccharide β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→$O(CH_2)_3NH_2$, trisaccharide β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→$O(CH_2)_3NH_2$, and tetrasaccharide β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→$O(CH_2)_3NH_2$ [6], representing fragments of the type 3 polysaccharide were conjugated to the cross-reacting material (CRM_{197}) of modified diphtheria toxin in different molar carbohydrate-protein ratios using the squarate coupling method [17]. The 3-aminopropyl glycoside (1 μmol) was dissolved in 0.1 M sodium phosphate buffer (75 μL, pH 6.95), and a diethyl squarate solution (76.2 μL, 1 μmol) in EtOH (from 24.9 μL diethyl squarate in 12.8 mL EtOH) was added. After stirring for16 h, EtOH was evaporated by flushing with N_2, and the waterlayer was

diluted with aq HOAc pH 4 (6 mL), and charged on a C18 Bakerbond spe™ column (500 mg). Rinsing with H_2O (10 mL), elution with MeOH (4 mL), and evaporation of the solvent by flushing with N_2 afforded the pure, elongated fragments. The elongated fragments were dissolved in 0.1 M borate buffer (400 µL, pH 9.55) and the appropriate volume of CRM_{197} solution (61 mg/mL, from Chiron vaccines, Siena, Italy) required for the target amount of oligosaccharide incorporation was added. After stirring for two to three days, the mixture was diluted with 50 mM sodium phosphate (pH 7.2) and dialyzed against the same buffer. The protein concentrations were determined by the Pierce assay [13] and the carbohydrate content by MALDI-TOF analysis. Schematic structures of the conjugates are shown in Figure 1 and the carbohydrate-protein ratios in Table 1.

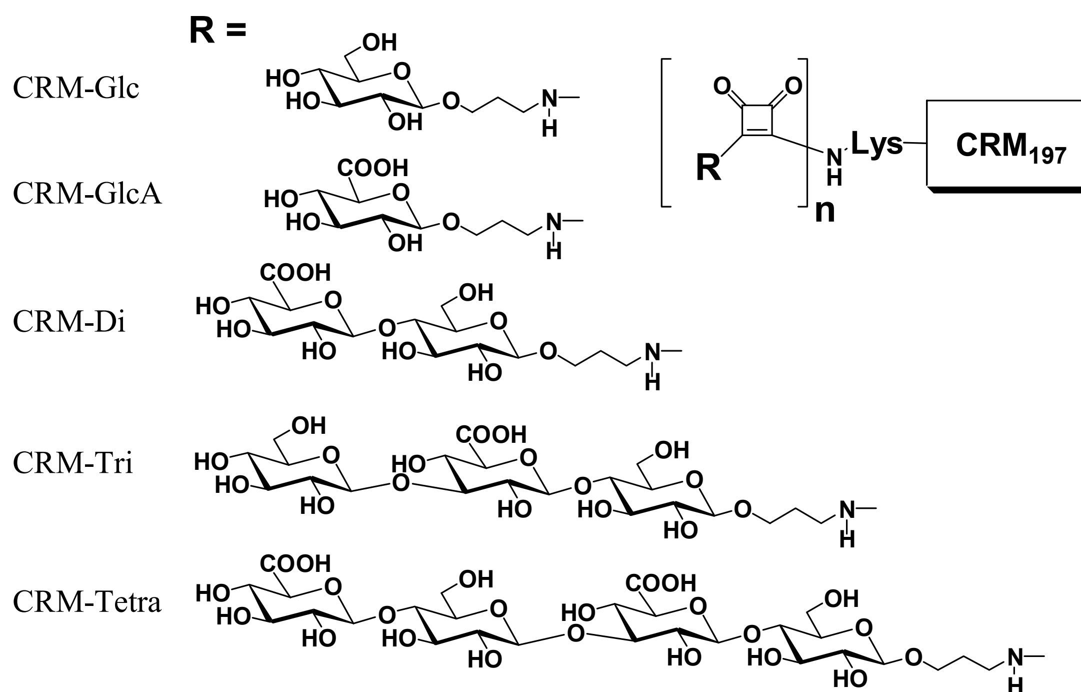

Figure 1. Schematic structure of the saccharide-CRM_{197} conjugates.
Presented are the spacer-containing monosaccharides glucose and glucuronic acid, and the di-, tri- and tetrasaccharide, representing fragments of the pneumococci type 3 polysaccharide conjugated to CRM_{197} using the squarate coupling method.

Table 1. Synthetic mono-, di-, tri-, and tetrasaccharide fragments of PS3 coupled to CRM_{197} with different carbohydrate-protein ratios.

Conjugate[a]	carbohydrate-protein ratios (μg/mg)	(mol/mol)
CRM-Glc	26	8.5
CRM-GlcA	22	6.6
CRM-Di 1	19	3.1
CRM-Di 2	41	6.7
CRM-Tri 1	44	4.9
CRM-Tri 2	61	6.8
CRM-Tri 3	107	12.0
CRM-Tetra 1	35	2.9
CRM-Tetra 2	80	6.7
CRM-Tetra 3	97	8.1

[a] After coupling and purification, conjugates were analyzed for protein content by the Pierce assay and for carbohydrate content by MALDI-TOF analysis.

For the protection study inbred female BALB/c mice were obtained from the Animal Laboratory of Utrecht University. The Ethics Committee on Animal Experimentation of University Medical Center-Utrecht approved the animal experiments described in this article. Mice of 8 to 10 weeks old were immunized with the conjugates subcutaneously at four sites (2.5 μg saccharide per mouse) and received a similar booster after 3 weeks. Control mice were injected with either CRM_{197} alone, the monosaccharides Glc and GlcA coupled to CRM_{197}, or the buffer used for preparation of the conjugates. Blood samples were taken 1 day before the booster and at week 2 and 5 after the booster. The mice were challenged intraperitoneally 2 weeks after the last blood sampling with a 20 LD_{50} (400 c.f.u.) dose of *S. pneumoniae* type 3 (ATTC 6303, Rockville, MD, USA). Survival of mice was recorded daily for 14 days after which blood was withdrawn for evaluation of the immune response after the infection.

Antibodies binding to polysaccharide 3 (PS 3) were measured by ELISA. Plates (Nunc Laboratories, Denmark) were coated with PS 3 (1 µg/mL in saline) overnight at 37°C. After blocking with phosphate buffer solution pH 7.4 (PBS)-3% gelatin, serum dilutions made in PBS, supplemented with 0.05% Tween 20 and 3% Protifar (Nutricia, Zoetermeer, The Netherlands), were transferred to the coated plates and incubated for 1 h at 37°C. After repeat washings the binding of IgM or IgG antibodies was determined with goat-anti- mouse IgM or IgG coupled to HRPO (Nordic Immunological Laboratories, CA, USA). The amount of bound peroxidase was visualized by incubating with a solution of 3,3',5,5'-tetramethylbenzidine (Sigma Chemicals Co, St. Louis, MO, USA) and H_2O_2. After 20 min, the reaction was stopped with 0.1 M H_2SO_4 and optical density was measured at 450 nm with a microplate reader (Bio-Rad, model 3550). Antibody titres were defined as the $\log_{10}$ of the dilution giving twice the absorbance value calculated against sera of control mice (immunized with the buffer), with a minimum value of 0.2.

Results

Groups of four mice were immunized subcutaneously with oligosaccharide-protein conjugates (2.5 µg carbohydrate per mouse). After the first immunization no IgM or IgG PS3 binding antibodies were detected (data not shown). IgG antibodies were present two weeks after the second immunization (data not shown) and titres did not change after another three weeks. As shown in Table 2 all mice immunized with the tri- and tetrasaccharide-CRM_{197} conjugates developed PS3 binding IgG antibodies. In each of the two disaccharide-CRM_{197} immunized groups there was one mouse in which antibodies were not detectable, even after the booster. The mice immunized with the tenfold lower dose of the tetrasaccharide-2 conjugate developed a slightly lower level of antibodies.

Upon intraperitoneal challenge with a lethal dose of *S. pneumoniae* type 3 all mice with PS3-specific antibodies survived (Table 2). All control mice died within four days, except two mice in the group injected with GlcA-CRM_{197}. Two mice in each of the disaccharide-CRM_{197} immunized groups, with no detectable antibodies, also died. There was no influence of the saccharide density on the immunogenic capacity of the conjugate vaccines.

Table 2 Type-specific antibody response and outcome of infection in individual mice.[a]

Conjugate	^{10}log IgG serum titers before infection in individual mice				survival time of individual mice in days			
CRM	<2	<2	<2	n.t.[b]	2	3	3	n.t.
CRM-Glc	<2	<2	<2	<2	3	3	3	3
CRM-GlcA	<2	<2	<2	<2	3	>14	>14	3
Buffer	<2	<2	<2	n.t.	3	3	3	n.t.
CRM-Di 1	4.0	4.3	<2	4.2	>14	>14	4	>14
CRM-Di 2	2.8	3.1	<2	3.2	>14	>14	2	>14
CRM-Tri 1	4.6	4.0	3.4	3.7	>14	>14	>14	>14
CRM-Tri 2	4.7	4.0	4.7	4.5	>14	>14	>14	>14
CRM-Tri 3	4.5	4.3	4.4	4.3	>14	>14	>14	>14
CRM-Tetra 1	4.5	4.6	4.1	4.3	>14	>14	>14	>14
CRM-Tetra 2	3.4	4.1	4.7	3.4	>14	>14	>14	>14
CRM-Tetra 2 (0.25 µg)	n.t.	<2.0	2.6	3.4	n.t.	>14	>14	>14
CRM-Tetra 3	4.5	4.4	3.9	3.8	>14	>14	>14	>14

[a] Groups of 4 female BALB/c mice were immunized subcutaneously with 2.5 µg saccharide as a protein conjugate. Control mice received CRM_{197} alone, CRM_{197} coupled to the monosaccharides Glc and GlcA or the buffer solution. One group received a tenfold lower dose of CRM-Tetra 2 (0.25 µg saccharide). A similar booster was given 3 weeks after the first injection. Blood samples were taken 2 weeks before the challenge and individual sera were tested for the presence of IgG antibodies recognizing PS3. An intraperitoneal challenge with a 20 LD_{50} dose of type 3 *Streptococcus pneumoniae* was given 7 weeks after the booster. Survival was recorded daily for 14 days. [b] n.t. = not tested

Discussion

In earlier studies, Snippe et al. [15] demonstrated that a hexasaccharide, coupled without spacer as a pentasaccharide to keyhole limpet haemocyanin (KLH), was able to induce protective immunity against type 3 pneumococci in mice. A low amount of IgM was measured one week after the first immunization and high levels of IgG were detected after booster injections. Furthermore, Jansen et al. [2] observed a protective immunity in mice after several injections with a synthetic

tetrasaccharide-KLH conjugate related to type 6B polysaccharide whereas the di- and trisaccharide-KLH conjugates induced a low antibody response with no or partial protection against a lethal dose of type 6B pneumococci. Rabbits, immunized with these conjugates developed antibodies, which could passively protect mice. Our results show that for type 3, the disaccharide-conjugate is already large enough to generate protection in mice without the use of adjuvant. The study of Laferrière et al. [5] demonstrated that conjugation of a different carbohydrate chain length (with an *average* length of 8, 16, 27 and 37 repeating units) derived from type 3 polysaccharide to tetanus toxoid (TT) did not result in a distinct immune response in rabbits. However, it should be noted that pools of average chain lengths were used, which complicates interpretation of the results. Rabbits immunized with those conjugates developed PS3 binding IgG antibodies but the sera showed a low opsonophagocytic capacity. This suggests the absence of protective antibodies since protection against pneumococci by type specific antibodies is mediated by opsonization and phagocytosis of the bacteria. In the previous study conjugates with carbohydrate chains of pneumococcal strains 6A, 18C, 19F and 23F induced in rabbits high levels of PS binding serum antibodies with an effective opsonophagocytic capacity. Only in the case of the 19F-TT conjugate a decrease in immunogenicity with increasing saccharide chain length was observed. It was also demonstrated by Paoletti et al. [10] that in rabbits immunized with on average 6, 14 and 25 repeating units of the polysaccharide of group B *Streptococcus* type III conjugated to TT an opsonophagocytic immune response could be evoked. These rabbit sera could passively protect mice against a lethal dose of type III group B *Streptococcus*, although full protection was seen only with the serum of 14-TT immunized rabbits. The study of Pozsgay et al. [12] on *Shigella dysenteriae* type 1 using precisely defined synthetic oligosaccharide-Human Serum Albumin (HSA) conjugates shows a different influence of the chain length with different oligosaccharide loading, stressing the importance of varying only one parameter. In our study we found a correlation between PS3 specific antibodies and protective capacity of all conjugates. The mechanism of the observed protection is not yet certain since the opsonophagocytic capacity of these sera is still under investigation. Surprisingly, two mice immunized with the GlcA-CRM_{197} conjugate did not develop detectable antibodies but were protected against a lethal dose of *S. pneumoniae*. Two weeks after the infection, PS3 binding IgG antibodies were present in serum taken from one of the protected mice (data not shown), indicating that the mouse was primed by injection of the GlcA conjugate. This suggests that GlcA is the immunodominant monosaccharide. A similar result with a disaccharide conjugate was obtained by Wood and Kabat [18]. A low immune response to dextran was observed in one out of four rabbits immunized

with an isomaltose-stearylamine conjugate (stearyl-IM2). The reducing monosaccharide was, however, lost during reductive amination, thus the terminal monosaccharide glucose should be considered as the immunogenic moiety.

In our study, a change in carbohydrate-protein ratio of the conjugate vaccines did not influence their immunogenic capacity. The study of Laferrière et al. [5] with polysaccharide type 3 derived oligosaccharide-TT conjugates showed no relation between the number of polysaccharide chains incorporated and the immunogenicity of the conjugates. Investigations of Pozsgay et al. [12] with synthetic tetra-, octa-, dodeca- and hexadecasaccharide fragments of the lipopolysaccharide (LPS) of *Shigella dysenteriae* type 1 coupled to HSA showed high levels of LPS binding IgG antibodies in mice after three injections except for the tetrasaccharide conjugate which showed a low immunogenicity and was not further evaluated. The influence of the carbohydrate-protein ratio was different for the three remaining conjugates. The octasaccharide-HSA conjugate with the highest density evoked a good immune response, while the dodeca- and hexadecasaccharide conjugates with the median density showed to be the optimal immunogen. This is explained by the authors by the 'covering' effect of the protein carrier by the saccharide chains, which is related to the size of the saccharide and influences the availability of antigenic peptides to associate with MHC class II molecules on B-cells and thus the stimulation of T-cells. This could explain our results with short synthetic saccharides that showed no differences in immunogenicity with different carbohydrate-protein ratios, although, in the present study a different carrier protein was used.

Studies performed with different oligosaccharide conjugate vaccines show that they are good immunogenic, but the immunogenicity of these conjugates is not clearly determined by chain length and saccharide density.

This indicates that the immunogenicity of saccharide conjugates depends on the immunological properties of the saccharide and protein used. Furthermore, oligosaccharides derived by acid hydrolysis of polysaccharides are mixtures of oligosaccharides, differing in reducing and non-reducing ends. Also the coupling methodology and the structure of the reducing monosaccharide after reduction can influence the immunogenic properties of the conjugate. Saccharides synthesized and coupled to the carrier protein in a well-controlled process, thereby varying only one parameter, like used in our study, makes it possible to study the influence of two parameters, chain length and density on the immunogenicity of conjugate vaccines.

Important questions concerning the observed immune response remain and will be subject for further study with regard to the nature of the protective immune response we measured, such as opsonophagocytic capacity, subclass distribution

and change in antigenic specificity, like the immune response against proteins and other pneumococcal components in sera taken before and after challenge with viable *S. pneumoniae.*

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4

Synthesis of a hexasaccharide fragment of the capsular polysaccharide of *Streptococcus pneumoniae* type 3

Dirk J. Lefeber, Eneko Aldaba Arévalo, Johannis P. Kamerling, and Johannes F.G. Vliegenthart

Abstract

In the framework of the development of a new generation of neoglycoconjugate vaccines against *Streptococcus pneumoniae*, the synthesis is described of a spacer-containing hexasaccharide fragment related to the capsular polysaccharide of *Streptococcus pneumoniae* type 3. β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$ (**1**), comprising three repeating units, was synthesised *via* a blockwise strategy employing suitably protected disaccharide building blocks. Carboxylic groups were introduced by selective oxidation with TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) in the last reaction steps. Deprotection afforded target hexasaccharide **1**.

Introduction

Diseases caused by encapsulated bacteria can be prevented by vaccination with neoglycoconjugate vaccines as proven for *Haemophilus influenzae* type b [1], *Neisseria meningitidis* type C [2] and *Streptococcus pneumoniae* serotypes [3]. These vaccines are prepared by conjugation of isolated polysaccharide or a mixture of polysaccharide-derived oligosaccharides to a protein carrier. For a detailed investigation of the immune response of this type of vaccines, neoglycoproteins with a well-defined carbohydrate structure need to be available.
Neoglycoconjugates related to *S. pneumoniae* type 3 have been prepared by coupling di-, tri- or tetrasaccharide fragments in several carbohydrate-protein ratios to a non-toxic mutant of diphtheria toxin (CRM_{197}) [4]. Whereas immunisation experiments with these conjugates in mice showed that increasing IgG antibody titres were found with increasing oligosaccharide chain length, no difference was found with respect to the oligosaccharide loading [5].
The use of conjugates that contain larger oligosaccharide fragments than in the study described above might lead to an improved immunogenicity. Furthermore, human antibodies can recognise larger epitopes on the polysaccharide than do animal antibodies, as shown for neoglycoproteins consisting of synthetic oligosaccharides related to the capsular polysaccharide of *S. pneumoniae* type 23F [6]. For these reasons, a program was started for the synthesis of a *S. pneumoniae* type 3 related hexasaccharide fragment (**1**), comprising three cellobiuronic acid repeating units (Figure 1).

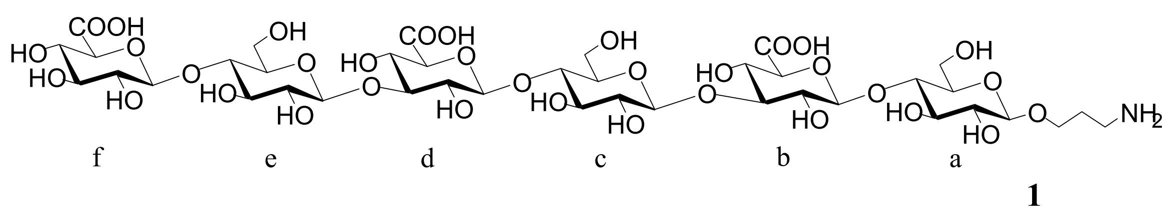
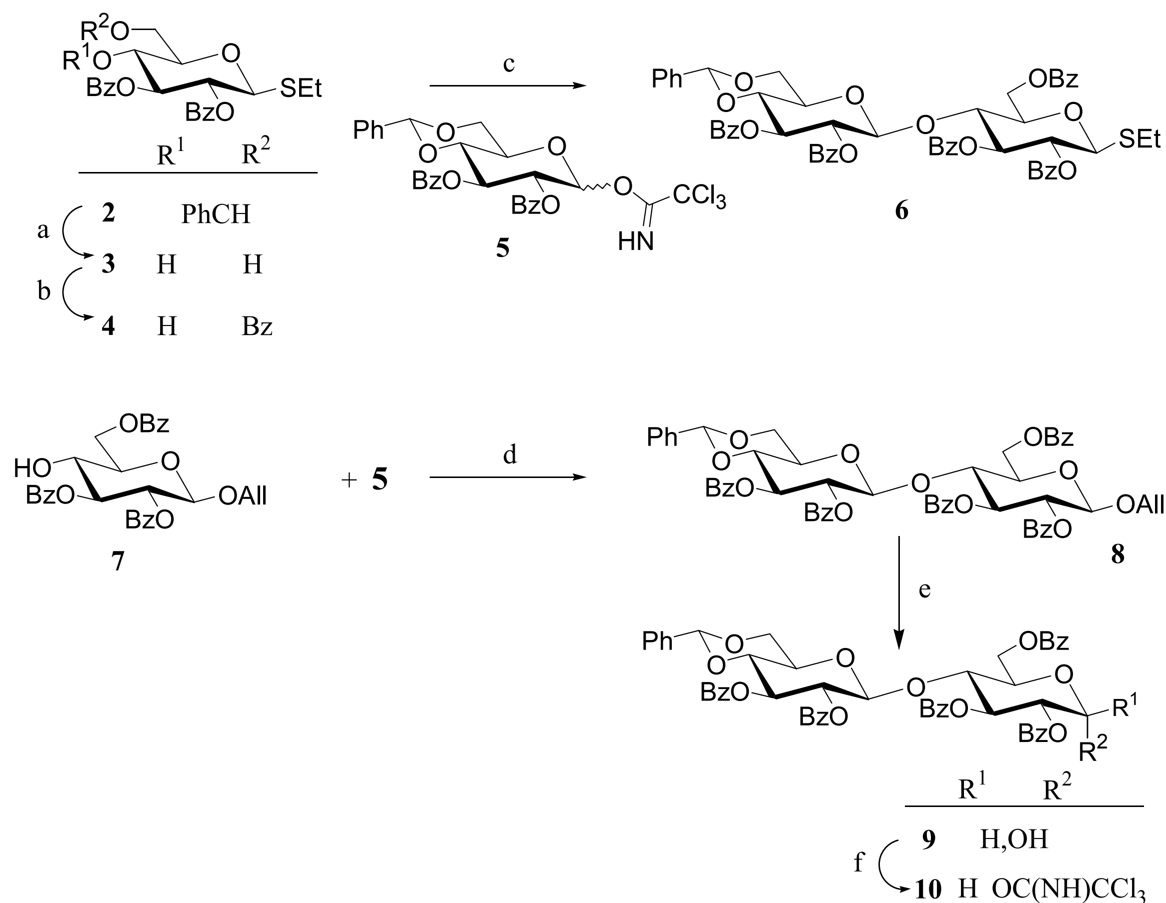


Figure 1. Target hexasaccharide **1** comprising three repeating units of the capsular polysaccharide of *Streptococcus pneumoniae* type 3.

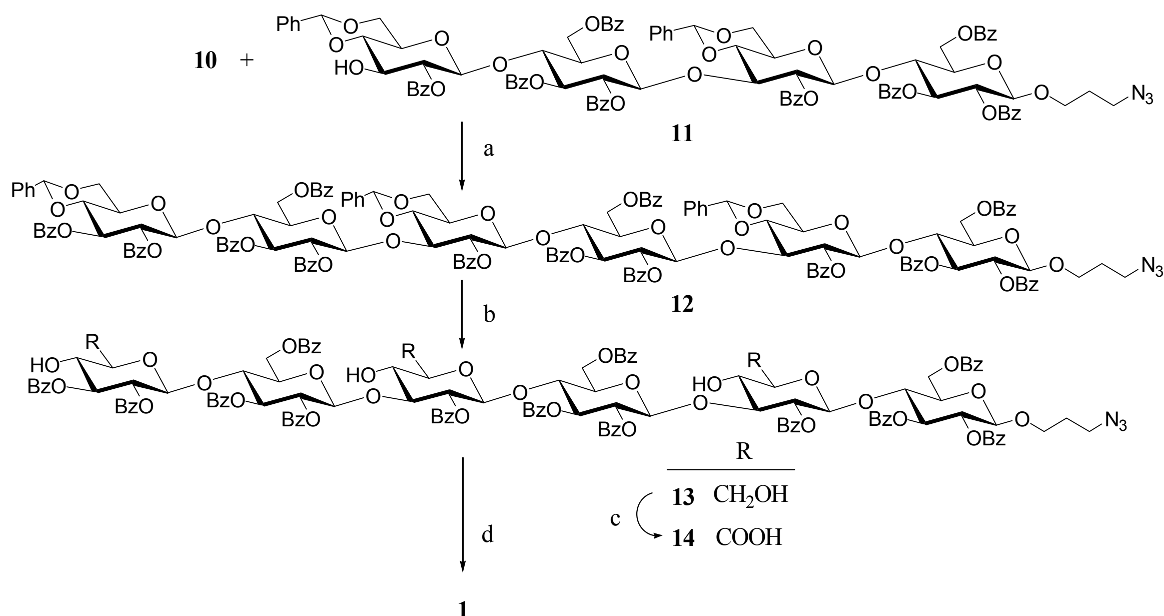
Results and discussion

Previously, the synthesis was described of tetrasaccharide acceptor **11** [4]. Two disaccharide donors (**6** and **10**, Scheme 1) were tested for the coupling with **11** to afford protected hexasaccharide **12** (Scheme 2).



Scheme 1. Synthesis of disaccharide donors **6** and **10**. Reagents and conditions: a) CF_3COOH, H_2O, CH_2Cl_2, 88%; b) PhCOCl, Et_3N, CH_2Cl_2, 50%; c) 10% TMSOTf, CH_2Cl_2, 75%; d) 11% TMSOTf, CH_2Cl_2, 83%; e) *i* $(PPh_3)_3Rh(I)Cl$, DABCO, $CH_3C_6H_5$/EtOH/CH_2Cl_2 10:5:1, *ii* NIS, H_2O, THF, 68%; f) Cl_3CCN, DBU, CH_2Cl_2, 86%.

For the synthesis of disaccharide thioethyl donor **6**, first monosaccharide acceptor **4** was prepared by debenzylidenation of **2** [7] using trifluoroacetic acid (→**3**, 88%), and subsequent selective benzoylation using benzoyl chloride (→**4**, 50%). Coupling of **4** with imidate donor **5** [4] using 10% TMSOTf as a promoter gave **6** in a yield of 75%. For the synthesis of disaccharide imidate donor **10**, first **8** was prepared in 83% yield by coupling of acceptor **7** [4] and donor **5** with 11% TMSOTf as a promoter. Then, deallylation of **8** using tris(triphenylphosphine)rhodium(I) chloride and *N*-iodosuccinimide (→ **9**, 68%) and subsequent trichloroacetimidation gave **10** (86%).



Scheme 2. Synthesis of hexasaccharide **1**. Reagents and conditions: a) 10% TMSOTf, CH_2Cl_2, 63%; b) CF_3COOH, H_2O, CH_2Cl_2, 75%; c) TEMPO, aqueous NaOCl, KBr, Bu_4NBr, aqueous NaCl, aqueous $NaHCO_3$, CH_2Cl_2, 70%; d) *i* NaOMe, MeOH (pH 10), *ii* $NaBH_4$, 10% Pd/C, 0.05 M NaOH, 61%.

For the preparation of protected hexasaccharide **12** (Scheme 2), tetrasaccharide acceptor **11** [4] was coupled with donor **6** using *N*-iodosuccinimide and triflic acid as a promoter system. Low yields of product (< 8%) were obtained when the reaction was performed at 0 °C or lower temperatures. Hexasaccharide **12** could, however, be obtained in a yield of 63% by coupling of **11** with disaccharide imidate donor **10** using 10% TMSOTf as a promoter at room temperature. After debenzylidenation of **12** using trifluoroacetic acid (→**13**, 75%), the free primary hydroxyl functions were oxidised. Treatment of **13** with TEMPO and sodium hypochlorite [8] was found to be efficient for the oxidation of the three primary hydroxyl groups in the presence of secondary functions, and **14** was obtained in 70% yield. ^{1}H NMR analysis after methylation with diazomethane showed three singlets at δ = 3.39, 3.30 and 3.23 (COOCH_3). Debenzoylation of **14** with sodium methoxide and reduction of the azide using sodium borohydride and 10% Pd/C afforded target hexasaccharide **1** in 61% yield. The identity of the compound was established by ^{1}H NMR spectroscopy (Table 1) and mass spectrometry.

Table 1. 500 MHz ^{1}H NMR data of **1** at 305K (in ppm)[a]. $J_{1,2}$ couplings are presented in parentheses.

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Glc[a]	4.50 (7.8)	3.33	3.64	3.61	n.d.[b]	3.98	3.80
GlcA[b]	4.53 (8.1)	3.56	3.79	n.d	n.d		
Glc[c]	4.82 (8.0)	3.38	3.65	3.62	n.d	3.98	3.80
GlcA[d]	4.53 (8.1)	3.56	3.79	n.d	n.d		
Glc[e]	4.82 (8.0)	3.38	3.65	3.62	n.d	3.98	3.80
GlcA[f]	4.50 (7.8)	3.36	3.51	3.51	3.75		

[a] The signals for the 3-aminopropyl spacer are: δ = 4.05 and 3.83 ($OCH_2CH_2CH_2NH_2$), 3.16 ($OCH_2CH_2CH_2NH_2$), 2.01 ($OCH_2CH_2CH_2NH_2$). [b] n.d. = not determined.

In conclusion, a versatile synthetic route was developed using a blockwise strategy for the preparation of hexasaccharide fragment **1**, representing three repeating units of the capsular polysaccharide of *S. pneumoniae* type 3. The carboxylic acids were efficiently introduced by selective oxidation using TEMPO.

Acknowledgements

This work was supported by the European Union program VACNET (grant ERB BIO 4CT960158).

Experimental section

General: All chemicals were of reagent grade, and were used without any further purification. Reactions were monitored by TLC on Silica gel 60 F_{254} (Merck); compounds were visualised, after examination under UV light, by heating with 10% (v/v) ethanolic H_2SO_4, orcinol (2 mg mL^{-1}) in 20% (v/v) methanolic H_2SO_4, or ninhydrin (1.5 mg mL^{-1}) in BuOH/H_2O/HOAc (38:1.75:0.25). In the work-up procedures of reaction mixtures, organic solutions were washed with appropriate amounts of the indicated aqueous solutions, then dried ($MgSO_4$), and concentrated under reduced pressure at 20-40 °C on a water-bath. Column chromatography was performed on Silica gel 60 (Merck, 0.063-0.200

mm). Optical rotations were measured in $CHCl_3$, unless stated otherwise, with a Perkin-Elmer 241 polarimeter, using a 10-cm l-mL cell. ^{1}H NMR spectra in $CDCl_3$ were recorded at 27 °C with a Bruker AC 300 spectrometer; the δ_H values are given in ppm relative to the signal for internal Me_4Si (δ = 0). ^{13}C (APT, 75 MHz) NMR spectra in $CDCl_3$ were recorded at 27 °C with a Bruker AC 300 spectrometer; indicated ppm values for δ_C are relative to the signal of $CDCl_3$ (δ = 76.9). The ^{1}H NMR spectrum of **1** was recorded in D_2O at 32 °C with a Bruker AMX 500 spectrometer, and the δ_H values are given in ppm relative to the signal for internal acetone (δ = 2.225). Two-dimensional TOCSY and ROESY spectra were recorded using a Bruker AMX 500 apparatus (500 MHz) to assign the spectra of compounds **1**, **12**, **13**, and **14**. Residues a-f (Figure 1) were assigned on the basis of interglycosidic ROESY cross-peaks. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) spectra were obtained on a Voyager-DE™ mass spectrometer using 2,4-dihydroxybenzoic acid (DHB) in H_2O as a matrix. Elemental analyses were carried out by H. Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany).

Ethyl 2,3-di-*O*-benzoyl-1-thio-β-D-glucopyranoside (3): To a solution of ethyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside **2** [7] (1.37 g, 2.63 mmol) in CH_2Cl_2 (36 mL) were added CF_3COOH (1.5 mL) and H_2O (0.2 mL). The mixture was stirred for 16 h, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHCO_3$ until neutral pH and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (CH_2Cl_2/MeOH 98:2) to obtain **3** (1.0 g, 88%). R_f 0.05 (CH_2Cl_2/EtOAc 9:1); $[\alpha]_D^{20}$= +76 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 7.97-7.93 and 7.53-7.34 (2 m, 10 H; 2 *Ph*CO), 4.74 (d, $J_{1,2}$ = 9.8 Hz, 1 H; H-1), 2.76-2.73 (m, 2 H; S$CH_2$$CH_3$), 1.26 (t, 3 H; SCH_2CH_3); ^{13}C NMR ($CDCl_3$): δ = 167.4 and 165.2 (2 Ph*C*O), 129.1 and 128.7 (2 *Ph*CO, quaternary C), 83.6 (C-1), 79.9, 78.2, 70.1, and 69.9 (C-2,3,4,5), 62.3 (C-6), 24.3 (SCH_2CH_3), 14.8 (SCH_2CH_3); elemental analysis calcd (%) for $C_{22}H_{24}O_7S$ (432.4): C 61.11, H 5.59; found C 60.99, H 5.74.

Ethyl 2,3,6-tri-*O*-benzoyl-1-thio-β-D-glucopyranoside (4): To a solution of **3** (0.95 g, 2.2 mmol) in CH_2Cl_2 (50 mL) and Et_3N (0.37 mL) at 0 °C was added dropwise a solution of benzoyl chloride (0.27 mL, 2.3 mmol) in CH_2Cl_2 (10 mL). After stirring for 24 h, the mixture was diluted with CH_2Cl_2, washed with 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography (CH_2Cl_2/EtOAc 95:5) to give **4** (0.6 g, 50%). R_f 0.51 (CH_2Cl_2/EtOAc 9:1); $[\alpha]_D^{20}$= +60 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.05-7.91 and 7.46-7.28 (2 m, 15 H; 3 *Ph*CO), 5.60 and 5.47 (2 t, $J_{2,3}$, $J_{3,4}$ = 9.5, 9.7 Hz, each 1 H; H-2,3), 4.82 (d, $J_{1,2}$ = 9.9 Hz, 1 H; H-1), 2.82-2.64 (m, 2 H; SCH_2CH_3), 1.24 (t, 3 H; SCH_2CH_3); ^{13}C NMR ($CDCl_3$): δ = 166.8, 166.7, and 165.2 (3 Ph*C*O), 83.4 (C-1), 78.2, 77.2, 70.2, and 69.3 (C-2,3,4,5), 63.6 (C-6), 24.1 (SCH_2CH_3), 14.8 (SCH_2CH_3); elemental analysis calcd (%) for $C_{29}H_{28}O_8S$ (536.5): C 64.92, H 5.26; found C 65.05, H 5.31.

Ethyl (2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-1-thio-β-D-glucopyranoside (6): A solution of **4** (50 mg, 0.09 mmol) and 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-D-glucopyranosyl trichloroacetimidate **5** [4] (87 mg, 0.14 mmol) in dry CH_2Cl_2 (2 mL), containing 4 Å molecular sieves, was stirred under Ar for 1 h. Then, TMSOTf (2.5 μL, 14 μmol) was added. After 30 min, the mixture was neutralised with dry pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **6** (67 mg, 75%). R_f 0.76 (CH_2Cl_2/EtOAc 9:1); $[\alpha]_D^{20}$= +41 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.05-7.85 and 7.49-7.31 (2 m, 30 H; *Ph*CH, 5 *Ph*CO), 5.74, 5.61, and 5.42 (3 t, $J_{2,3}$, $J_{3,4}$, $J_{3',4'}$ = 9.3, 9.6, 9.8 Hz, each 1 H; H-2,3,3'), 5.44 (dd, $J_{1',2'}$ = 7.7, $J_{2',3'}$ = 9.5 Hz, 1 H; H-2'), 5.20 (s, 1 H; PhC*H*), 4.83 (d, 1 H; H-1'), 4.68 (d, $J_{1,2}$ = 9.9 Hz, 1 H; H-1), 4.49 (dd, $J_{5,6a}$ = 2.1, $J_{6a,6b}$ = 12.2 Hz, 1 H; H-6a), 4.40 (dd, $J_{5,6b}$ = 4.8 Hz, 1 H; H-6b), 3.77 (ddd, 1 H; H-5), 3.61 (dd, $J_{5',6a'}$ = 4.9, $J_{6a',6b'}$ = 10.6 Hz, 1 H; H-6a'), 3.32 (ddd, $J_{5',6b'}$ = 10.4 Hz, 1 H; H-5'), 2.82 (dd, 1 H; H-6b'), 2.70-2.56 (m, 2 H; SCH_2CH_3), 1.16 (t, 3 H; SCH_2CH_3); ^{13}C NMR ($CDCl_3$): δ = 136.4 (*Ph*CH, quaternary C), 101.7 and 101.1 (Ph*C*H, C-1'), 83.3 (C-1), 67.5 (C-6'), 62.5 (C-6), 24.2 (SCH_2CH_3), 14.8 (SCH_2CH_3); elemental analysis calcd (%) for $C_{56}H_{50}O_{15}S$ (995.0): C 67.59, H 5.06; found C 67.56, H 5.01.

Allyl (2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (8): A solution of allyl 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside **7** [4] (0.48 g, 0.93 mmol) and 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-D-glucopyranosyl trichloroacetimidate **5** [4] (1.02 g, 1.64 mmol) in dry CH_2Cl_2 (15 mL), containing 4 Å molecular sieves, was stirred under Ar for 1 h. Then, TMSOTf (35 μL, 0.19 mmol) was added. After 45 min, the mixture was neutralised with dry pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **8** (0.76 g, 83%). R_f 0.37 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +27 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 8.05-7.85 and 7.50-7.25 (2 m, 30 H; *Ph*CH, 5 *Ph*CO), 5.78-5.65 (m, 1 H; $OCH_2CH{=}CH_2$), 5.72 and 5.60 (2 t, $J_{2,3}$ = $J_{2',3'}$ = 9.6, $J_{3,4}$ = $J_{3',4'}$ = 9.5 Hz, each 1 H; H-3,3'), 5.43 and 5.40 (2 dd, $J_{1,2}$, $J_{1',2'}$ = 7.7, 7.8 Hz, each 1 H; H-2,2'), 5.21 (s, 1 H; PhC*H*), 5.17-5.10 and 5.08-5.03 (2 m, each 1 H; $OCH_2CH{=}CH_2$), 4.83 and 4.72 (2 d, each 1 H; H-1,1'), 4.49 (dd, $J_{5,6a}$ = 2.1, $J_{6a,6b}$ = 12.1 Hz, 1 H; H-6a), 4.39 (dd, $J_{5,6b}$ = 4.5 Hz, 1 H; H-6b), 4.27-4.20 and 4.07-3.99 (2 m, each 1 H; $OCH_2CH{=}CH_2$), 4.11 and 3.63 (2 t, $J_{4,5}$, $J_{4',5'}$ = 9.2, 9.5 Hz, each 1 H; H-4,4'), 3.76 (ddd, 1 H; H-5), 3.61 (dd, $J_{5',6a'}$ = 4.6, $J_{6a',6b'}$ = 10.6 Hz, 1 H; H-6a'), 3.31 (ddd, $J_{5',6b'}$ = 10.6 Hz, 1 H; H-5'), 2.83 (t, 1 H; H-6b'); ^{13}C NMR ($CDCl_3$): δ = 165.4, 165.2, 165.0, 164.9, and 164.7 (5 Ph*C*O), 136.4 (P*h*CH, quaternary C), 117.5 ($OCH_2CH{=}CH_2$), 101.7, 101.0, and 99.2 (Ph*C*H, C-1,1'), 69.7 ($OCH_2CH{=}CH_2$), 67.4 (C-6'), 62.2 (C-6). Elemental analysis calcd (%) for $C_{57}H_{50}O_{16}$ (991.0): C 69.08, H 5.08; found C 69.16, H 5.05.

(2,3-Di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-D-glucopyranose (9): To a solution of **8** (1.5 g, 1.5 mmol) in absolute EtOH (40 mL), toluene (80 mL), and CH_2Cl_2 (8 mL) were added a catalytic amount of diazabicyclo[2.2.2]octane (DABCO) and tris(triphenylphosphine)rhodium(I) chloride (0.4 g). After stirring at boiling under reflux for 4 h, the mixture was concentrated. The residue was dissolved in THF (75 mL), and water (10 mL) and NIS (0.6 g) were added. After 20 min, the mixture was concentrated, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHSO_3$ (2 x) and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude residue was purified by column chromatography. Impurities were eluted with toluene/EtOAc (95:5), and **9** was obtained as a light brown syrup by elution with toluene/EtOAc (9:1) (0.99 g, 68%). R_f $0.17^{\alpha}/0.08^{\beta}$ (toluene/EtOAc 8:2); ^{1}H NMR ($CDCl_3$): δ = 8.08-7.85 and 7.49-7.23 (2 m, 30 H; *Ph*CH, 5 *Ph*CO), 5.63 (t, $J_{3',4'}$ = 9.6 Hz, 1 H; H-3'), 5.46 (dd, $J_{1',2'}$ = 7.7, $J_{2',3'}$ = 9.5 Hz, 1 H; H-2'), 5.23 (s, 1 H; PhC*H*), 4.89 (d, 1 H; H-1'), 4.49 (dd, $J_{5,6a}$ = 2.0, $J_{6a,6b}$ = 12.3 Hz, 1 H; H-6a), 4.40 (dd, $J_{5,6b}$ = 3.8 Hz, 1 H; H-6b), 3.63 (dd, $J_{5',6a'}$ = 4.9, $J_{6a',6b'}$ = 10.6 Hz, 1 H; H-6a'), 3.32 (dt, $J_{5',6b'}$ = 10.4 Hz, 1 H; H-5'), 2.94 (t, 1 H; H-6b'); ^{13}C NMR ($CDCl_3$): δ = 165.8, 165.4, 165.0 (2 C), and 164.9 (5 Ph*C*O), 136.5 (*Ph*CH, quaternary C), 101.9 and 101.1 (Ph*C*H, C-1'), 95.5 and 90.1 (C-1^{α},1^{β}), 67.7 (C-6'), 62.0 (C-6); elemental analysis calcd (%) for $C_{54}H_{46}O_{16}$ (951.0): C 68.21, H 4.87; found C 68.16, H 5.02.

(2,3-Di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate (10): To a solution of **9** (1.0 g, 1.1 mmol) in dry CH_2Cl_2 (14 mL) were added Cl_3CCN (1.2 mL) and DBU (40 μL). After stirring for 16 h, the mixture was concentrated and the residue was purified by column chromatography (toluene/EtOAc 88:12) to yield **10** (0.99 g, 86%). R_f 0.32 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +83 (c = 1); ^{1}H NMR ($CDCl_3$): δ = 8.55 (s, 1 H; N*H*), 8.08-7.85 and 7.47-7.16 (2 m, 30 H; *Ph*CH, 5 *Ph*CO), 6.68 (d, $J_{1,2}$ = 3.7 Hz, 1 H; H-1), 6.12 (dd, $J_{2,3}$ = 10.2, $J_{3,4}$ = 8.6 Hz, 1 H; H-3), 5.66 (t, $J_{2',3'}$ = $J_{3',4'}$ = 9.6 Hz, 1 H; H-3'), 5.49 (dd, 1 H; H-2), 5.48 (dd, $J_{1',2'}$ = 7.7 Hz, 1 H; H-2'), 5.25 (s, 1 H; PhC*H*), 4.95 (d, 1 H; H-1'), 4.54 (dd, $J_{5,6a}$ = 2.0, $J_{6a,6b}$ = 12.2 Hz, 1 H; H-6a), 4.44 (dd, $J_{5,6b}$ = 3.8 Hz, 1 H; H-6b), 4.30 (ddd, $J_{4,5}$ = 10.0 Hz, 1 H; H-5), 4.22 (dd, 1 H; H-4), 3.69 (t, $J_{4',5'}$ = 9.5 Hz, 1 H; H-4'), 3.64 (dd, $J_{5',6a'}$ = 4.8, $J_{6a',6b'}$ = 10.5 Hz, 1 H; H-6a'), 3.34 (dt, $J_{5',6b'}$ = 10.3 Hz, 1 H; H-5'), 2.99 (t, 1 H; H-6b'); ^{13}C NMR ($CDCl_3$): δ = 165.4, 165.3 (2 C), and 164.8 (2 C) (5 Ph*C*O), 160.3 (O*C*(NH)CCl_3), 136.4 (*Ph*CH, quaternary C), 101.8 and 101.1 (Ph*C*H, C-1'), 92.8 (C-1), 67.6 (C-6'), 61.6 (C-6).

3-Azidopropyl (2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl) - (1→4) - (2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl) - (1→4) - (2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl) - (1→4) - 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (12): A solution of **10** (70 mg, 63 μmol) and **11** [4] (65 mg, 35 μmol) in dry CH_2Cl_2 (1 mL), containing 4 Å molecular sieves, was stirred under Ar for 1 h. Then, TMSOTf (1.1 μL, 6 μmol) was added. After 30 min, the mixture was neutralised with dry

pyridine, filtered over cotton, diluted with CH_2Cl_2, and washed with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 95:5) to obtain **12** (59 mg, 63%). R_f 0.46 (toluene/EtOAc 9:1); $[\alpha]_D^{20}$= +59 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 5.56 (t, 1 H; H-3^a), 5.45 (t, 1 H; H-3^f), 5.38 (t, 1 H; H-3^e), 5.29 (t, 1 H; H-3^c), 5.26 (H-2^a,2^f), 5.16 (H-2^e), 5.15 (H-2^b), 5.14 (H-2^c), 5.12 (2 H) and 5.09 (2 s, 3 PhC*H*), 5.06 (H-2^d), 4.67 (d, $J_{1,2}$ = 7.8 Hz, 1 H; H-1^e), 4.64 (d, $J_{1,2}$ = 7.8 Hz, 1 H; H-1^c), 4.59 (d, $J_{1,2}$ = 7.8 Hz, 1 H; H-1^f), 4.54 (d, $J_{1,2}$ = 7.8 Hz, 1 H; H-1^a), 4.50 (d, $J_{1,2}$ = 7.8 Hz, 1 H; H-1^b), 4.34 (d, $J_{1,2}$ = 7.8 Hz, 1 H; H-1^d), 3.97 (t, 1 H; H-4^e), 3.90 (t, 2 H; H-4^a,3^b), 3.85 (t, 1 H; H-3^d), 3.84 (t, 1 H; H-4^c), 3.77 and 3.45 ($OCH_2CH_2CH_2N_3$, with first CH_2 italic), 3.55 (H-5^a), 3.25 (H-5^e), 3.21 (H-5^c), 3.12 ($OCH_2CH_2\mathit{CH_2}N_3$), 3.05 (H-5^b), 2.93 (H-5^d), 2.66 (H-6^f), 2.65 (H-6^b), 2.53 (H-6^d), 1.72-1.57 (m, 2 H; $OCH_2\mathit{CH_2}CH_2N_3$); ^{13}C NMR ($CDCl_3$): δ = 136.4 (*Ph*CH, quaternary C), 101.4, 101.3 (2 C), 101.1, 100.9 (2 C), 100.6, 99.8, and 99.7 (3 Ph*C*H, C-1^a,1^b,1^c,1^d,1^e,1^f), 66.2 ($O\mathit{C}H_2CH_2CH_2N_3$), 47.6 ($OCH_2CH_2\mathit{C}H_2N_3$), 28.7 ($OCH_2\mathit{C}H_2CH_2N_3$); MS (MALDI-TOF): *m/z* 2713 $[M+Na]^+$.

3-Azidopropyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyl) - (1→4) - (2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-β-D-glucopyranosyl) - (1→4) - (2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-β-D-glucopyranosyl) - (1→4) - 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (13): To a solution of **12** (84 mg, 31 μmol) in CH_2Cl_2 (0.85 mL) were added CF_3COOH (75 μL) and H_2O (75 μL). The mixture was stirred for 4 h, diluted with CH_2Cl_2, washed with 10% (w/v) aqueous $NaHCO_3$ until neutral pH and 10% (w/v) aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene/EtOAc 6:4→4:6) to obtain **13** (55 mg, 75%). R_f 0.21 (toluene/EtOAc 1:1); $[\alpha]_D^{20}$= +45 (*c* = 1); ^{1}H NMR ($CDCl_3$): δ = 5.57 (t, 1 H; H-3^e), 5.50 (t, 1 H; H-3^a), 5.39 (t, 1 H; H-3^c), 5.33 (dd, 1 H; H-2^f), 5.31 (dd, 1 H; H-2^a), 5.30 (dd, 1 H; H-2^e), 5.25 (t, 1 H; H-3^f), 5.23 (dd, 1 H; H-2^c), 5.03 (bt, 2 H; H-2^b,2^d), 4.76 (d, $J_{1,2}$ = 7.7 Hz, 1 H; H-1^f), 4.63 (2 H; H-1^e,6^e), 4.53 (d, 1 H; H-1^a), 4.52 (d, 1 H; H-1^c), 4.43 (d, $J_{1,2}$ = 8.0 Hz, 1 H; H-1^b), 4.42 (d, $J_{1,2}$ = 8.0 Hz, 1 H; H-1^d), 4.31 (3 H; H-6a^a,6a^c,6^e), 4.18 (dd, 1 H; H-6b^a), 4.14 (dd, 1 H; H-6b^c), 3.99 (t, 1 H; H-4^e), 3.98 (t, 1 H; H-4^a), 3.83 (t, 1 H; H-4^c), 3.82 (H-5^e), 3.78 and 3.45 ($O\mathit{CH_2}CH_2CH_2N_3$), 3.73 (H-4^f), 3.58 (t, 1 H; H-3^d), 3.56 (H-5^c), 3.54 (H-5^a), 3.51 (t, 1 H; H-3^b), 3.36 (H-4^d), 3.32 (H-4^b), 3.23 (H-5^f), 3.15-3.09 (m, 2 H; $OCH_2CH_2\mathit{CH_2}N_3$), 3.00 (H-5^d), 2.96 (H-5^b), 1.72-1.55 (m, 2 H; $OCH_2\mathit{CH_2}CH_2N_3$); ^{13}C NMR ($CDCl_3$): δ = 101.1 (2 C), 100.6 (3 C), and 100.5 (C-1^a,1^b,1^c,1^d,1^e,1^f), 66.2 ($O\mathit{C}H_2CH_2CH_2N_3$), 62.2, 62.1, 62.0 (2 C), 61.8, and 61.3 (C-6^a,6^b,6^c,6^d,6^e,6^f), 47.6 ($OCH_2CH_2\mathit{C}H_2N_3$), 28.7 ($OCH_2\mathit{C}H_2CH_2N_3$); MS (MALDI-TOF): *m/z* 2440 $[M+Na]^+$.

3-Azidopropyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyluronic acid) - (1→4)-(2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-β-D-glucopyranosyluronic acid) - (1→4) - (2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl) - (1→3) - (2-*O*-benzoyl-β-D-glucopyranosyluronic acid) - (1→4) - 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (14):

To a solution of **13** (50 mg, 21 μmol) in CH_2Cl_2 (0.22 mL) were added TEMPO (catalytic amount), and 40 μL of a solution of KBr (2.4 mg) and Bu_4NBr (3.2 mg) in saturated aqueous $NaHCO_3$ (0.4 mL). The mixture was stirred vigorously at 0 °C, when 122 μL of a solution of saturated aqueous NaCl (0.44 mL), saturated aqueous $NaHCO_3$ (0.22 mL), and aqueous NaOCl (13% Cl active; 0.56 mL) was added dropwise. After 45 min, the mixture was acidified with 4 M HCl (pH 4), diluted with CH_2Cl_2, and the organic layer was washed with 10% (w/v) aqueous NaCl, dried, filtered, and concentrated. The residue was purified by column chromatography. Impurities were eluted with CH_2Cl_2/acetone (8:2), and **14** (35 mg, 70%) was eluted with CH_2Cl_2/acetone/HOAc (8:2:0.5). R_f 0.05 (CH_2Cl_2/acetone/HOAc 8:2:1); MS (MALDI-TOF): *m/z* 2490 $[M+Na]^+$, 2512 $[M-H+2Na]^+$, 2534 $[M-2H+3Na]^+$, 2556 $[M-3H+4Na]^+$.
A small amount was methylated with diazomethane in methanol for analysis. ^1H NMR ($CDCl_3$): δ = 5.55 (t, 1 H; H-3^e), 5.54 (t, 1 H; H-3^a), 5.39 (t, 1 H; H-3^c), 5.36 (2 H; H-2^f,3^f), 5.26 (dd, $J_{1,2}$ = 7.8, $J_{2,3}$ = 9.6 Hz, 1 H; H-2^e), 5.20 (dd, $J_{1,2}$ = 7.8, $J_{2,3}$ = 9.5 Hz, 1 H; H-2^a), 5.15 (dd, $J_{2,3}$ = 9.7 Hz, 1 H; H-2^c), 5.08 (2 t, each 1 H; H-2^b,2^d), 4.83 (d, $J_{1,2}$ = 7.6 Hz, 1 H; H-1^f), 4.65 (d, 1 H; H-1^e), 4.61 (dd, 1H; H-6^e), 4.53 (d, $J_{1,2}$ = 8.0 Hz, 2 H; H-1^b,1^c), 4.52 (d, 1 H; H-1^a), 4.48 (d, $J_{1,2}$ = 7.9 Hz, 1 H; H-1^d), 4.38 (dd, 1 H; H-6a^a), 4.33 (dd, 1 H; H-6a^c), 4.28 (dd, 1 H; H-6b^e), 4.17 (dd, 1 H; H-6b^a), 4.13 (dd, 1 H; H-6b^c), 4.10 (t, 1 H; H-4^e), 4.04 (t, 1 H; H-4^a), 3.91 (H-4^f), 3.90 (t, 1 H; H-4^c), 3.81 (H-5^e), 3.78 and 3.44 ($OCH_2CH_2CH_2N_3$), 3.76 (H-4^d), 3.74 (H-4^b), 3.69 (H-5^f), 3.63 (t, 1 H; H-3^d), 3.57 (t, 1 H; H-3^b), 3.56 (H-5^c), 3.55 (H-5^a), 3.51 (d, $J_{4,5}$ = 9.6 Hz, 1 H; H-5^d), 3.49 (d, $J_{4,5}$ = 9.6 Hz, 1 H; H-5^b), 3.39, 3.30, and 3.23 (3 s, each 3 H; $COOCH_3$), 3.09 ($OCH_2CH_2CH_2N_3$), 1.70-1.59 (m, 2 H; $OCH_2CH_2CH_2N_3$).

3-Aminopropyl (β-D-glucopyranosyluronic acid)-(1→4)-(β-D-glucopyranosyl)-(1→3)-(β-D-glucopyranosyluronic acid)-(1→4)-(β-D-glucopyranosyl)-(1→3)-(β-D-glucopyranosyluronic acid)-(1→4)-β-D-glucopyranoside (1): To a solution of **14** (20 mg, 8 μmol) in MeOH (3 mL) was added NaOMe until pH 10, and after 5 h, water (1 mL) was added. After stirring for 16 h, TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of a new spot (R_f 0.1), and the mixture was neutralised with Dowex H^+, filtered, and concentrated. A solution of the residue in water was washed with CH_2Cl_2 (3x), and the aqueous layer was concentrated. Then, a solution of the residue in 0.1 M NaOH (0.4 mL) was added dropwise to a suspension of 10% Pd/C (0.8 mg) and $NaBH_4$ (3.5 mg) in bidistilled water (0.3 mL). After 2 h, when TLC (EtOAc/MeOH/H_2O 12:5:3) showed the formation of a ninhydrin-positive spot on the baseline, the mixture was acidified with Dowex H^+ (pH 4), then loaded on a short column of Dowex 50 W × 2 (H^+, 200-400 mesh). After elution of contaminants with water, elution with 1% NH_4OH afforded **1** after concentration and lyophilisation from water (2x) (5.4 mg, 61%). $[\alpha]_D^{20}$= -17 (*c* = 0.4, H_2O); MS (MALDI-TOF): *m/z* 1091 $[M+H]^+$, 1113 $[M+Na]^+$, 1135 $[M-H+2Na]^+$, 1157 $[M-2H+3Na]^+$. For ^1H NMR data, see Table 1.

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Isolation of oligosaccharides from a partial acid hydrolysate of pneumococcal type 3 polysaccharide for use in conjugate vaccines

Dirk J. Lefeber[1], Ricardo Gutiérrez Gallego[1], Christian H. Grün[1], Daniela Proietti[2], Sandro D'Ascenzi[2], Paolo Costantino[2], Johannis P. Kamerling[1], and Johannes F.G. Vliegenthart[1]

[1]Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80.075, NL-3508 TB Utrecht, The Netherlands; [2]Chiron Vaccines SpA, Via Fiorentina 1, 53100 Siena, Italy

Abstract

A series of well-defined oligosaccharide fragments of the capsular polysaccharide of *Streptococcus pneumoniae* type 3 has been generated. Partial acid hydrolysis of the capsular polysaccharide, followed by fractionation of the oligosaccharide mixture by Sepharose Q ion-exchange chromatography yielded fragments containing one to seven [→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→] repeating units. The isolated fragments were analysed for purity by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an IonPac AS11 column, and their structures were verified by ^{1}H NMR spectroscopy and nano-electrospray mass spectrometry. The oligosaccharides can be used to produce neoglycoprotein vaccines with a defined carbohydrate part. These products are easier to analyse and will facilitate the investigation of the effect of the saccharide part on the immune response of conjugate vaccines.

Introduction

The Gram-positive pathogenic bacterium *Streptococcus pneumoniae* causes diseases like otitis media, pneumonia and meningitis. Particularly young children, the elderly and immunocompromised patients are at high risk towards pneumococcal infections. Protection against pneumococci is conferred by antibodies against the polysaccharide capsule surrounding the bacterium. Carbohydrate material derived from this capsule is used in neoglycoprotein vaccines that induce a serotype-specific antibody response against the polysaccharide. Currently, vaccines exist against *Haemophilus influenzae* type b [1], *Neisseria meningitidis* type C [2], and *Streptococcus pneumoniae* serotypes [3]. These neoglycoproteins are prepared by conjugation of isolated capsular polysaccharides or a mixture of polysaccharide-derived oligosaccharides to a protein carrier. The polysaccharide-protein conjugates have complex structures, whereas the oligosaccharide-protein conjugates contain mixtures of carbohydrate with respect to chain length and presented epitope. Especially in the case of a pneumococcal conjugate vaccine, wherein many serotypes have to be included, the presence of mixtures complicates the analysis of the conjugates. Such analyses are increasingly important for product control. Furthermore, the lack of defined saccharide fragments hampers the investigation of the immune response at a molecular level. For these reasons, pure carbohydrate fragments with a unique structure are needed. Thus, methods for the isolation of pure fragments and for screening of the purity need to be developed [4,5]. Especially, methods like NMR spectroscopy and mass spectrometry [6], combined with chromatographic techniques like HPAEC-PAD [7] are suitable for characterising carbohydrate material used in conjugate vaccines [8].

The capsular polysaccharide (CPS) of *S. pneumoniae* type 3 consists of [→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→] repeating units [9,10]. Partial acid hydrolysis of the polysaccharide has been described before [11-13] yielding fragments of increasing degree of oligomerisation with glucuronic acid at the nonreducing end, *i.e.* epitope A (Figure 1). Enzymatic digestion of the polysaccharide using an enzyme from *Bacillus palustris* [11,12,14-16] generates fragments of increasing chain lengths having the alternative epitope B (Figure 1). Chemical synthesis has been performed to obtain short defined fragments [17-20]. Oligosaccharides have been conjugated to a carrier protein as mixtures of different chain lengths via their reducing end [21] or via multiple point attachment [22], forming lattice type structures. A more chemically defined conjugate consisted of a hexasaccharide coupled to a protein via reductive amination [23].

The use of conjugates containing oligosaccharides with uniform chain length and unique structure will facilitate product control and enable the investigation of the

immunological effect of the specific carbohydrate structure. Here, the isolation and characterisation of a series of oligosaccharide fragments are reported, containing one to seven repeating units from the CPS of *S. pneumoniae* type 3.

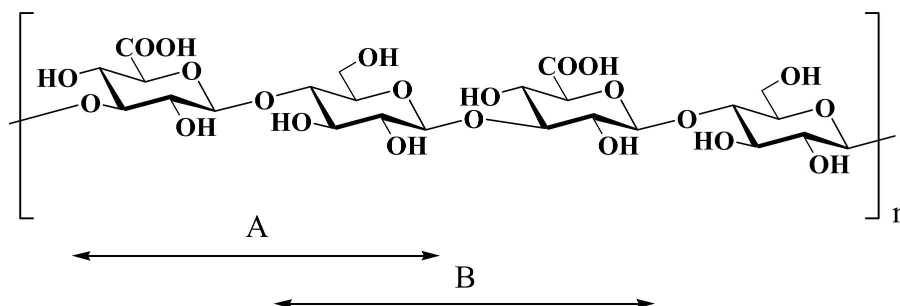

Figure 1. Two repeating units of the CPS of *Streptococcus pneumoniae* type 3. Epitope A of increasing oligomerisation can be obtained by partial acid hydrolysis, and epitope B of increasing oligomerisation by depolymerisation with an enzyme from *Bacillus palustris*.

Materials and methods

Reagents

Capsular polysaccharide (CPS) of *S. pneumoniae* type 3 (Pn 3) was obtained from Chiron Vaccines (Siena, Italy). Trifluoroacetic acid and sulfuric acid (conc.) were purchased from Merck, and phenol from Fluka. All chemicals were of the highest purity commercially available.

Partial acid hydrolysis

To a solution of Pn 3 CPS (50 mg) in bidistilled water (25 mL) was added 0.6 M trifluoroacetic acid (25 mL), and the mixture was heated for 3 h at 100°C, then lyophilised. Lyophilisation from bidistilled water was repeated three times.

FPLC ion-exchange chromatography using Mono Q or Sepharose Q

The oligosaccharide mixtures were fractionated by ion-exchange chromatography on a Pharmacia Biotech FPLC system equipped with a 500 µL or 10 mL sample loop. Before use, solvents were filtered over a 0.22 µm filter and degassed by flushing with helium. Samples were dissolved in bidistilled water and injected on the column through a 0.22 µm filter. Analytical scale fractionation of the oligosaccharide mixture (1 mg/mL) was performed by injection of 2 mL on a prepacked 1 mL gelbed Mono Q® HR 5/5 column (Pharmacia). Fractions were eluted with a flow rate of 0.5 mL/min, by isocratic elution with water for 4 mL, followed by a gradient of 0-500 mM NaCl from 4-84 mL. Fractions of 0.5 mL were collected and analysed for carbohydrate content by a phenol-sulfuric acid assay [24].

Preparative scale fractionation of the oligosaccharide mixture (5.0 mg/mL) was performed by injection of 10 mL on a 20 mL gelbed Sepharose Q® HR 16/10 column (Pharmacia). Fractions were eluted with a flow rate of 5.0 mL/min, by isocratic elution with water for 80 mL, followed by a gradient of 0-213 mM NaCl from 80-750 mL. Fractions of 5.0 mL were collected and analysed for carbohydrate content by a phenol-sulfuric acid assay. Fractions containing pure oligosaccharides as observed by HPAEC-PAD and fractions containing a side-product (*vide infra*) were collected separately, lyophilised, redissolved in water, and desalted by Toyopearl® HW-40S (TosoHaas) size-exclusion chromatography in 5 mM ammonium acetate.

High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The oligosaccharide mixture, fractions from ion-exchange chromatography, and the pooled fractions were analysed by injection with an AS 3500 autosampler on a Dionex DX-500 chromatographic system fitted with a GP40 gradient pump and an ED40 electrochemical detector (PAD detection) with a working gold electrode. Detection was performed using the following pulse potentials and durations: E_1 = 0.1 V (400 ms); E_2 = -0.2 V (20 ms); E_3 = 0.6 V (10 ms); E_4 = -0.1 V (60 ms). Integration occurred from 200 to 400 ms during E_1 application. The system was equipped with a CarboPac™ PA10 or an IonPac™ AS11 column (4 x 250 mm) in combination with a CarboPac™ PA10 or an IonPac™ AS11 guard column, respectively, and operated at room temperature. The eluent was degassed with helium and pressurised continuously with the eluent degas module of Dionex. Samples injected on a PA10 column were run with 100 mM NaOH at a flow rate of 1.0 mL/min and a gradient of sodium acetate from 0-700 mM in 10.5 min, from 700-800 mM in 9.5 min, and isocratically at 800 mM for 10 min. Samples injected on an AS11 column were run in 10 mM NaOH at a flow rate of 1.0 mL/min with a gradient of 10-100 mM NaOH in 54 min.

^{1}H NMR Spectroscopy

Prior to analysis, oligosaccharide samples were dissolved in ^2H_2O (99.9 atom % ^{2}H) to a concentration of 5 mg/mL. The polysaccharide sample was dissolved in ^2H_2O (99.9 atom % ^{2}H) containing 100 mM NaCl to a concentration of 0.5 mg/mL. Resolution-enhanced 1D ^{1}H NMR spectra were recorded on a Bruker AMX-500 spectrometer at probe temperatures of 278, 300 or 353 K. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 2.225).

Nano-electrospray mass spectrometry

Electrospray ionisation (ESI) mass spectrometric analyses were performed on a Thermoquest / Finnigan LC-Q ion trap mass spectrometer equipped with a Protana

nanoES sample probe. Samples in methanol-water (1:1) with an approximate concentration of 10 to 30 pmol/µL were prepared as follows. From a solution of the oligosaccharide in bidistilled water (2 µg/µL), 5 µL aliquots were diluted to 500 µL with methanol-water (1:1). For each experiment, 2 µL were loaded into the capillary. The capillary temperature was set to 180 °C. Spectra were taken in the negative-ion mode with a cone voltage of 1.5 kV and a capillary voltage of -46.0 V.

Results

Partial acid hydrolysis and fractionation of the oligosaccharide mixture by ion-exchange chromatography

The partial acid hydrolysis of Pn 3 CPS with 0.3 M trifluoroacetic acid was followed in time. The composition of the oligosaccharide mixture after 3 h of hydrolysis as analysed by HPAEC-PAD is shown in Figure 2.

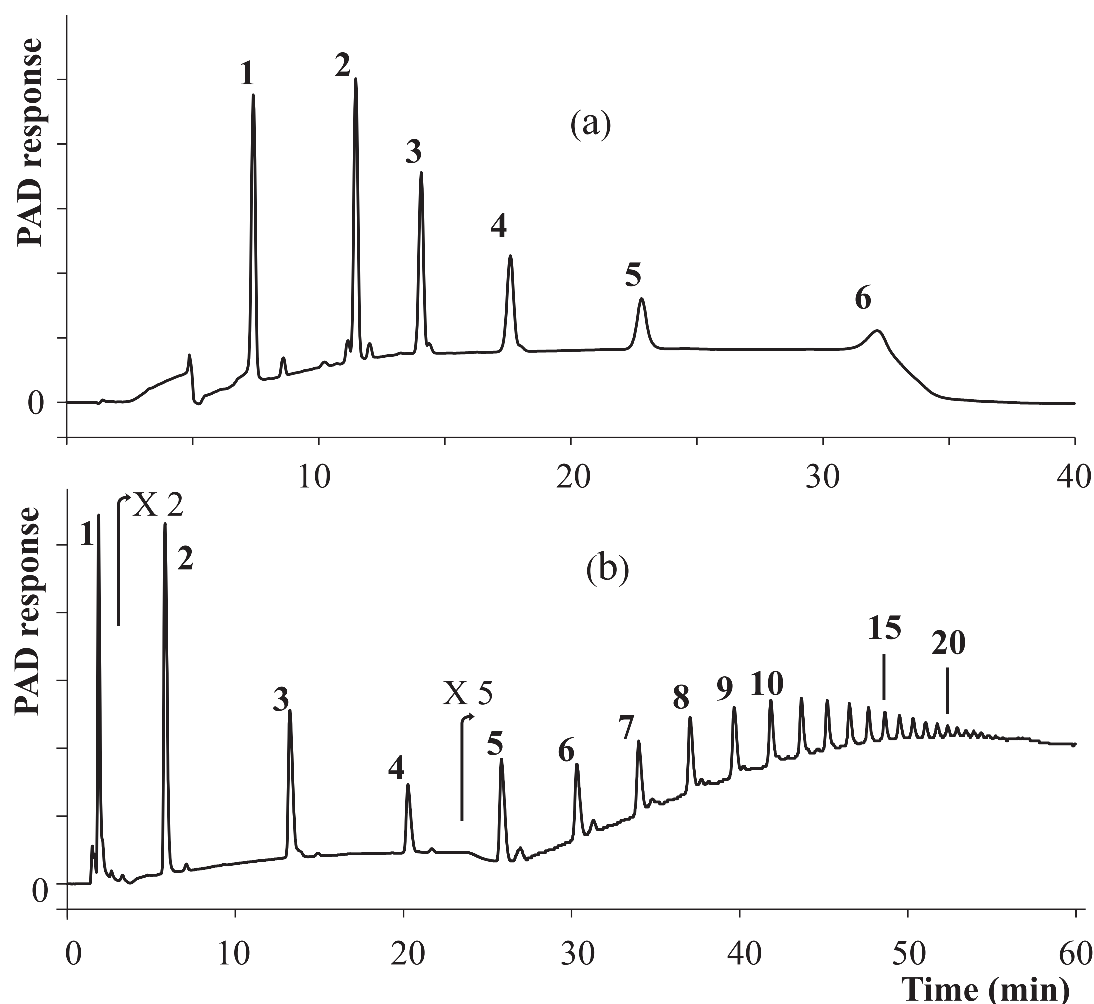

Figure 2. HPAEC-PAD profiles of the oligosaccharide mixture obtained by partial acid hydrolysis of Pn 3 CPS using (a) CarboPac PA10 or (b) IonPac AS11. Numbers indicate the size of the oligosaccharide fragments expressed in repeating units.

Using a CarboPac PA10 column, a high salt gradient was needed for the elution of fragments up to six repeating units (Figure 2a). Better results were obtained when using an IonPac AS11 column (Figure 2b). Fragments of over 20 repeating units were shown as isolated peaks. Small additional peaks for fragments of 1-9 repeating units indicated the presence of side-products (*vide infra*).

In Figure 3a, the fractionation of the oligosaccharide mixture by ion-exchange chromatography on analytical scale with a Mono Q HR 5/5 ion-exchange column is shown. The composition of the individual fractions of a peak was analysed by HPAEC-PAD on IonPac AS11. The side-products as mentioned above were shown to reside in the faster eluting fractions of a MonoQ peak. A similar fractionation pattern of the oligosaccharide mixture as observed for Mono Q was obtained on a preparative scale using a Sepharose Q HR 16/10 ion-exchange column (Figure 3b). Fractions belonging to one peak were collected thereby omitting the fractions contaminated with side-product, which were collected separately.

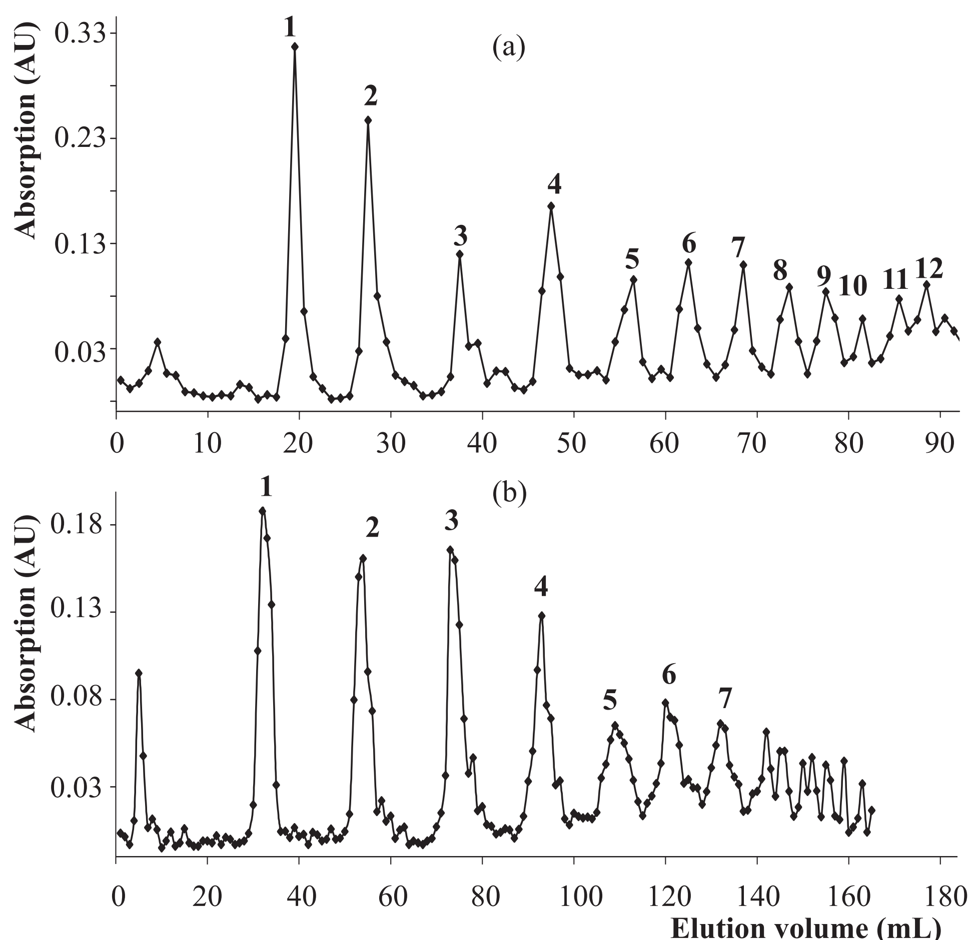

Figure 3. Ion-exchange chromatograms of the oligosaccharide mixture obtained by partial acid hydrolysis of Pn 3 CPS on (a) Mono Q and (b) Sepharose Q. Numbers correspond to the size of the oligosaccharide fragments expressed in repeating units.

Purity analysis of the isolated fragments from Sepharose Q by HPAEC-PAD on IonPac AS11

The purity of the pooled fractions was analysed by HPAEC-PAD. In Figure 4, the IonPac AS11 profiles are shown of the pure and of the side-product-containing fractions.

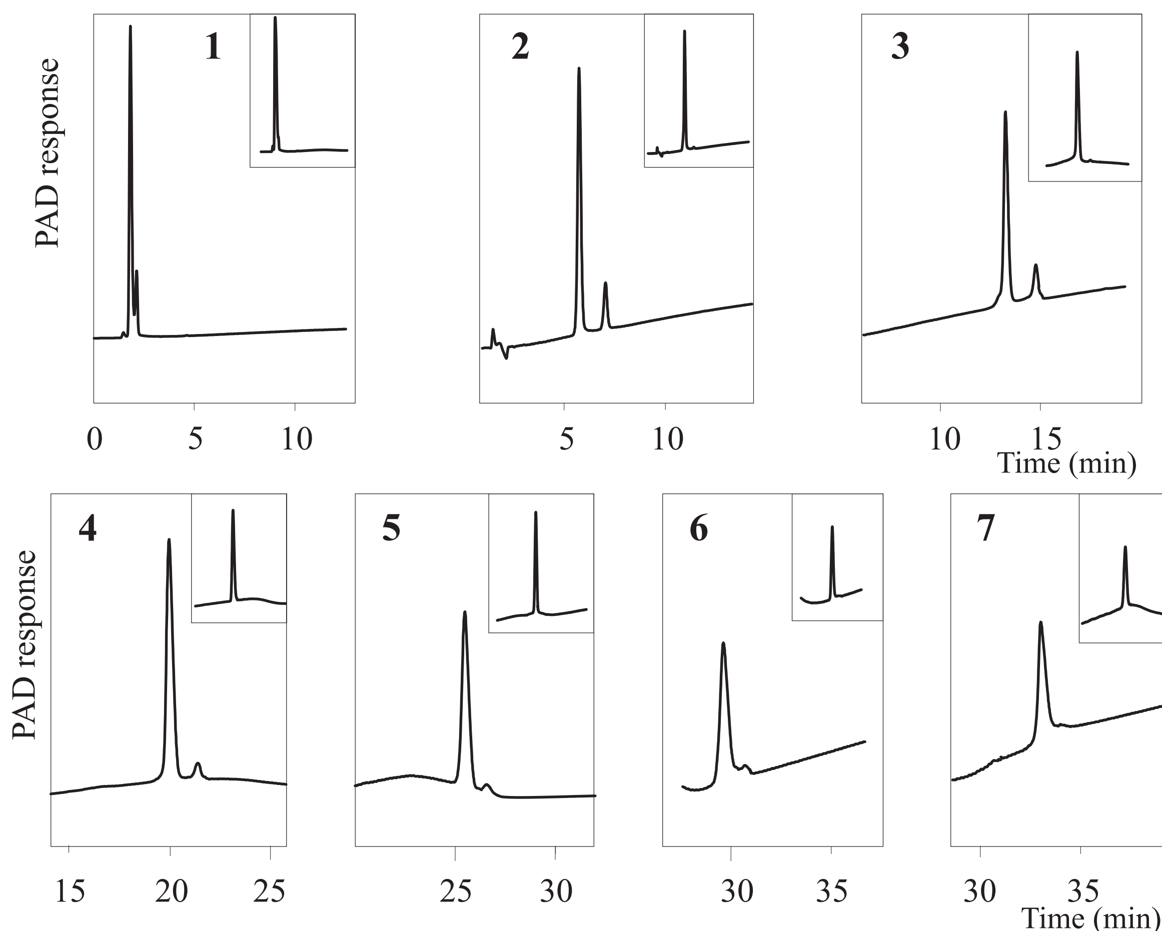

Figure 4. HPAEC-PAD profiles of pooled side-product containing Sepharose Q fractions on IonPac AS11. Pooled fractions containing pure products are shown as inserts, 2.5x reduced in size. Numbers correspond to the size of the oligosaccharide fragments expressed in repeating units.

Characterisation of the oligosaccharides by ^{1}H NMR spectroscopy and nano-electrospray mass spectrometry

^{1}H NMR spectroscopy and nano-electrospray mass spectrometry were used for the characterisation of the oligosaccharides. The 1D ^{1}H NMR spectra of the oligosaccharides having a glucuronic acid residue at the non-reducing end and a glucose residue at the reducing end (e.g. tetradecasaccharide, Figure 5a) and the polysaccharide (Figure 5b) were in good agreement. Due to the high viscosity of the

polysaccharide, proton signals were only resolved upon addition of sodium chloride (100 mM) and at a probe temperature of 353 K.

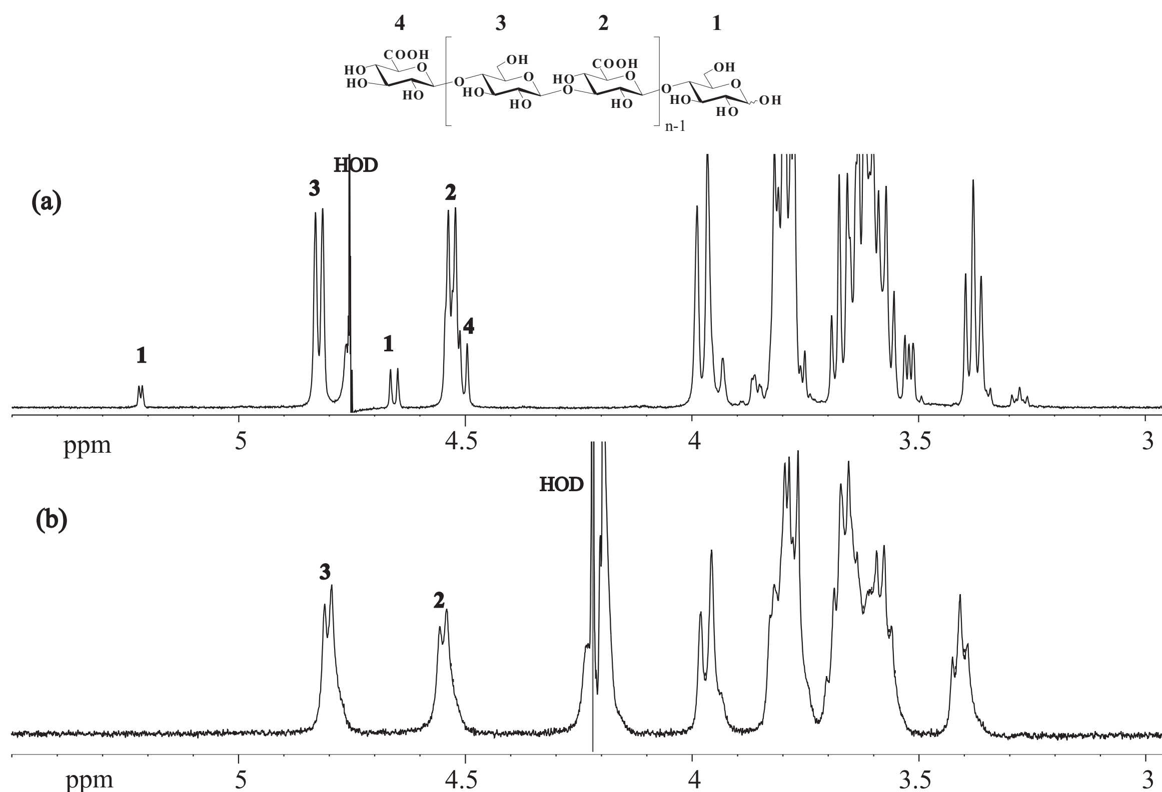

Figure 5. 500 MHz ^{1}H NMR spectra of (a) the tetradecasaccharide fragment at 300K and (b) the native polysaccharide at 353K. The anomeric protons are indicated.

In Figure 6, the anomeric regions of the 1D ^{1}H NMR spectra of the $(GlcA\text{-}Glc)_n$ oligosaccharides (n = 1-7) are shown. The positions of the anomeric signals were in good agreement with literature data of comparable synthetic saccharides [19], and their chemical shifts are reported in Table 1. Nano-electrospray mass spectrometry confirmed the molecular masses of the oligosaccharide fragments. In Table 2, their molecular masses are reported.

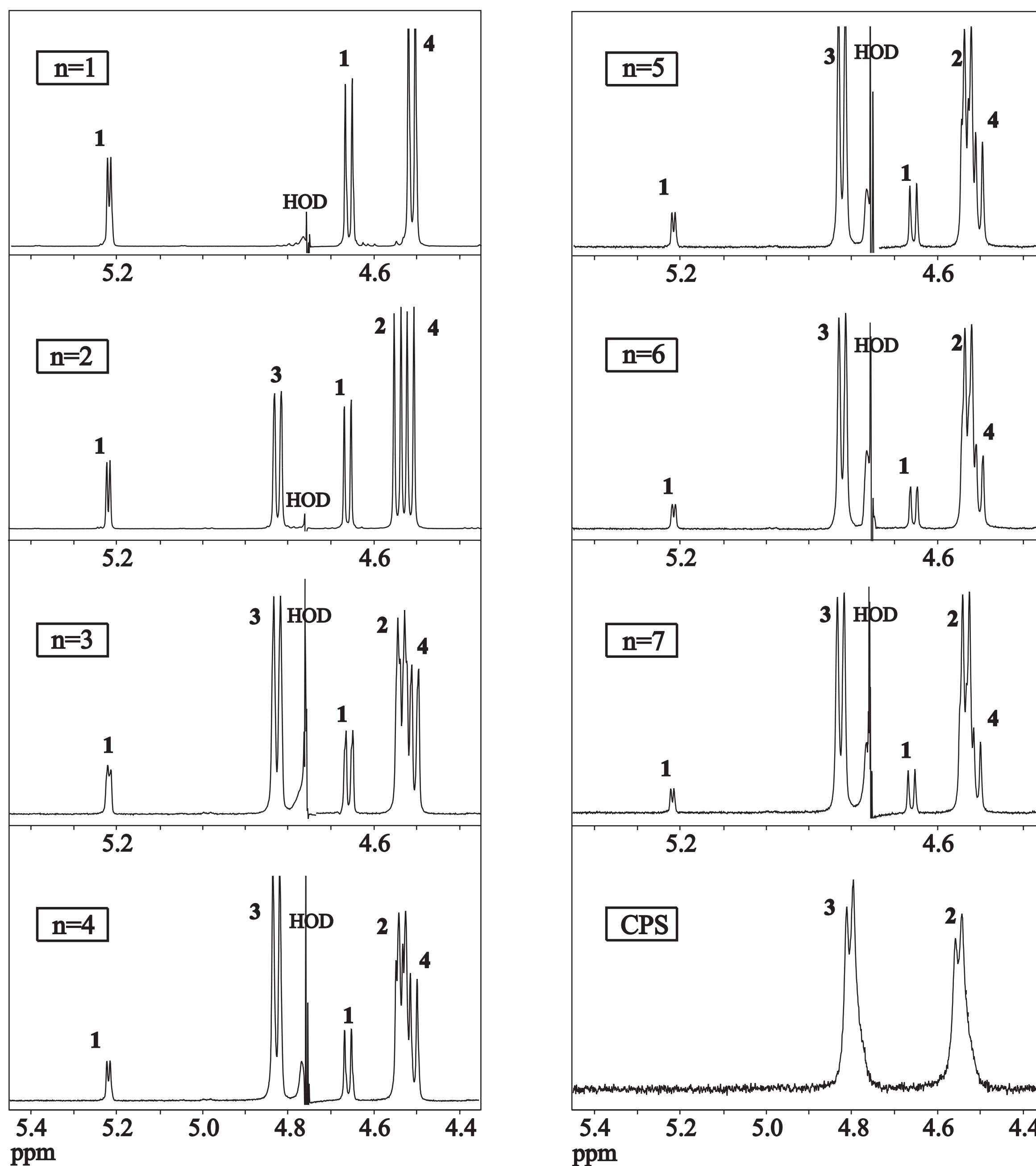

Figure 6. Anomeric regions of the ^{1}H NMR spectra at 300K of the di- to tetradecasaccharide fragments (n=1-7) and the capsular polysaccharide (353K). Numbers refer to the structure in Figure 5.

Table 1. 500 MHz ^{1}H NMR data of the anomeric protons of the $(GlcA\text{-}Glc)_n$ oligosaccharide fragments (n = 1-7) at 300K and of the CPS at 353K. Chemical shifts are given in ppm, coupling constants (in parentheses) are in Hz. Bold numbers correspond to Figure 6.

	n=1	n=2	n=3	n=4	n=5	n=6	n=7	CPS
1α	5.218	5.217	5.217	5.217	5.216	5.216	5.216	–
	(3.7)	(3.7)	(3.7)	(3.7)	(3.7)	(3.7)	(3.7)	
1β	4.659	4.657	4.657	4.658	4.657	4.656	4.657	–
	(8.2)	(7.9)	(7.9)	(7.9)	(8.0)	(8.3)	(7.9)	
3	–	4.820	4.825	4.826	4.823	4.824	4.822	4.803
		(8.0)	(8.0)	(7.9)	(8.0)	(8.0)	(8.0)	(7.6)
4	4.512	4.510	4.503	4.505	4.503	4.503	4.504	–
	(8.0)	(8.0)	(8.0)	(8.0)	(7.9)	(8.0)	(7.9)	
2	–	4.541	4.536	4.538	4.536	4.535	4.536	4.549
		(8.0)	(8.0)	(7.9)	(8.0)			(7.7)
	–	–	4.531	4.531	4.529	4.530	4.530	–
			(7.9)	(8.0)	(8.0)	(8.0)	(7.7)	

Table 2. Molecular masses of the $(GlcA\text{-}Glc)_n$ oligosaccharide fragments (n = 1-7), as determined by nano-electrospray mass spectrometry in the negative-ion mode.

n	Calculated	$[M\text{-}H]^-$
1	356.09	355.2
2	694.18	693.3
3	1032.26	1031.3
4	1370.35	1369.4
5	1708.43	1707.4
6	2046.51	1022.3 $[M\text{-}2H]^{2-}$
7	2384.60	1191.5 $[M\text{-}2H]^{2-}$

In Figure 7, the structures of the possible side-products (*vide supra*) resulting from partial acid hydrolysis of Pn 3 CPS are shown. The fractions containing as major product the fragments of one and two repeating units, contaminated with side-product as observed by HPAEC-PAD, were characterised by 1D ^{1}H NMR spectroscopy (Figures 8a and 8b, respectively). With respect to the side-product, the signals at 4.803 ppm (Figure 8a) and 4.805 ppm (Figure 8b) represent glucose

at the nonreducing end, the signal at 4.534 ppm (Figure 8a) stems from internal glucuronic acid. This indicates the presence of Glc-GlcA-Glc and Glc-GlcA-Glc-GlcA-Glc sequences (structures I, Figure 7). These side-products originate from cleavage of the β-D-Glc*p*A-(1→4)-β-D-Glc*p* linkage. In the mass spectrum of the side-product-containing fraction (n = 2, Figure 9a), besides the ion at *m*/*z* 693.5 an additional ion at *m*/*z* 855.3 is shown. This corresponds to the molecular ion ($[M\text{-}H]^-$) of $GlcA_2Glc_3$, which confirms the identity of the side-products having a glucose residue at the nonreducing end (Figure 7; structure I).

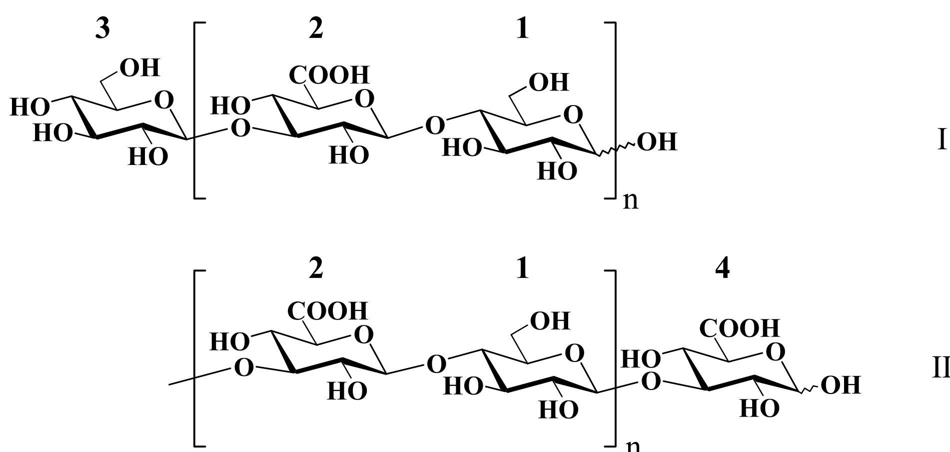

Figure 7. Structures of the possible side products.

Careful inspection of the 1D ^1H NMR spectrum of the pure tetrasaccharide shows a minor additional signal at 5.231 ppm (Figure 8c), indicating the α-anomeric proton of glucuronic acid, and thus, the presence of a GlcA-Glc-GlcA sequence with structure II (Figure 7). In the mass spectrum of the pure tetrasaccharide (Figure 9b) an ion of low abundance is observed at *m*/*z* 531.1, corresponding to the molecular ion ($[M\text{-}H]^-$) of $GlcA_2Glc$. This confirms the molecular mass and structure of the trisaccharide.

The amounts of the two side-products co-eluting with the tetrasaccharide fragment, *i.e.* Glc-GlcA-Glc-GlcA-Glc and GlcA-Glc-GlcA, was estimated to be ~2% each, relative to the total amount of tetrasaccharide as derived from ^1H NMR.

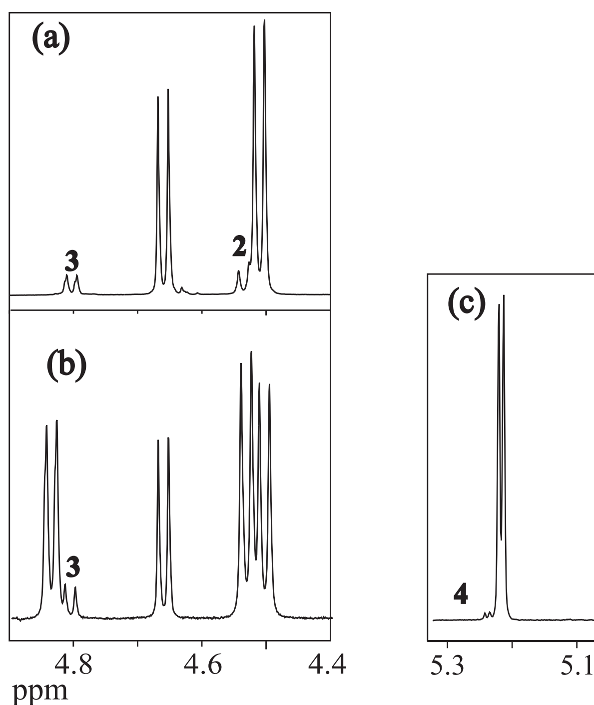

Figure 8. ^{1}H NMR spectra at 278K of the β-anomeric protons of the fractions of (a) one and (b) two repeating units containing a side-product; (c) ^{1}H NMR spectrum at 300K of the α-anomeric protons of the tetrasaccharide fragment. Numbers refer to structures in Figure 7.

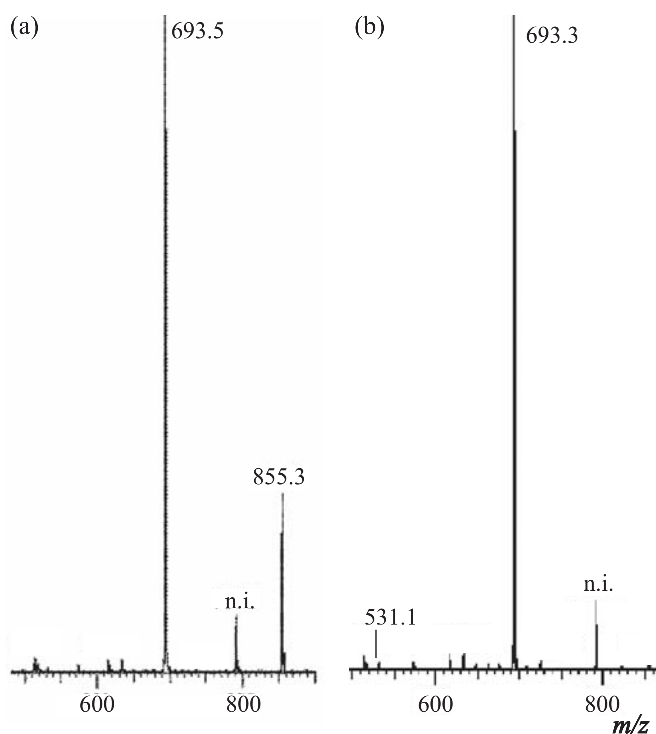

Figure 9. Nano-electrospray mass spectra of the fractions containing (a) the tetrasaccharide fragment and (b) the tetrasaccharide fragment and the side-product. n.i. = not identified.

Conclusion

We have reported the isolation of a series of $(GlcA\text{-}Glc)_n$ oligosaccharides of increasing degree of oligomerisation (n = 1-7) from a partial acid hydrolysate of the capsular polysaccharide of *S. pneumoniae* type 3. The purity of the pooled fragments was determined by HPAEC-PAD using an IonPac AS11 column. The structure of the oligosaccharide fragments was assigned by nano-electrospray mass spectrometry and ^{1}H NMR spectroscopy. In earlier studies, partial acid hydrolysis of the capsular polysaccharide of *S. pneumoniae* type 3 was found to lead to a specific cleavage of the (1→3)-linkage. However, the use of modern analytical and physicochemical methods permitted the identification of side-products originating from cleavage of the β-D-Glc*p*A-(1→4)-β-D-Glc*p* linkage. The oligosaccharides can be used as standards in analytical assays and for the preparation of neoglycoproteins with a defined carbohydrate part that can be used for immunisation studies and to investigate the immunological effect of the saccharide in conjugate vaccines.

Acknowledgements

This work was supported by the European Union program VACNET (grant ERB BIO 4CT960158).

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6

Synthesis and antibody binding properties of glycoclusters related to the capsular polysaccharide of *Streptococcus pneumoniae* type 3

Dirk J. Lefeber[1], Wouter T.M. Jansen[2], H. Snippe[2], Johannis P. Kamerling,[1] and Johannes F.G. Vliegenthart[1]

[1]Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University; [2]Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation, Vaccines Section, Utrecht Medical Center, The Netherlands

Abstract

The recognition of capsular polysaccharide antigens by immunoglobulins on the B-cell surface is the first step in the immune response against encapsulated bacteria. To investigate the carbohydrate-immunoglobulin interaction, glycoclusters were synthesised containing one, two or three copies of the disaccharide repeating unit of the capsular polysaccharide of *Streptococcus pneumoniae* type 3. To this end, β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$ was synthesised and coupled to 4-(2-carboxyethyl)-4-nitropimelic acid as a scaffold. Mono-, di-, and trivalent structures were isolated and their interaction with the monoclonal antibody IgG 3.3 was studied with surface plasmon resonance. The di- and trivalent compounds showed a slight increase in binding compared to the monovalent one, whereas the uncoupled disaccharide showed a low affinity.

Introduction

The first step in the immune response against encapsulated bacteria is the interaction of antibodies with carbohydrate epitopes on the capsular polysaccharide. Previously, we have shown that CRM_{197}-conjugates of the synthetic disaccharide β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$, representing one repeating unit of the capsular polysaccharide of *Streptococcus pneumoniae* type 3, were able to generate a protective immune response in mice[1]. In this study, the disaccharides on the surface of the carrier protein were randomly presented in a multivalent fashion with an oligosaccharide loading of 3.1 or 6.7. Glycodendrimers or glycodendrons [2] can be used as more defined structures to explore the influence of multivalency in the binding of the disaccharide to immunoglobulins. Such multivalent arrays of oligosaccharides have been developed for studying the spatial requirements for binding of oligosaccharides to lectins [3,4], and in a few cases for studying carbohydrate-antibody interactions [5,6].

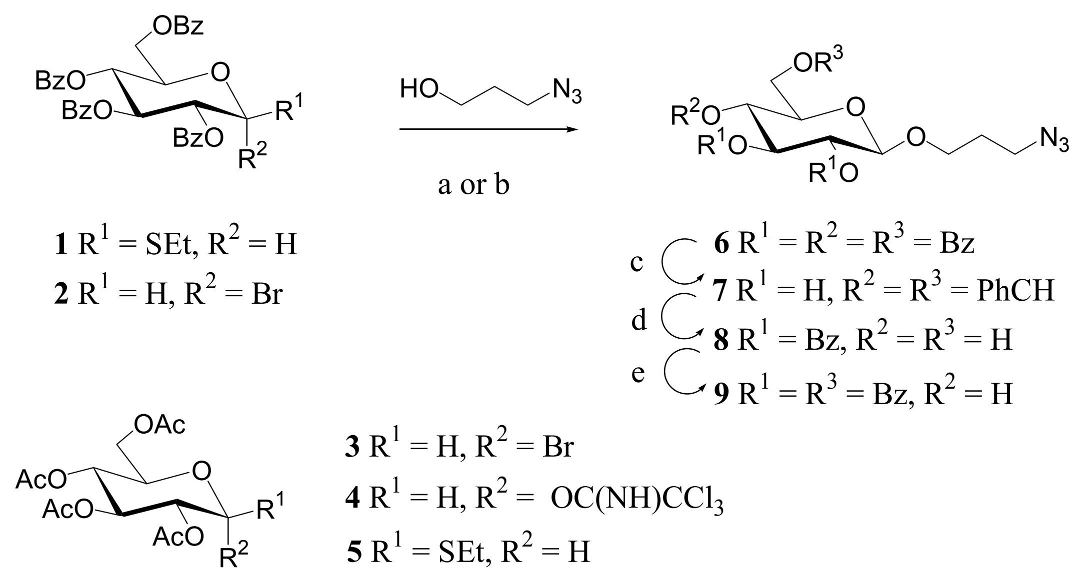
In the present study, we prepared glycoclusters containing the repeating unit β-D-Glc*p*A-(1→4)-β-D-Glc*p* of the CPS ([→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→]) of *S. pneumoniae* type 3 for investigating their interaction with a polysaccharide-specific antibody. Glycosidic linkages can be applied to connect carbohydrates to a core structure [7]. More often, however, a spacer-containing oligosaccharide is attached to a scaffold. As a first approach, we synthesised mono-, di-, and trivalent structures with 4-(2-carboxyethyl)-4-nitropimelic acid [8] as the core molecule. This compound has three carboxylic groups available for the formation of amide bonds. Reduction of the nitro group [9] allows coupling to spacers, carrier proteins, peptides or surfaces.

Results

Synthesis of glycoclusters

For the preparation of the mono-, di-, and trivalent structures **16-18**, first, the spacer-containing disaccharide β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$ (**14**) was synthesised *via* a precursor disaccharide involving the coupling of glucopyranosyl donor **11** and 3-azidopropyl glucopyranoside acceptor **9**. For the introduction of the 3-azidopropyl spacer, differently activated glucose derivatives were coupled to 3-azido-1-propanol [10] (Scheme 1). Ethyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-glucopyranoside (**1**) reacted with 3-azido-1-propanol in 50% yield (→**6**) using *N*-iodosuccinimide and triflic acid as a promoter, whereas 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (**2**) gave **6** in a high isolated yield of 84% using silver triflate as a promoter. It should be noted that coupling of 3-azido-

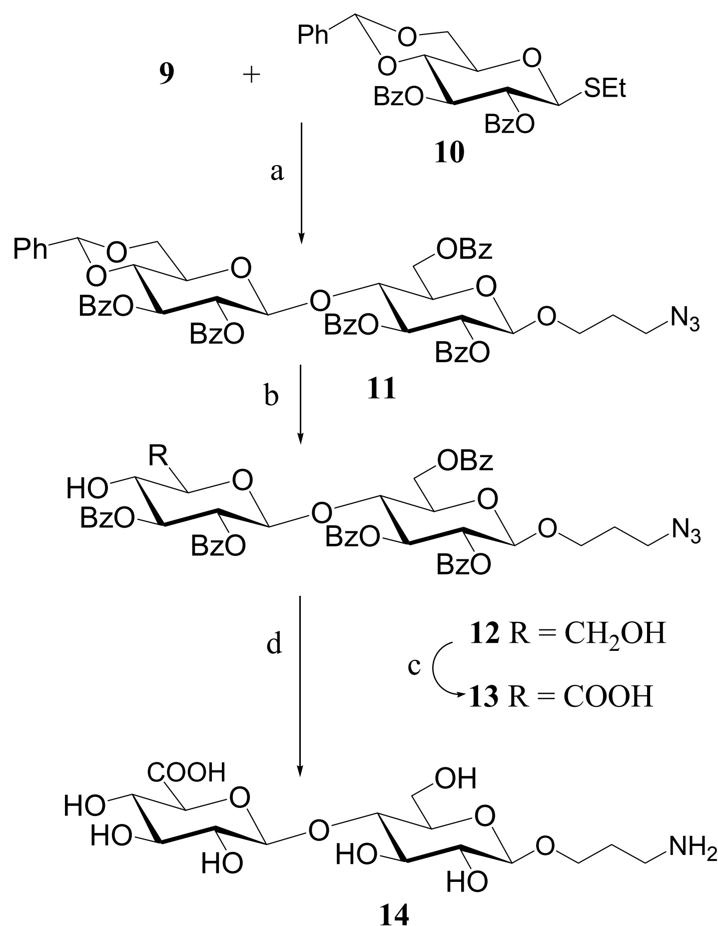
1-propanol with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**3**) using silver triflate as a promoter or with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl imidate (**4**) using TMSOTf as a promoter, or with the corresponding thioethyl glucoside (**5**) promoted by *N*-iodosuccinimide and triflic acid resulted mainly in the formation of orthoester and hydrolysis products of the donor and therefore in low yields of product (<20%).



Scheme 1. *Reagents and conditions*: (a) **1**, NIS, AgOTf, CH_2Cl_2–toluene (1:8), 50%. (b) **2**, AgOTf, CH_2Cl_2–toluene (1:8), 84%. (c) *i* NaOMe, MeOH, *ii* $PhCH(OMe)_2$, *p*TsOH, DMF, 70%. (d) *i* PhCOCl, Et_3N, CH_2Cl_2, *ii* CF_3COOH, H_2O, CH_2Cl_2, 92%. (e) PhCOCl, imidazole, CH_2Cl_2, 96%.

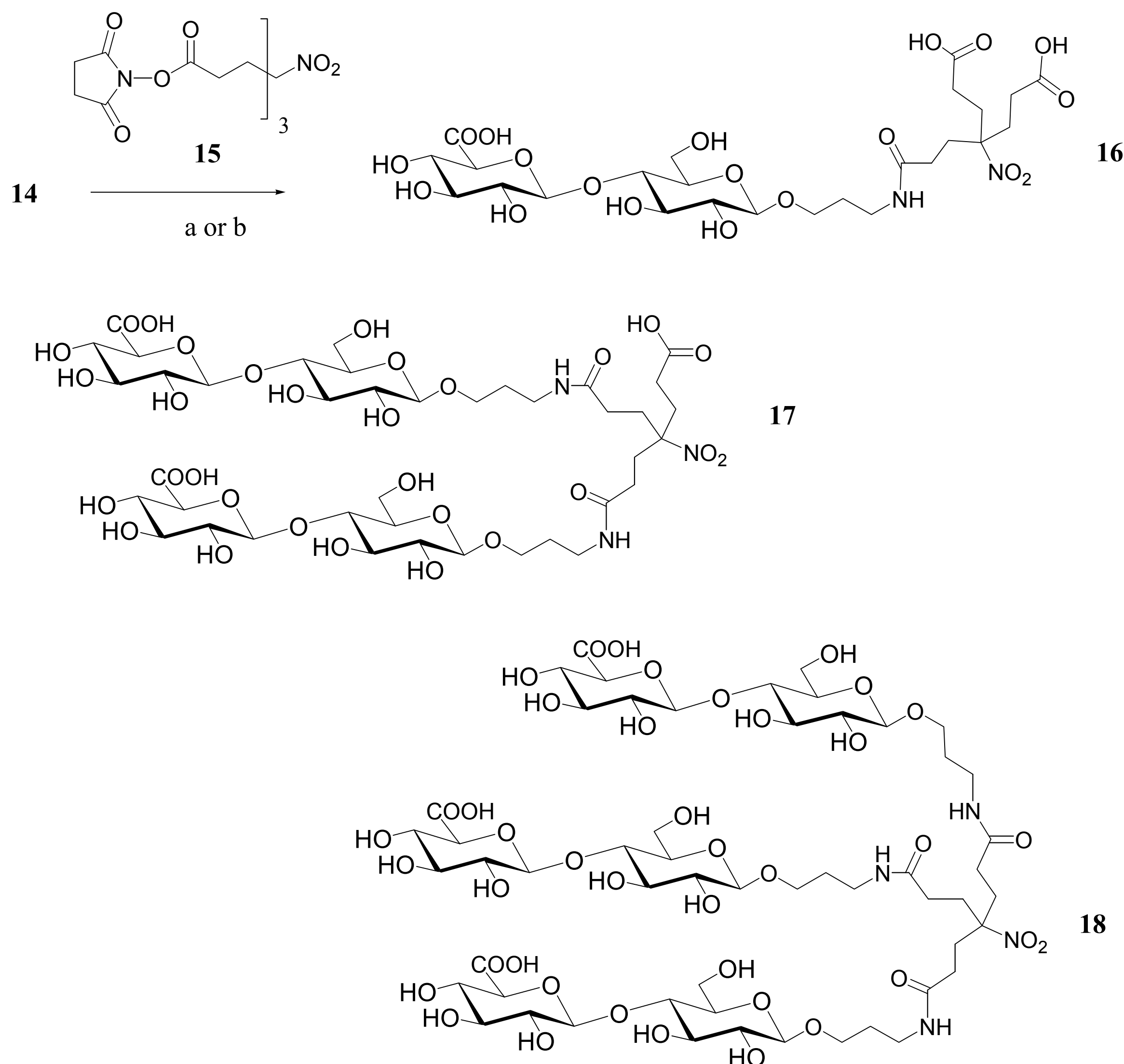
Debenzoylation of **6** with sodium methoxide and benzylidenation using benzaldehyde dimethyl acetal afforded **7** (70%). Subsequent benzoylation with benzoyl chloride and debenzylidenation with trifluoroacetic acid gave **8** in a yield of 92%. Treatment of **8** with benzoyl imidazole as described before [10] afforded **9** (96%).

Coupling of **9** with thioethyl donor **10** [11] (Scheme 2) promoted by *N*-iodosuccinimide and triflic acid gave disaccharide **11** in a yield of 65%. Debenzylidenation of **11** with trifluoroacetic acid (→**12**, 87%), and subsequent selective oxidation of HO-6 in the presence of HO-4 using TEMPO [12] and sodium hypochlorite afforded **13** (85%). Deprotection of **13** was performed in two steps. First, debenzoylation was achieved with sodium methoxide, then, the azide was reduced using sodium borohydride and 10% Pd/C to give **14** in a yield of 88%.



Scheme 2. *Reagents and conditions*: (a) NIS, TfOH, CH_2Cl_2-Et_2O (3:1), 65%. (b) CF_3COOH, H_2O, CH_2Cl_2, 87%. (c) TEMPO, aqueous NaOCl, KBr, Bu_4NBr, aqueous NaCl, aqueous $NaHCO_3$, CH_2Cl_2, 85%. (d) *i* NaOMe, MeOH (pH 11), *ii* $NaBH_4$, 10% Pd/C, 0.1 M NaOH, 88%.

For the preparation of glycoclusters (Scheme 3), disaccharide **14** was coupled to succinimide activated 4-(2-carboxyethyl)-4-nitropimelic acid (**15**) in pyridine at 65 °C for 8 h or 2 days, respectively. When the reaction was allowed to proceed for 8 h, the mono- (**16**), di- (**17**) and trisubstituted (**18**) products were formed in a ratio of 3:3:1. After stirring for 2 days, a ratio of 0:1:3 was obtained. The compounds could be isolated by Sephadex LH-20 size-exclusion chromatography in methanol.



Scheme 3. *Reagents and conditions*: (a) C_6H_5N, 65 °C, 8 h. (b) C_6H_5N, 65 °C, 2 days.

Surface plasmon resonance analysis

The binding of the synthesised compounds (**14**, **16-18**) to a monoclonal antibody (IgG 3.3), specific for the capsular polysaccharide of *S. pneumoniae* type 3, was studied with surface plasmon resonance (SPR). Since the coupling of the IgG antibody to a CM5 chip did not result in an active surface, the sensor chip was

coated with a synthetic neoglycoprotein. This conjugate was prepared by coupling of the synthetic tetrasaccharide β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$, containing two repeating units of the polysaccharide of *S. pneumoniae* type 3, to a non-toxic mutant of diphtheria toxin (cross-reacting material, CRM_{197}) with an oligosaccharide loading of 6.7 [10]. A glucose-CRM_{197} conjugate (loading 8.1) was used as the reference surface. Low capacity surfaces were generated of both conjugates (160-170 response units) to avoid artefacts that could complicate the analysis [13].

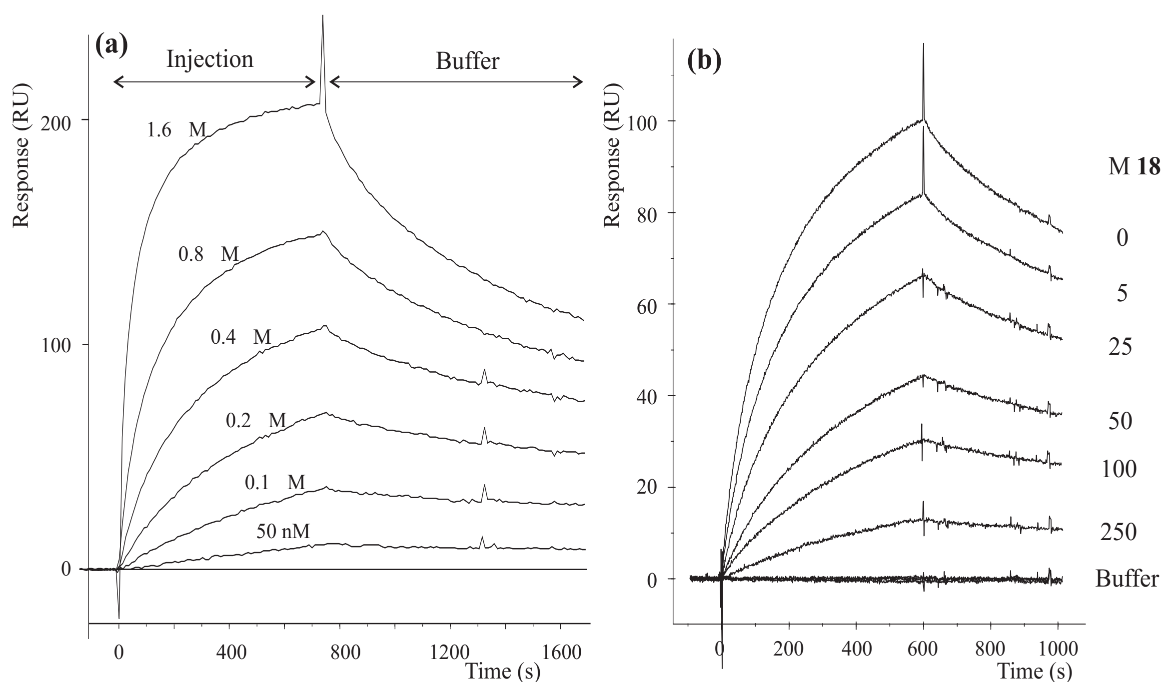


Figure 1. SPR response curves obtained for binding to a tetrasaccharide-CRM_{197} surface of (a) IgG 3.3 in various concentrations and (b) 0.5 μM IgG 3.3 in the presence of trivalent **18** at increasing concentrations.

In Figure 1a, a typical sensorgram is shown for the binding of IgG 3.3 in various concentrations to the tetrasaccharide-CRM_{197} surface. For studying the interaction of the monoclonal antibody with the synthesised structures (**14**, **16-18**), IgG 3.3 was passed over the neoglycoprotein surface at a fixed concentration of 0.5 μM, premixed with various concentrations of synthetic ligand. In Figure 1b, the response curves for the binding of IgG 3.3 to the tetrasaccharide-CRM_{197} surface is shown in the presence of increasing concentrations **18**. The maximum binding level at the end of injection was plotted against the ligand concentration (Figure 2).

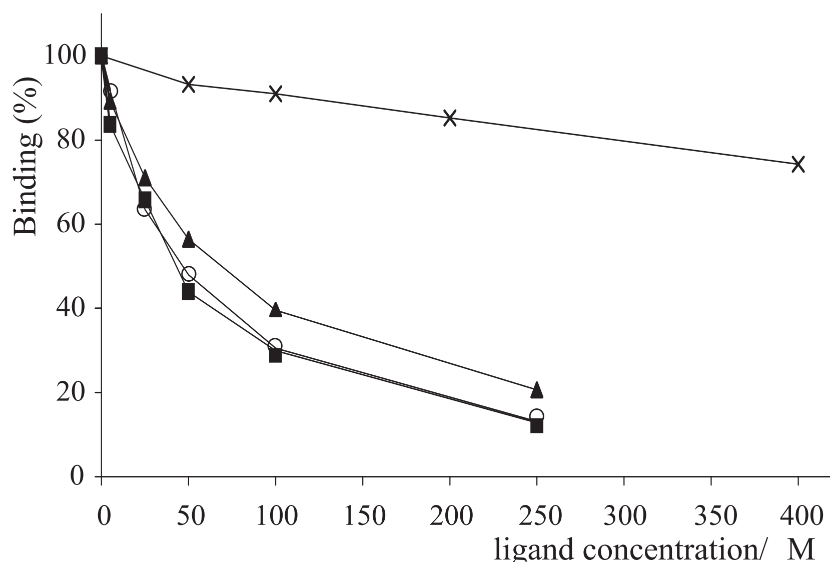


Figure 2. Binding level at the end of injection for binding of IgG 3.3 to a tetrasaccharide-CRM_{197} surface at different concentrations of synthetic ligand. The mono- (**16**, π), di- (**17**, □), and trivalent (**18**, ′) structures and disaccharide **14** (×) were tested.

The scaffold-containing structures showed concentrations at 50% inhibition (IC_{50}) of 70 μM for **16**, 47 μM for **17**, and 43 μM for **18**, respectively. Thus, the di- and trivalent compounds showed a 1.5 times higher affinity compared to the monovalent one. The free disaccharide (**14**) affected the binding of IgG 3.3 to the tetrasaccharide-CRM_{197} surface only slightly (IC_{50} >> 400 μM). To assure that the difference between the conjugated structures (**16-18**) and the free disaccharide (**14**) was not caused by aspecific interactions of the scaffold with the monoclonal antibody, 4-(2-carboxyethyl)-4-nitropimelic acid was tested as a ligand. This compound, however, did not influence the binding of IgG 3.3 to the tetrasaccharide-CRM_{197} surface at concentrations of 0.5 or 1.0 mM.

Conclusions

Compounds have been synthesised containing one, two or three copies of the disaccharide β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O-$(CH_2)_3NH_2$, which is the repeating unit of the capsular polysaccharide of the bacterium *S. pneumoniae* type 3. The interactions of disaccharide **14** and the mono-, di-, and trivalent molecules **16-18** with the monoclonal antibody IgG 3.3 were studied using surface plasmon resonance.

As compared by their IC_{50} values, divalent **17** showed a higher affinity for IgG 3.3 than monovalent **16** by a factor of about 1.5. Increasing the valency as in trivalent **18** did not further increase the affinity. A comparison of the binding of structures

16-18 (IC_{50} 43-70 μM) with the binding of the free disaccharide **14** (IC_{50} >> 400 μM) showed that introduction of the hydrophobic scaffold caused a large increase in affinity of the disaccharide for IgG 3.3. Previous studies have shown that increased binding of oligosaccharides to lectins could be achieved by the introduction of a hydrophobic spacer, as for example the coupling of a nitrophenyl group to mannose [14], the use of aromatic core molecules [15] or by varying the spacer length [16].
In the current study the binding of glycoclusters to antibody has been investigated in solution. As it has been shown that binding effects can be dependent on the test system used [17,18] and on the mode of presentation of the receptor, *i.e.* in solution or on a surface [19], future studies will deal with the interaction of the synthesised compounds with surface bound immunoglobulins using a different type of assay.

Acknowledgements

This work was supported by the European Union program VACNET (grant ERB BIO 4CT960158).

Experimental

General procedures

All reagents were used as obtained commercially, without any further purification. All solvents were distilled from appropriate drying agents. Reactions were monitored by TLC on Silica gel 60 F_{254} (Merck); after examination under UV light, compounds were visualised by heating with 10% (v/v) ethanolic H_2SO_4, orcinol (2 mg mL^{-1}) in 20% (v/v) methanolic H_2SO_4, or ninhydrin (1.5 mg mL^{-1}) in BuOH-H_2O-HOAc (38:1.75:0.25). In the work-up procedures of reaction mixtures, organic solutions were washed with appropriate amounts of the indicated aqueous solutions, then dried with $MgSO_4$, and concentrated under reduced pressure at 20-40 °C on a water-bath. Column chromatography was performed on Silica gel 60 (Merck, 0.063-0.200 mm). Optical rotations were measured with a Perkin-Elmer 241 polarimeter, using a 10-cm l-mL cell, in H_2O for **14**, **16-18** and in $CHCl_3$ for all other compounds; $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. ^1H NMR spectra (300 MHz) and ^{13}C (APT, 75 MHz) NMR spectra were recorded in $CDCl_3$ at 300 K with a Bruker AC 300 spectrometer; indicated values for δ_H are given in ppm relative to the signal for internal Me_4Si (δ 0) and for δ_C relative to the signal of $CDCl_3$ (δ 76.9). ^1H NMR data of **14**, **16-18** were derived from 2D TOCSY and ROESY spectra, measured on a Bruker AMX 500 apparatus (500 MHz) at 300 K in D_2O, and δ_H values are given in ppm relative to internal acetone (δ 2.225). All *J* values are given in Hz. Mass spectral data were obtained from a Thermoquest / Finnigan LC-Q ion trap mass spectrometer equipped with a Protana nanoES sample probe. Spectra were taken in the negative-ion mode. From a 2 μg μL^{-1} solution of the oligosaccharide fragment in bidistilled water was taken 5 μL that were further diluted to 500 μL with methanol-water

(1:1). For each experiment, 2 μL were loaded into the capillary. Elemental analyses were carried out by H. Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany).

3-Azidopropyl 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranoside (6): A solution of silver triflate (3.95 g, 15.4 mmol) in dry toluene (66 mL) was added to a dried (2 h *in vacuo*) mixture of 3-azido-1-propanol [10] (5.7 g, 56 mmol) and 4Å molecular sieves. After 30 min, a solution of 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (**2**) (8.9 g, 13.2 mmol) in dry CH_2Cl_2 (8.2 mL) was added, and the mixture was stirred for 1 h. Then, the mixture was neutralised with pyridine, diluted with EtOAc, washed with 5% aqueous $NaHSO_3$ and 5% aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene-EtOAc 95:5) to afford **6** (7.7 g, 84%). $[\alpha]_D^{20}$= +21 (*c* 1.1); R_f 0.53 (toluene-EtOAc 85:15); (Found: C, 65.5; H, 4.8. Calc. for $C_{37}H_{33}O_{10}N_3$: C, 65.4; H, 4.9%); δ_H($CDCl_3$) 8.05-7.82 and 7.55-7.28 (20 H, 2 m, 4 *Ph*CO), 5.90 and 5.67 (each 1 H, 2 t, $J_{3,4}$, $J_{4,5}$ 9.7, H-3,4), 5.52 (1 H, dd, $J_{1,2}$ 7.8, $J_{2,3}$ 9.8, H-2), 4.84 (1 H, d, H-1), 4.65 (1 H, dd, $J_{6a,6b}$ 12.1, $J_{5,6a}$ 3.2, H-6[a]), 4.50 (1 H, dd, $J_{5,6b}$ 5.1, H-6[b]), 4.16 (1 H, ddd, H-5), 4.03-3.96 and 3.66-3.60 (each 1 H, 2 m, $OCH_2CH_2CH_2N_3$), 3.24 (2 H, dt, $OCH_2CH_2CH_2N_3$), 1.85-1.72 (2 H, m, $OCH_2CH_2CH_2N_3$); δ_C($CDCl_3$) 165.7, 165.1, and 165.0 (2 C) (4 Ph*C*O), 101.2 (C-1), 66.6 ($OCH_2CH_2CH_2N_3$), 63.0 (C-6), 47.8 ($OCH_2CH_2CH_2N_3$), 28.9 ($OCH_2CH_2CH_2N_3$).

3-Azidopropyl 4,6-*O*-benzylidene-β-D-glucopyranoside (7): To a solution of **6** (6.2 g, 9.1 mmol) in MeOH (60 mL) was added solid sodium methoxide until pH 9 was reached. After 2 h, when TLC (CH_2Cl_2-MeOH 9:1) showed the formation of a new spot (R_f 0.13), the mixture was neutralised with Dowex H^+, filtered, and concentrated. To a solution of the residue in DMF (40 mL) were added benzaldehyde dimethyl acetal (BDMA) (2.7 ml, 18 mmol) and *p*-TsOH (until pH 4) under argon. After stirring for 25 h, the mixture was neutralised with solid Na_2CO_3, filtered, and co-concentrated with toluene. The residue was purified by column chromatography (toluene-EtOAc 6:4) to obtain **7** (2.24 g, 70%). $[\alpha]_D^{20}$= -38 (*c* 0.2); R_f 0.15 (toluene-EtOAc 6:4); (Found: C, 54.5; H, 6.1. Calc. for $C_{16}H_{21}O_6N_3$: C, 54.7; H, 6.0%); δ_H($CDCl_3$) 7.50-7.46 and 7.38-7.34 (5 H, 2 m, *Ph*CH), 5.51 (1 H, s, PhC*H*), 4.37 (1 H, d, $J_{1,2}$ 7.7, H-1), 4.33 (1 H, dd, $J_{6a,6b}$ 10.2, $J_{5,6a}$ 4.8, H-6[a]), 3.96 and 3.67 (each 1 H, 2 dt, $OCH_2CH_2CH_2N_3$), 3.77 and 3.52 (each 1 H, 2 t, $J_{3,4}$, $J_{4,5}$ 9.1, H-3,4), 3.76 (1 H, t, $J_{5,6b}$ 9.9, H-6[b]), 3.47 (1 H, $J_{2,3}$ 9.0, dd, H-2), 3.41 (2 H, t, $OCH_2CH_2CH_2N_3$), 1.93-1.85 (2 H, m, $OCH_2CH_2CH_2N_3$); δ_C($CDCl_3$) 136.8 (*Ph*CH, quaternary C), 129.2, 128.2 (2 C), and 126.2 (2 C) (*Ph*CH), 103.1 (Ph*C*H), 101.8 (C-1), 68.5 (C-6), 67.0 ($OCH_2CH_2CH_2N_3$), 48.2 ($OCH_2CH_2CH_2N_3$), 28.9 ($OCH_2CH_2CH_2N_3$).

3-Azidopropyl 2,3-di-*O*-benzoyl-β-D-glucopyranoside (8): To a solution of **7** (1.7 g, 4.8 mmol) in CH_2Cl_2 (30 mL) and Et_3N (4 mL) was added dropwise benzoyl chloride (1.7 mL, 14.5 mmol). After stirring for 4 h, the mixture was diluted with CH_2Cl_2, washed twice with 10% aqueous NaCl, and the organic layer was dried, filtered, and concentrated

(R_f 0.72, toluene-EtOAc 6:4). To a solution of the residue in CH_2Cl_2 (7 mL) were added CF_3COOH (2.7 mL) and H_2O (0.4 mL). After 4 h, the mixture was diluted with CH_2Cl_2, washed with 10% aqueous $NaHCO_3$ until neutral pH and 10% aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (CH_2Cl_2-EtOAc 9:1) to obtain **8** (2.1 g, 92%). $[\alpha]_D^{20}$= +33 (*c* 1); R_f 0.15 (toluene-EtOAc 6:4); (Found: C, 58.7; H, 5.3. Calc. for $C_{23}H_{25}O_8N_3$: C, 58.6; H, 5.3%); $\delta_H(CDCl_3)$ 8.10-7.94 and 7.51-7.33 (10 H, 2 m, 2 *Ph*CO), 5.45 (1 H, t, $J_{3,4}$ 9.8, H-3), 5.39 (1 H, dd, $J_{1,2}$ 7.6, $J_{2,3}$ 9.7, H-2), 4.73 (1 H, d, H-1), 3.28-3.21 (2 H, m, $OCH_2CH_2CH_2N_3$), 1.79-1.74 (2 H, m, $OCH_2CH_2CH_2N_3$); $\delta_C(CDCl_3)$ 167.3 and 165.2 (2 Ph*C*O), 100.9 (C-1), 66.3 ($OCH_2CH_2CH_2N_3$), 62.0 (C-6), 47.7 ($OCH_2CH_2CH_2N_3$), 28.8 ($OCH_2CH_2CH_2N_3$).

3-Azidopropyl (2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (11): A mixture of 3-azidopropyl 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside **9** [10] (0.20 g, 0.35 mmol), ethyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside **10** [11] (0.28 g, 0.52 mmol), and 4Å molecular sieves in CH_2Cl_2 (1.8 mL) was stirred for 30 min under argon. Then, 1.8 mL of a solution of NIS (0.44 g, 1.95 mmol) and TfOH (12.5 μl) in CH_2Cl_2-Et_2O (10 mL, 1:1) was added at 0 °C. After 15 min, the mixture was neutralised with pyridine, diluted with CH_2Cl_2, washed with 10% aqueous $NaHSO_3$ (2x) and 10% aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The crude product was purified by column chromatography (toluene-EtOAc 9:1) to give **11** (0.23 g, 65%). $[\alpha]_D^{20}$= +15 (*c* 0.6); R_f 0.33 (toluene-EtOAc 9:1); (Found: C, 65.9; H, 5.15. Calc. for $C_{57}H_{51}O_{16}N_3$: C, 66.2; H, 5.0%); $\delta_H(CDCl_3)$ 8.06-7.85 and 7.50-7.25 (30 H, 2 m, 5 *Ph*CO, *Ph*CH), 5.72 (dd) and 5.61 (t) (each 1 H, $J_{2,3}$, $J_{2',3'}$ 9.5, 9.8, H-3,3'), 5.43 and 5.36 (each 1 H, 2 dd, $J_{1,2}$, $J_{1',2'}$ 7.7, 7.9, H-2,2'), 5.21 (1 H, s, PhC*H*), 4.84 and 4.66 (each 1 H, 2 d, H-1,1'), 4.51 (1 H, dd, $J_{6a,6b}$ 12.0, $J_{5,6a}$ 1.9, H-6^a), 4.39 (1 H, dd, $J_{5,6b}$ 4.5, H-6^b), 3.88-3.81 and 3.55-3.48 (each 1 H, 2 m, $OCH_2CH_2CH_2N_3$), 3.16 (2 H, dt, $OCH_2CH_2CH_2N_3$), 1.78-1.63 (2 H, m, $OCH_2CH_2CH_2N_3$); $\delta_C(CDCl_3)$ 165.6, 165.3, 165.1, 165.0, and 164.8 (5 Ph*C*O), 136.5 (*Ph*CH, quaternary C), 101.8, 101.1, and 100.8 (PhC*H*, C-1,1'), 67.6 (C-6'), 66.4 ($OCH_2CH_2CH_2N_3$), 62.1 (C-6), 47.8 ($OCH_2CH_2CH_2N_3$), 28.8 ($OCH_2CH_2CH_2N_3$).

3-Azidopropyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (12): To a solution of **11** (0.23 g, 0.22 mmol) in CH_2Cl_2 (13 mL) were added CF_3COOH (0.15 mL) and H_2O (0.2 mL). After stirring for 3 h, the mixture was diluted with CH_2Cl_2, washed with 10% aqueous $NaHCO_3$ (2 x) and 10% aqueous NaCl, and the organic layer was dried, filtered, and concentrated. The residue was purified by column chromatography (toluene-EtOAc 9:1→1:1) to obtain **12** (0.19 g, 87%). $[\alpha]_D^{20}$= +61 (*c* 1); R_f 0.11 (toluene-EtOAc 8:2); (Found: C, 63.6; H, 5.1. Calc. for $C_{50}H_{47}O_{16}N_3$: C, 63.5; H, 5.0%); $\delta_H(CDCl_3)$ 8.04-7.88 and 7.50-7.33 (25 H, 2 m, 5 *Ph*CO), 5.71 and 5.24 (each 1 H, 2 t, $J_{2,3}$, $J_{2',3'}$ 9.6, 9.7, H-3,3'), 5.38 and 5.37 (each 1 H, 2 dd, $J_{1,2} = J_{1',2'} =$ 7.8, H-2,2'), 4.80 and 4.66 (each 1 H, 2 d, H-1,1'), 4.60 (1 H, dd, $J_{6a,6b}$ 12.1, $J_{5,6a}$ 2.1, H-6^a), 4.41 (1 H, dd, $J_{5,6b}$ 4.8, H-6^b), 3.89-3.82 and 3.55-3.48 (each 1 H, 2 m,

$OCH_2CH_2CH_2N_3$), 3.17 (2 H, dt, $OCH_2CH_2CH_2N_3$), 1.74-1.67 (2 H, m, $OCH_2CH_2CH_2N_3$); $\delta_C(CDCl_3)$ 100.8 and 100.7 (C-1,1'), 66.4 ($OCH_2CH_2CH_2N_3$), 62.3 and 61.4 (C-6,6'), 47.8 ($OCH_2CH_2CH_2N_3$), 28.8 ($OCH_2CH_2CH_2N_3$).

3-Azidopropyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyluronic acid)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (13): To a solution of **12** (0.14 g, 0.15 mmol) in CH_2Cl_2 (1.4 mL) were added a catalytic amount of TEMPO, KBr (1.7 mg), Bu_4NBr (2.3 mg) and saturated aqueous $NaHCO_3$ (0.3 mL). The mixture was stirred vigorously at 0 °C, when a mixture of saturated aqueous NaCl (0.3 mL), saturated aqueous $NaHCO_3$ (0.17 mL) and aqueous NaOCl (13% Cl active; 0.39 mL) was added dropwise over 15 min. After stirring for 45 min, the mixture was acidified to pH 4 with 4 M HCl, diluted with CH_2Cl_2, and the organic layer was washed with 10% aqueous NaCl, dried, filtered, and concentrated. The residue was purified by column chromatography. Impurities were eluted with CH_2Cl_2-acetone (8:2), and **13** (0.12 g, 85%) was eluted with CH_2Cl_2-acetone-HOAc (8:2:0.5). $[\alpha]_D^{20}$= +63 (*c* 2.1); R_f 0.50 (CH_2Cl_2-acetone-HOAc 8:2:1); (Found: C, 62.5; H, 4.9. Calc. for $C_{50}H_{45}O_{17}N_3$: C, 62.6; H, 4.7%); $\delta_H(CDCl_3)$ 7.97-7.82 and 7.55-7.31 (25 H, 2 m, 5 *Ph*CO), 5.86 and 5.47 (each 1 H, 2 t, $J_{2,3}$, $J_{2',3'}$ 9.0, 9.3, H-3,3'), 5.39 and 5.32 (each 1 H, 2 t, $J_{1,2}$, $J_{1',2'}$ 7.2, 7.5, H-2,2'), 5.00 and 4.74 (each 1 H, 2 d, H-1,1'), 3.14 (2 H, br t, $OCH_2CH_2CH_2N_3$), 1.75-1.60 (2 H, m, $OCH_2CH_2CH_2N_3$); $\delta_C(CDCl_3)$ 101.1 and 100.6 (C-1,1'), 66.4 ($OCH_2CH_2CH_2N_3$), 62.1 (C-6), 47.8 ($OCH_2CH_2CH_2N_3$), 28.8 ($OCH_2CH_2CH_2N_3$).

3-Aminopropyl β-D-glucopyranosyluronic acid-(1→4)-β-D-glucopyranoside (14): To a solution of **13** (0.12 g, 0.12 mmol) in MeOH (12 mL) was added NaOMe until pH 11. After stirring for 1.5 h, TLC (EtOAc-MeOH-H_2O 12:5:3) showed the formation of a new spot (R_f 0.35), and the mixture was neutralised with Dowex H^+, filtered, and concentrated. A solution of the residue in water was washed with CH_2Cl_2 (3x), and the aqueous layer was concentrated. MALDI-TOF analysis of the residue showed a peak at *m/z* 462 $(M+Na)^+$. Then, a solution of the residue in 0.1 M NaOH (1.3 mL) was added dropwise to a suspension of 10% Pd/C (2.5 mg) and $NaBH_4$ (10 mg) in bidistilled water (0.6 mL). After 4 h, when TLC (EtOAc-MeOH-H_2O 12:5:3) showed the formation of a ninhydrin-positive spot on the baseline, the mixture was acidified with Dowex H^+ (pH 4) and charged on a short column of Dowex 50 W × 2 (H^+, 200-400 mesh). After elution of contaminants with water, **14** was eluted with 1% NH_4OH. After concentration of the solvent and co-concentration of the residue with water (2x), **14** was obtained (49 mg, 88%). $\delta_H(D_2O)$ 4.51 (2 H, d, $J_{1,2} = J_{1',2'} = 7.7$, H-1,1'), 4.05 and 3.82 ($OCH_2CH_2CH_2NH_2$), 3.98 (1 H, br d, $J_{5,6a} < 1$, $J_{6a,6b}$ 12.1, H-6^a), 3.81 (H-6^b), 3.75 (1 H, d, $J_{4',5'}$ 8.8, H-5'), 3.66 (H-3), 3.61 (H-4), 3.52 (H-3',4'), 3.36 (1 H, t, H-2'), 3.33 (1 H, t, H-2), 3.16 (2 H, t, $OCH_2CH_2CH_2NH_2$), 2.04-1.96 (2 H, m, $OCH_2CH_2CH_2NH_2$); *m/z* (MALDI-TOF) 414 $(M+H)^+$, 436 $(M+Na)^+$.

Preparation of 16, 17 and 18

A suspension of disaccharide **14** (32 mg, 77 μmol) and 4-(2-carboxyethyl)-4-nitroheptane-1,7-dicarboxylic acid-tris-(*N*-hydroxysuccinimide) ester **15** (6.3 mg, 11.1 μmol) in pyridine (7 mL) was stirred at 65 °C for 8 h or 2 days, respectively. Then, the mixture was concentrated, dissolved in aqueous HOAc (2 mL, pH 4) and charged on a C18 Bakerbond spe™ column (500 mg). After elution of **14** with water (10 mL), the scaffold-containing compounds were obtained by elution with MeOH (5 mL), concentration and lyophilisation from water. Reaction for 8 h afforded **16** (3.2 mg, 35 %), **17** (5.1 mg, 35%), and **18** (2.3 mg, 12 %). Reaction for 2 days afforded **17** (1.5 mg, 16%) and **18** (13 mg, 63 %).

16: $[\alpha]_D^{20}$= -17° (*c* 0.4); $\delta_H(D_2O)$ see **18**, 2.35-2.31 (4 H, m, 2 HOC(O)CH_2CH$_2$C); *m/z* (ES LC-Q) 671.5 (M-H)$^-$.

17: $[\alpha]_D^{20}$= -18° (*c* 0.5); $\delta_H(D_2O)$ see **18**, 2.35-2.32 (2 H, m, HOC(O)CH_2CH$_2$C); *m/z* (ES LC-Q) 1066.3 (M-H)$^-$.

18: $[\alpha]_D^{20}$= -24° (*c* 0.5); $\delta_H(D_2O)$ 4.51 (3 H, d, $J_{1',2'}$ 8.0, 3 H-1'), 4.48 (3 H, $J_{1,2}$ 8.0, 3 H-1), 3.97 (3 H, br d, $J_{5,6a}$ < 1, $J_{6a,6b}$ 11.9, 3 H-6^a), 3.94 and 3.71 (each 3 H, 2 dt, 3 OCH_2CH$_2$), 3.81 (3 H-6^b), 3.77 (3 H-5'), 3.65 (3 H-3), 3.60 (3 H-4), 3.53 (3 H-3',4') 3.36 (3 H, t, 3 H-2'), 3.31 (3 H, t, 3 H-2), 3.28 (6 H, t, 3 CH$_2$CH_2NH), 2.26 (12 H, m, 3 C(O)CH_2CH_2C), 1.82 (6 H, m, 3 OCH$_2$CH_2); *m/z* (ES LC-Q) 1461.5 (M-H)$^-$.

SPR analysis

Instrumentation and reagents

A BIAcore 2000 instrument, BIAevaluation software 3.0 and sensor chip CM5 were from Pharmacia Biosensor AB (Uppsala, Sweden). The monoclonal antibody IgG 3.3, specific for the capsular polysaccharide of *S. pneumoniae* type 3, was prepared by Jansen *et al.* [20].

Immobilisation of the conjugates on the chip

After equilibration of the sensor surface with PBS buffer (pH 7.4) at a flow rate of 5 μL min^{-1}, the surface was activated with a 35-μL pulse of a mixture of freshly prepared 0.1 M *N*-hydroxysuccinimide and 0.1 M *N*-ethyl-*N*'-(dimethylaminopropyl) carbodiimide (1:1). A 2.0 μM neoglycoprotein solution in 100 mM NaOAc buffer (pH 5.0) was passed over the surface until sufficient incorporation was reached (< 5 μL). Remaining *N*-hydroxysuccinimide esters were blocked by injection of 1.0 M ethanolamine hydrochloride (pH 8.5) for 10 min. The level of bound neoglycoprotein was found to be 170 response units (RU) for the tetrasaccharide-CRM$_{197}$ conjugate and 160 RU for the glucose-CRM$_{197}$ conjugate.

SPR detection

All analyses were performed in duplo at a flow rate of 20 μL min^{-1} using PBS buffer (pH 7.4) as eluent in a Method run. As indicated in Figure 1a, various concentrations of IgG 3.3 were injected for 12 min (720 μL) over the tetrasaccharide-CRM$_{197}$ surface using the INJECT command. Regeneration was performed with a 50-μL pulse of saturated aqueous NaCl. As indicated in Figure 2, IgG 3.3 (0.5 μM) was premixed with various

concentrations of synthetic ligand, and 200 μL were passed over the surface using the INJECT command. Regeneration was performed with a 50-μL pulse of saturated aqueous NaCl.

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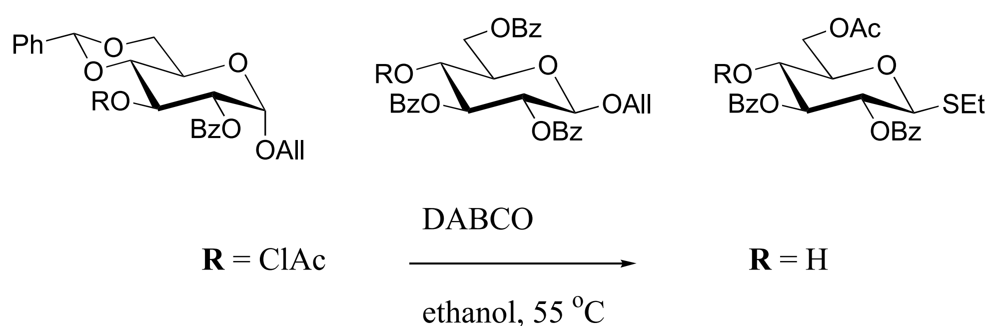
7

The use of diazabicyclo[2.2.2]octane as a novel highly selective dechloroacetylation reagent

Dirk J. Lefeber, Johannis P. Kamerling, and Johannes F.G. Vliegenthart

Abstract



In a study directed toward the use of the chloroacetyl protecting group in carbohydrate synthesis, the sterically hindered tertiary amine diazabicyclo[2.2.2]octane (DABCO) was found to give complete and selective cleavage of the chloroacetyl group in the presence of other ester functions such as benzoyl and acetyl groups at primary and/or secondary positions.

Introduction

The chloroacetyl group is used for the temporary protection of hydroxyl groups, as it is a very base-sensitive group that can be selectively cleaved in the presence of other acyl functions by a number of methods. In addition to, for example, aqueous ammonia [1] or hydrazine acetate [2], the most widely used deprotection reagent is thiourea [3]. The latter reaction is easy to perform but requires relatively long reaction times. The nucleophilic sulfur of thiourea displaces the chloride atom of the chloroacetyl group, and one of the amine functions subsequently attacks the ester bond intramolecularly [4]. Deprotection using hydrazine dithiocarbonate (HDTC), introduced by van Boeckel et al. [5], is based on the same reaction mechanism. It gives rise to fast and clean reactions but requires the preparation of the reagent just before use, since it is only stable for 15-20 min [6]. This paper reports a novel dechloroacetylation reagent, diazabicyclo[2.2.2]octane (DABCO), which turned out to be highly selective, fast, and easy to handle.

Results and discussion

In the course of the synthesis of oligosaccharides, we were confronted with problems in the deallylation of compound **1** to give **2** (Figure 1). The yield of the isomerization of the double bond using tristriphenylphosphine rhodium(I) chloride and DABCO (3 equiv) in refluxing ethanol was low, due to the formation of the dechloroacetylated monosaccharide derivative **3**, as shown by ^{1}H NMR (Figure 1) [7]. This observation stimulated further investigations toward the use of DABCO as a general dechloroacetylation reagent.

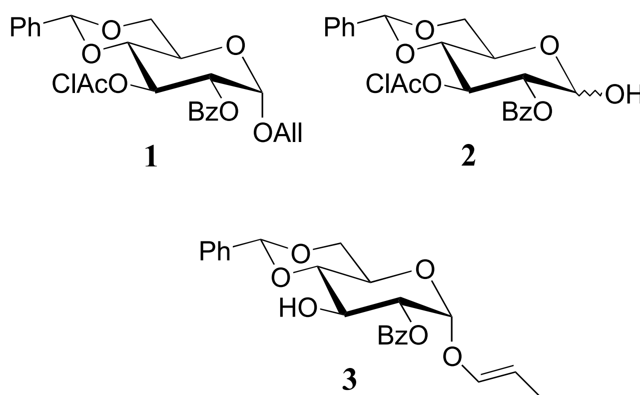


Figure 1. Deallylation of **1** (→**2**) and the formed side product **3**.

Complete dechloroacetylation of **1** to give **4** (Figure 2) was achieved by incubating 0.02 M **1** in ethanol in the absence of Wilkinson's catalyst with 5 equiv of DABCO

at room temperature for 6 h [8]. Isomerization of the double bond without affecting the chloroacetyl group was achieved by reaction of 5 mM **1** with Wilkinson's catalyst without DABCO in refluxing ethanol or by using only a catalytic amount of DABCO in either refluxing ethanol or refluxing 1:1 ethanol–toluene, the latter solvent system slowing down the dechloroacetylation. After reaction of the intermediate with *N*-iodosuccinimide and water, **2** was obtained in 77% yield [9].

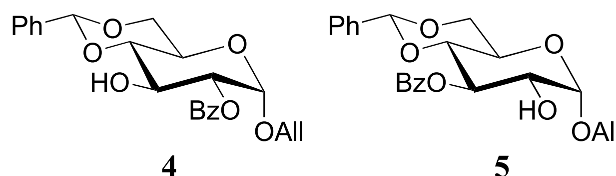


Figure 2. Dechloroacetylation of **1** afforded **4** and in some cases the migrated product **5**.

To gain insight into the factors influencing the reaction time needed for complete dechloroacetylation of **1** in ethanol, several parameters were varied (Table 1).

Table 1. Effect of the amount of DABCO, temperature, and concentration on the reaction time of the dechloroacetylation of **1** as carried out in ethanol.

Entry	DABCO (equiv)	Temp (°C)	Time (min)	Concn (M)
1	5	20	360	0.02
2	20	20	120	0.02
3	10	20	240	0.02
4	10	55	40	0.02
5	10	70	15	0.02
6	15	55	30	0.02
7	15	55	<10	0.06

By increasing the amount of DABCO, the reaction time was shortened from 6 to 2 h (Table 1, entries 1 and 2). Furthermore, the temperature of the reaction mixture had a marked influence on the reaction rate (entries 3, 4, and 5), and thereby on the reaction time. Using 15 equiv of DABCO at 55 °C, a sufficiently short reaction time was obtained (entry 6). By increasing the concentration of **1** from 0.02 to 0.06 M (Table 1, entries 6 and 7), the reaction time was further decreased from 30 min to less than 10 min. In all cases a quantitative conversion was detected from TLC

and ^{1}H NMR, after neutralization of the reaction mixtures with Dowex H^+, filtration, and concentration. No migration of the benzoyl group from O-2 to O-3 was observed. Only after 3 days of stirring, in the case of entries 1 and 2, about 5% migration (**4**→**5**, Figure 2) could be seen [10]. This migration also started to occur after more than 4 h of stirring for entries 4-6. Only in the case of entry 7, some migration was already found after 30 min. Therefore, in view of reaction time and practical ease, we preferred the reaction conditions described for entry 6.
To compare the effectiveness of DABCO with thiourea, the following experiments were performed. According to the commonly used dechloroacetylation with thiourea, 3 equiv of reagent, either thiourea or DABCO, was reacted with 0.03 M **1** in 5:1 ethanol–pyridine at 70 °C. Although in both cases excellent isolated yields were obtained, the use of DABCO resulted in much faster dechloroacetylation (Table 2, entries 1 and 2). Also following the established procedure (Table 1, entry 6), DABCO was found to give a much faster dechloroacetylation (Table 2, entries 3 and 4).

Table 2. Comparison of DABCO and thiourea in the dechloroacetylation of **1**.

Entry	Reagent	Solvent	Time (min)	Yield (%)
1	thiourea	ethanol/pyridine	90	93
2	DABCO	ethanol/pyridine	<10	95
3	thiourea	ethanol	270	97
4	DABCO	ethanol	30	94

In additional studies, two other tertiary amines, triethylamine (TEA) and *N*,*N*-diisopropylethylamine (DIPEA), were examined for their dechloroacetylating properties (Table 3).

Table 3. Dechloroacetylation of **1** using the tertiary amines TEA or DIPEA.

Entry	Base	Solvent	Temp (°C)	Time (h)	Yield (%)[a] (1:4:5)
1	TEA	ethanol	20	16	25:65:10
2	TEA	dichloromethane	20	1.5	no reaction
3	DIPEA	ethanol	20	16	25:65:10
4	DIPEA	dichloromethane	20	1.5	no reaction

[a] estimated from TLC

Incubation of **1** with 15 equiv of TEA or DIPEA in ethanol at room temperature resulted in partial dechloroacetylation. Before the dechloroacetylation was complete, about 10% migration of the benzoyl group had occurred from O-2 to O-3, resulting in the formation of **5** (Table 3, entries 1 and 3). In dichloromethane, no reaction was observed within 1.5 h at room temperature (Table 3, entries 2 and 4). These results clearly show that the selectivity of DABCO in the dechloroacetylation of **1** does not result solely from being a tertiary amine.
To further explore the generality of the procedure, compounds **6** and **8** (Figure 3) were subjected to the following reaction conditions [11]: (A) 0.02 M **6** or **8**, 15 equiv of DABCO, 55 °C, ethanol; and (B) 0.04 M **6** or **8**, 1 equiv of DABCO, 55 °C, 5:1 ethanol–pyridine.

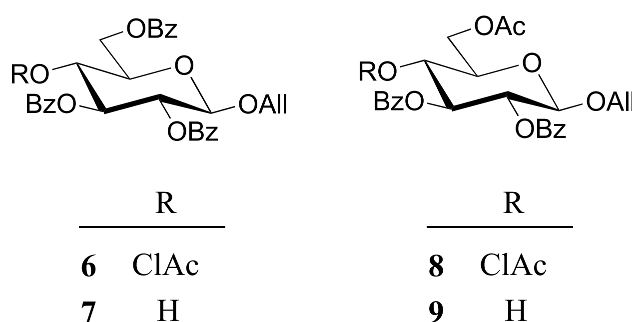


Figure 3. Dechloroacetylation of **6** and **8**.

Dechloroacetylation of **6** was found to be complete in 45 min using method A. Using method B, which is much milder, 2 h were needed for the conversion of **6** to **7**. In both cases the isolated yield was 95% [12]. Compound **8** was dechloroacetylated in 75 min using method A and in 2.5 h using method B. The isolated yields were 95% and 97%, respectively [13]. No migration could be observed by ^{1}H NMR. Although method A is much faster than method B, method B may be used when more basic conditions are expected to cause problems.
Performing the reaction according to method B without DABCO, complete dechloroacetylation of **6** and **8** required 6 and 7 h, respectively. This observation indicates that pyridine alone is also able to affect the chloroacetyl group but that DABCO greatly accelerates the reaction. Ethylenediamine, *o*-phenylenediamine, and 2-mercaptoethylamine [14] have also been mentioned as dechloroacetylation reagents. However, those reactions were performed with a large excess of pyridine and TEA in methanol. Our findings with pyridine and TEA show that these reagents themselves might not be the actual dechloroacetylating agents.

Conclusions

The results clearly show that the use of DABCO as dechloroacetylating agent provides a good method in addition to the commonly used reagents. Benzoyl and acetyl groups at primary and/or secondary positions are stable under the reaction conditions employed. The procedure is fast, highly selective, and easy to perform.

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[7] ^{1}H NMR analysis showed the removal of the chloroacetyl group (δ 4.00, 3.94: 2 d, each 1 H), a shift of H-3 from 5.87 to 4.40 ppm, and the presence of a 1-propenyl group (δ 6.11: dq, 1 H, $OCH{=}CHCH_3$; 5.19: dq, 1 H, $OCH{=}CHCH_3$; 1.52: dd, 3 H, $OCH{=}CHCH_3$).

[8] Characteristic ^{1}H NMR data of **4**: δ 5.87-5.74 (m, 1 H, $OCH_2CH{=}CH_2$), 5.57 (s, 1 H, PhC*H*), 5.21 (d, 1 H, H-1), 5.30-5.22, 5.15-5.10 (2 m, each 1 H, $OCH_2CH{=}CH_2$), 5.06 (dd, 1 H, H-2), 4.40 (t, 1 H, H-3).

[9] Characteristic NMR data of **2**: ^{1}H, δ 5.54 (s, 1 H, $PhCH^{\alpha}$), 5.52 (s, 1 H, $PhCH^{\beta}$), 5.88 (t, 1 H, H-3), 5.57 (d, 1 H, H-1$^{\alpha}$), 5.19 (dd, 1 H, H-2$^{\beta}$), 5.09 (dd, 1 H, H-2$^{\alpha}$), 4.92 (d, 1 H, H-1$^{\beta}$); ^{13}C, δ 101.5 (Ph*C*H), 95.8 (C-1$^{\alpha}$), 90.7 (C-1$^{\beta}$), 40.4 ($ClCH_2CO^{\alpha}$), 40.3 ($ClCH_2CO^{\beta}$).

[10] All signals in the ^{1}H NMR spectrum were slightly shifted. Strong shifts were observed for H-3, from 4.40 to 5.61 ppm, and for H-2, from 5.06 ppm to the bulk region.

[11] Purification: after complete reaction, the mixture was diluted with ethyl acetate, poured into 0.05 M HCl, and washed. The organic layer was dried ($MgSO_4$), filtered, and concentrated.

[12] Characteristic ^{1}H NMR data for **6**: δ 5.76 (t, 1 H, H-4), 4.86 (d, 1 H, H-1), 3.95 and 3.89 (2 d, each 1 H, $ClCH_2CO$). **7**: δ 4.81 (d, 1 H, H-1), 3.93 (dt, 1 H, H-4), 3.58 (d, 1 H, HO-4).

[13] Characteristic ^{1}H NMR data for **8**: δ 5.72 (t, 1 H, H-4), 4.76 (d, 1 H, H-1), 3.97 and 3.90 (2 d, each 1 H, $ClCH_2CO$), 2.12 (s, 3 H, CH_3CO). **9**: δ 4.73 (d, 1 H, H-1), 3.83 (t, 1 H, H-4), 2.12 (s, 3 H, CH_3CO).

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Summary

Bacteria containing a polysaccharide capsule e.g. *Streptococcus pneumoniae, Neisseria menigitidis* and *Haemophilus influenzae* can cause severe diseases like meningitis, pneumonia and otitis media. Especially young children are susceptible. A vaccine that consists of isolated polysaccharides from several *S. pneumoniae* serotypes affords protection in adults, but it is not effective in high risk groups such as young children. Neoglycoprotein vaccines against several encapsulated bacteria are now available, containing carbohydrate material derived from the polysaccharide capsule conjugated to a protein carrier. It has been well established that these neoglycoprotein vaccines have a marked impact on reduction of diseases, also in the high risk groups. However, the mechanisms underlying the immune response of this type of vaccines is still poorly understood. This is mainly due to the heterogeneity of the commercial vaccines, which hampers the investigation of the immune response at a molecular level. Furthermore, this heterogeneity complicates the analysis of the products, which is needed for routine product control. Finally, the use of carbohydrate material isolated from biological sources has the risk of being contaminated. To address these problems, neoglycoconjugates with a defined structure have to become available.
In this research program, neoglycoproteins have been prepared according to the structure of the capsular polysaccharide (CPS) of *Streptococcus pneumoniae* type 3. This serotype is present in both the 23-valent polysaccharide and 11-valent conjugate vaccines. Furthermore, it is often used to study the protective capacity of experimental vaccines because of its high virulence in mice. The capsular polysaccharide of this type is built up from disaccharide repeating units with the structure [→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→]. Both chemical synthesis and partial acid hydrolysis of the CPS have been carried out to obtain well-defined oligosaccharides. Neoglycoproteins were prepared containing the synthetic carbohydrates and their immunogenicity in mice was studied. Furthermore, glycoclusters were synthesised as an interesting class of defined compounds and their binding to the monoclonal antibody IgG 3.3 was investigated using surface plasmon resonance. Finally, a dechloroacetylation procedure was explored, needed for the synthesis of the oligosaccharides.

In **Chapter 1** the background of the bacterium *Streptococcus pneumoniae* is presented, including the course of infection, epidemiology, and vaccination. Furthermore, the preparation and analysis of neoglycoproteins are discussed and

the structural factors of the vaccine that might influence its immunogenicity. Finally, the research on experimental *S. pneumoniae* type 3 vaccines is reviewed.

In **Chapter 2** the development of a versatile synthetic strategy is described for the preparation of oligosaccharide fragments related to the CPS of *S. pneumoniae* type 3. Furthermore, their conjugation to carrier proteins in varying carbohydrate-protein ratios is reported. In this study, spacer-containing mono-, di-, tri-, and tetrasaccharide fragments have been synthesised. To this end, suitably protected D-glucose derivatives were coupled to obtain disaccharide building blocks for the stepwise construction of a protected oligosaccharide. For the coupling reactions, trichloroacetimidate donors were used with trimethylsilyl trifluoromethanesulfonate as a promoter. Benzylidene groups were introduced to protect HO-6 for introduction of carboxylic groups. Protected glucose moieties were converted into glucuronic acid by oxidation with TEMPO. Deprotected compounds were conjugated to CRM_{197}, TT or KLH *via* diethyl squarate, whereby the CRM_{197}-conjugates varied in oligosaccharide loading ranging from 3-12.

In **Chapter 3** the results are reported obtained by immunisation of mice with the CRM_{197}-conjugates. Groups of four mice were immunised twice subcutaneously, with a 3-week interval with each of the CRM_{197}-conjugates without adjuvant. Antibody titers were determined by a phagocytosis assay. A challenge experiment with a lethal dosis of *S. pneumoniae* type 3 was used to establish the protective capacity of the raised antibodies. The tri- and tetrasaccharide conjugates afforded complete protection, while the disaccharide conjugates afforded 75% protection. No difference in protective immunity was observed for conjugates with different oligosaccharide loading ranging from 3-12.

In **Chapter 4** the synthesis is described of a hexasaccharide fragment related to the capsular polysaccharide of *S. pneumoniae* type 3 to investigate the influence of a further increase of the saccharide chain length on the immunogenicity of the conjugates (Chapter 3). The synthesis of the hexasaccharide was achieved by using the synthetic strategy as described in Chapter 2, employing disaccharide building blocks, and TEMPO oxidation to convert protected glucose derivatives into glucuronic acid.

In **Chapter 5** an approach is presented for the analysis and isolation of pure fragments from a mixture obtained by partial acid hydrolysis of the CPS of *S. pneumoniae* type 3. Oligosaccharides of 1-7 repeating units were isolated on basis of their charge using anion-exchange chromatography on a Sepharose Q column.

High-pH anion-exchange chromatography using an AS11 column was used for the analysis of mixture composition and purity control. The purity and identity of the products was confirmed by ^{1}H NMR spectroscopy and nano-electrospray mass spectrometry.

In **Chapter 6** the preparation of glycoclusters is reported containing a disaccharide repeating unit of the CPS of *S. pneumoniae* type 3 to study the interaction of antibody with multivalent structures. To this end, the spacer-containing disaccharide repeating unit was synthesised and coupled to 4-(2-carboxyethyl)-4-nitropimelic acid. Mono-, di-, and trivalent compounds were isolated and their interaction with the monoclonal antibody IgG 3.3 was studied by surface plasmon resonance. Clustering of the disaccharides in this way resulted in a slightly higher affinity, whereas introduction of the hydrophobic scaffold caused a large increase in affinity.

In **Chapter 7** a novel dechloroacetylation procedure is described developed during the synthesis of the saccharide fragments as described in Chapter 2. The scope of this reaction, based on the use of diazabicyclo[2.2.2]octane (DABCO), was explored. Efficient removal of the chloroacetyl group in the presence of acetate or benzoate esters was accomplished in 30 min using 15 equivalents of DABCO in ethanol at 55 °C.

Samenvatting

Bacteriën met een polysacharide kapsel, bijvoorbeeld *Streptococcus pneumoniae, Neisseria menigitidis* en *Haemophilus influenzae*, kunnen ernstige ziektes veroorzaken zoals meningitis, longontsteking en middenoorontsteking. Vooral jonge kinderen zijn vatbaar voor deze ziektes. Een vaccin, dat bestaat uit geïsoleerde polysachariden van verschillende serotypes van *S. pneumoniae*, biedt volwassenen bescherming, maar is niet effectief in groepen met verhoogd risico zoals jonge kinderen. Tegen verschillende van deze ingekapselde bacteriën zijn nu glycoproteïne vaccins beschikbaar, die opgebouwd zijn uit koolhydraten afkomstig uit het kapsel gekoppeld aan een drager eiwit. Deze neoglycoproteïne vaccins hebben een dramatische daling van het aantal ziektes tot gevolg gehad, ook in de risicogroepen. Echter, de mechanismen die ten grondslag liggen aan de immuunrespons van dit type vaccins worden nog maar slecht begrepen. De hoofdreden hiervan is de heterogeniteit van de commerciële vaccins, die de bestudering van de immuunrespons op moleculair niveau belemmerd. Ook bemoeilijkt deze heterogeniteit de analyse van producten, hetgeen nodig is voor routinematige controles. Ten slotte bestaat bij het gebruik van koolhydraten uit biologisch materiaal het risico van verontreinigingen. Om deze problemen aan te pakken moeten neoglycoconjugaten met een gedefinieerde structuur beschikbaar komen.

In dit onderzoek zijn neoglycoproteïnen gemaakt naar de structuur van het polysacharide kapsel van *Streptococcus pneumoniae* type 3. Dit serotype is aanwezig in de 23-valente polysacharide vaccins en in de 11-valente conjugaat vaccins. Bovendien wordt dit type vaak gebruikt om het beschermend vermogen van experimentele vaccins te bestuderen, aangezien het sterk virulent is in muizen. Het polysacharide kapsel is opgebouwd uit repeterende eenheden van het disacharide [→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→]. Zowel chemische synthese als partiële zure hydrolyse van het polysacharide zijn uitgevoerd om goed gedefinieerde oligosachariden te verkrijgen. Met de synthetische koolhydraten werden neoglycoproteïnen gemaakt, waarvan de immunogeniciteit werd bestudeerd in muizen. Ook werden glycoclusters gesynthetiseerd als een interessante klasse van gedefinieerde verbindingen en de binding van deze structuren aan het monoklonale antilichaam IgG 3.3 werd onderzocht met surface plasmon resonantie. Ten slotte werd een methode voor dechloroacetylering bestudeerd, die ontwikkeld werd voor de synthese van de oligosacharide fragmenten.

In **Hoofdstuk 1** wordt een achtergrond gegeven over de bacterie *Streptococcus pneumoniae*. Deze omvat het verloop van een infectie, de epidemiologie en vaccinatie. Bovendien worden de bereiding en analyse van neoglycoproteïnen bediscussieerd alsmede de structuurelementen van het vaccin die de immunogeniciteit kunnen beïnvloeden. Ten slotte wordt een samenvatting gegeven van de experimentele vaccins met betrekking tot *S. pneumoniae* type 3.

In **Hoofdstuk 2** wordt de ontwikkeling beschreven van een flexibele syntheseroute om oligosacharide fragmenten te maken gerelateerd aan het polysacharide kapsel van *S. pneumoniae* type 3. Ook wordt de koppeling van deze fragmenten aan drager eiwitten vermeld in verschillende koolhydraat-eiwit verhoudingen. In deze studie zijn spacer-bevattende mono-, di-, tri- en tetrasacharide fragmenten gesynthetiseerd. Daartoe werden beschermde D-glucose derivaten gekoppeld tot disacharide bouwstenen voor de stapsgewijze opbouw van een beschermd oligosacharide. Voor de koppelingsreacties werden trichloroacetimidaat donoren gebruikt met trimethylsilyl trifluoromethaansulfonaat als promoter. Benzylideen groepen werden geïntroduceerd om de HO-6 positie te beschermen waar carboxyl groepen geïntroduceerd moeten worden. Beschermde glucose eenheden werden in glucuronzuur omgezet door oxidatie met TEMPO. Ontschermde verbindingen werden geconjugeerd aan CRM_{197}, TT of KLH via diethyl squaraat, waarbij de CRM_{197} conjugaten in oligosacharide belading varieerden van 3-12.

In **Hoofdstuk 3** worden de resultaten gepresenteerd die verkregen zijn met de immunisatie van muizen met de CRM_{197} conjugaten. Groepen van vier muizen werden twee keer subcutaan geïmmuniseerd met een tussenperiode van 3 weken met elk van de CRM_{197} conjugaten zonder adjuvans. Antilichaamtiters werden bepaald met een phagocytose assay. Een challenge experiment met een lethale dosis van *S. pneumoniae* type 3 werd gebruikt om het beschermend vermogen van de opgewekte antilichamen te bepalen. De tri- en tetrasacharide conjugaten gaven volledige bescherming, terwijl de disacharide conjugaten 75% bescherming boden. Er werd geen verschil in beschermende immuniteit waargenomen tussen conjugaten met verschillende oligosacharide belading variërend van 3-12.

In **Hoofdstuk 4** wordt de synthese beschreven van een hexasacharide fragment gerelateerd aan het polysacharide kapsel van *S. pneumoniae* type 3 om de invloed te onderzoeken van een grotere ketenlengte op de immunogeniciteit van de conjugaten (Chapter 3). De synthese van het hexasacharide werd bereikt door gebruik te maken van de syntheseroute die beschreven staat in Hoofdstuk 2,

waarbij gebruik wordt gemaakt van disacharide bouwstenen en oxidatie met TEMPO om beschermde glucose derivaten in glucuronzuur om te zetten.

In **Hoofdstuk 5** wordt een methode beschreven om zuivere fragmenten te verkrijgen van het polysacharide kapsel van *S. pneumoniae* type 3 door partiële zure hydrolyse. Oligosachariden van 1-7 repeterende eenheden werden geïsoleerd op basis van hun lading door gebruik te maken van anionenwisselingschromatografie op een Sepharose Q kolom. Hoge pH anionenwisselingschromatografie met een AS11 kolom werd gebruikt voor de analyse van de samenstelling van mengsels en voor de zuiverheidscontrole. De zuiverheid en identiteit van de producten werden bevestigd met ^{1}H NMR spectroscopie en nano-electrospray massa spectrometrie.

In **Hoofdstuk 6** wordt de bereiding van glycoclusters met een disacharide repeterende eenheid van het polysacharide kapsel van *S. pneumoniae* type 3 besproken om de interactie van een antilichaam met multivalente stoffen te bestuderen. Daartoe werd een spacer-bevattend disacharide gesynthetiseerd en gekoppeld aan 4-(2-carboxyethyl)-4-nitropimelic acid. Mono-, di- en trivalente stoffen werden geïsoleerd en hun interactie met het monoklonale antilichaam IgG 3.3 werd bestudeerd met surface plasmon resonantie. Het op deze manier clusteren van de disachariden resulteerde in een minimale verhoging van de affiniteit, terwijl introductie van de hydrophobe spacer een sterke verhoging veroorzaakte.

In **Hoofdstuk 7** wordt een nieuwe dechloroacetyleringsreactie beschreven die ontwikkeld werd tijdens de synthese van de oligosacharide fragmenten zoals beschreven in Hoofdstuk 2. De mogelijkheden van deze reactie die gebaseerd is op het gebruik van diazabicyclo[2.2.2]octaan (DABCO) werden verkend. Een efficiënte verwijdering van de chloroacetyl groep in aanwezigheid van acetaat of benzoaat esters werd tot stand gebracht in 30 min met behulp van 15 equivalenten DABCO in ethanol bij 55 °C.

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D.J. Lefeber, E. Aldaba Arévalo, J.P. Kamerling, and J.F.G. Vliegenthart, Synthesis of a hexasaccharide fragment of the capsular polysaccharide of *Streptococcus pneumoniae* type 3, submitted.

Dankwoord

Joyce, per se wilde ik jouw naam hier als eerste noemen. Ook de afgelopen vier jaar was je er weer altijd voor me, om me uit verschillende dips te halen, door je interesse in mijn werk en om mijn vele vragen over medische achtergronden te beantwoorden. Dank je wel.
Hans en Hans, ik wil jullie bedanken voor de mogelijkheid om een promotietraject in het Europese VACNET-programma te doorlopen. Vooral tijdens de verschillende buitenlandse bezoeken was er tijd voor discussies en ontspanning. Jullie interesse in het onderzoek heb ik altijd zeer gewaardeerd, waarbij ik vooral tijdens de laatste fase heb geleerd om nog kritischer naar het werk te kijken.
Mijn enthousiasme voor de bio-organische chemie is ontstaan in de groep van Rob Liskamp en John Kruijtzer, alhoewel de nadruk vanaf nu wat meer op "bio" zal liggen. Voor deze beginperiode wil ik jullie graag bedanken.
Koen, jou wil ik bedanken voor je enthousiaste begeleiding tijdens mijn hoofdvakperiode.
Tijdens mijn promotieperiode heb ik veel mensen meegemaakt bij de BOC. John, jouw relativeringsvermogen en loyaliteit op het lab waren belangrijk voor de "niet-te-erasen" sfeer bij de synthese. De etentjes met jou, Koen, Eric, half-syntheet Yuri en later ook Adriana waren altijd erg geslaagd (van gekruide gehaktballetjes tot Spaanse kip en eieren met aardappel!). De andere syntheten, Jutta, Britta, Dirk en Hiromi wil ik ook bedanken voor de sfeer op het lab en voor suggesties tijdens moeilijke synthesestappen.
Van de analyse wil ik met name Ricardo, Gerrit, Christian en Paul bedanken voor hun hulp bij verschillende analysetechnieken en Simon voor het opstarten van de Biacore-experimenten. Ook de mensen van de biokatalyse en eiwitvouwing zijn natuurlijk van belang geweest voor de gezelligheid op labdagen en tijdens sportwedstrijden, maar zeker ook voor de wederzijdse interesse in het onderzoek.
Anne Marie, Mieke en Merel wil ik bedanken voor al het (on)zichtbare werk waardoor de vakgroep zo goed draait.
Ingrid, Jan en Alloys wil ik bedanken voor de professionele uitstraling van mijn posters en presentaties, waarvoor op congressen altijd veel bewondering is.
So now and then, a Hungarian joined our group. They all knew many synthetic tricks and contributed to the atmosphere of the lab. Janos Kerékgyártó, Janos Hajkó and Karoly Ágoston, I would like to thank you for those periods.
Furthermore, I would like to thank all the people I met during the VACNET meetings. Besides the enjoyable time, we had valuable discussions about the

research. Especially, I would like to thank Daniela Proietti who helped me during my visit to Siena.
Harm Snippe, Barry Benaissa-Trouw, Kees Kraaijeveld en Wouter Jansen hebben mijn interesse gewekt in de immunologische achtergronden van dit proefschrift. Ik ben dan ook zeer dankbaar dat ik de mogelijkheid heb gekregen om me in die richting verder te ontwikkelen.
Door alle studievernieuwingen heb ik de mogelijkheid gekregen om veel studenten op de BOC te begeleiden voor twee tot drie weken: Mark, Daphne, Lisette, Mark, Ruben, Alexander, Ferrence en Peter. Een drietal studenten, Don, Nicole en Frank, heeft zich 10 weken ingezet voor het onderzoek en twee die-hards, Peter Steunenberg en Eneko Aldaba Arévalo hebben wel negen maanden geholpen bij het onderzoek. Ik wil jullie allemaal bedanken voor jullie inbreng en voor het bijdragen aan de sfeer op de synthesezaal.
Naast het drukke promotieleven bleef er gelukkig nog tijd over voor anderen. Bart, tijdens onze studie een leuke tijd als huisgenoten, bedankt voor je belangstelling en voor het feit dat je paranimf wilt zijn. De etentjes met jou, Machiel en Tjerk zorgden voor de nodige ontspanning. Ik hoop dat deze worden voortgezet ondanks de geografische afstand.
Tot slot wil ik natuurlijk mijn familie bedanken voor hun interesse en steun tijdens de afgelopen vier jaar.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 3 maart 1974 te Lent (gemeente Elst). In juni 1992 werd het gymnasium β diploma behaald aan het Johan de Witt Gymnasium te Dordrecht. Aan de Universiteit Utrecht werd in september 1992 begonnen met de studie scheikunde. De eerste onderzoeksstage, waarin de synthese van ureumpeptoïden in oplossing en op een vaste drager werd bestudeerd, werd uitgevoerd van april-december 1995 bij de vakgroep Medicinal Chemistry (Farmacie, Prof. Dr. R.M.J. Liskamp en Dr. J.A.W. Kruijtzer). In 1997 werd voor dit werk een nominatie voor de Organon research prijzen 1997 verkregen. De afstudeerstage, waarin gewerkt werd aan de chemo-enzymatische synthese van suikers van de parasiet *Schistosoma mansoni*, werd uitgevoerd van januari 1996-februari 1997 bij de vakgroep Bio-Organische Chemie (Scheikunde, Prof. Dr. J.P. Kamerling, Prof. Dr. J.F.G. Vliegenthart en Dr. K.M. Halkes). In september 1997 werd voor de afstudeerstage de Unilever research prijs verkregen. In april 1997 werd het doctoraal diploma behaald (*cum laude*).
Als assistent in opleiding werd in het kader van het europese project VACNET onderzoek gedaan bij de vakgroep Bio-Organische Chemie (Prof. Dr. J.P. Kamerling en Prof. Dr. J.F.G. Vliegenthart) naar de synthese van goed gedefinieerde neoglycoconjugaten ten behoeve van anti-pneumokokken vaccins. De resultaten hiervan zijn in dit proefschrift beschreven. Onderzoek uit deze periode werd gepresenteerd op de congressen 9th European Carbohydrate Symposium (Eurocarb$_9$, Utrecht, 1997), the first EuroConference on Carbohydrates in Drug Research (ECCDR1, Sardinië, 1999) en the 20th International Carbohydrate Symposium (ICS2000, Hamburg, 2000), alsmede bij de VACNET bijeenkomsten in Londen, Stockholm en Milaan.
De auteur is sinds augustus 2001 in dienst als onderzoeker bij het Eijkman-Winkler instituut voor microbiologie, sectie Vaccins.