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Preparation of synthetic oligosaccharide-conjugates of poly-β-(1→6)-*N*-acetyl glucosamine

Anikó Fekete [a,*], Dániel Eszenyi [c], Mihály Herczeg [a], Vince Pozsgay [b], Anikó Borbás [c]

[a] Department of Organic Chemistry, University of Debrecen, PO Box 20, H-4010 Debrecen, Hungary
[b] National Institute of Child Health and Human Development, National Institutes of Health, 31 Center Dr., MSC 2423 Bethesda, MD 20892-2423, United States
[c] Department of Pharmaceutical Chemistry, Medical and Health Center, University of Debrecen, PO Box 70, H-4010 Debrecen, Hungary

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ABSTRACT

Staphylococcus aureus and *Staphylococcus epidermidis* are prominent bacterial pathogens of nosocomial infections. Both microorganisms colonize medical devices by forming adherent biofilms. Poly-β-D-(1→6)-*N*-acetyl-glucosamine (PNAG) is a surface polysaccharide antigen which was found on both *S. aureus* and *S. epidermidis*. Animal studies have proved that PNAG can elicit antibodies which protect against staphylococcal infections. We have presented the synthesis of di-, tetra- and hexasaccharide fragments of PNAG with formyl-heptyl aglycone and their attachment to bovine serum albumin (BSA) by reductive amination.

1. Introduction

In the last few decades *Staphylococcus aureus* and *Staphylococcus epidermidis* have become the most dominant microorganisms that cause nosocomial infections in association with indwelling medical devices like intravascular catheters, pacemakers, prosthetic heart valves, peritoneal dialysis catheters and prosthetic joints, resulting in life-threatening illnesses. Both pathogens colonize biomaterials in adherent biofilms composed of multilayered cell clusters embedded in a slime matrix, which make the organisms more resistant to antibiotics.[1–3] The emergence of antibiotic resistance among staphylococcal isolates has made the prevention of staphylococcal infections by immunization more important.[4] A major component of the *S. aureus*[5] and *S. epidermidis*[6] biofilm matrix is a poly-β-D-(1→6)-*N*-acetyl-glucosamine (PNAG) surface polysaccharide antigen. Some animal studies have established that purified PNAG can elicit protective immunity against both species, suggesting that PNAG is a vaccine candidate for many clinically important strains of staphylococci.[5,7]

The use of vaccines based on carbohydrates against bacterial infections has become widespread by now. Heidelberger and Avery reported in their early work that a 'soluble specific substance' isolated from pneumococci was immunogenic in animals.[8] This substance consisted of mostly polysaccharides, namely capsular polysaccharides of pneumococci possessing serotype-specificity. Francis and Tillett demonstrated that purified pneumococcal capsular polysaccharides elicited anti-polysaccharide antibodies in humans.[9] First, McLeod et al. used these polysaccharides as vaccines.[10] However, the effective application of antibiotics against bacterial pathogens postponed the development of polysaccharide vaccines. Increasing occurrence of antibiotic resistance has resulted in renewed interest for prevention by vaccination in the late 1960s. Then several polysaccharide vaccines have been developed and licensed. Despite their success in adults, this type of vaccines has limitations. They are poorly immunogenic in infants under the age of two years old and elderly because polysaccharides are T cell independent antigens. In 1931 Avery and Goebel described that covalent attachment of capsular polysaccharides to proteins increased their immunogenicity.[11] Since glycoproteins are T cell dependent antigens therefore glycoconjugate vaccines are effective in both young children and elderly. The recognition, that low molecular weight fragments of capsular polysaccharides termed haptens in the form of protein conjugates can produce polysaccharide-specific antibodies, resulted in the development of oligosaccharide-conjugate vaccines.[12] Oligosaccharides obtained by chemical synthesis are homogeneous and their attachment to protein affords well-defined glycoconjugates, as well as their use as haptens makes possible the investigation of the influence of chain length and oligosaccharide loading of the protein into glycoconjugate on the immune response.[13]

Pier and his co-workers have conjugated purified PNAG and N-deacetylated derivative of PNGA termed dPNAG (degree of N-acetylation ~15%) to the carrier protein diphtheria toxoid (DT) and used them to immunize animals. They have found both conjugates were very immunogenic in mice and rabbits. Antibodies

* Corresponding author. Tel.: +36 52 512 900; fax: +36 52 512 744.
E-mail address: fekete.aniko@science.unideb.hu (A. Fekete).

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raised to the conjugates in rabbits mediated the opsonic killing of various staphylococcal strains, but the specificity of the opsonic killing was primarily to dPNGA. Passive immunization of mice with anti-dPGNA-DT rabbit sera showed significant levels of clearance of *S. aureus* from blood, whereas PNGA-specific antibodies were ineffective at clearing *S. aureus*.[14]

We planned to realize the synthesis of di-, tetra-, hexa- and octasaccharide fragments of PNAG bearing a formyl heptyl aglycone and their attachment to bovine serum albumin (BSA) by reductive amination to investigate the immunological properties of synthetic oligosaccharide-conjugates of PNAG. Meanwhile, Nifantiev and Pier, as well as their co-workers have synthesized penta- and nonasaccharide fragments of both poly-β-D-(1→6)-glucosamine and poly-β-D-(1→6)-*N*-acetyl-glucosamine. These oligosaccharides were conjugated to tetanus toxoid and used to immunize animals. They have found the N-acetylated oligosaccharide-conjugates elicited high titres of nonopsonic antibodies in mice, whereas the non-N-acetylated oligosaccharide-conjugates elicited highly active opsonic antibodies in mice and rabbits. In addition, they have realized that the antibodies arising from latter species showed excellent passive protective efficacy against *S. aureus* skin infections and *Escherichia coli* peritonitis.[15] Investigation of the oligosaccharide-BSA conjugates of PNAG that we synthesized is expected to provide further information for the development of vaccine against staphylococcal infections.

2. Results and discussion

Several syntheses of β-D-1,6-linked *N*-acetyl-glucosamine oligosaccharides have been published to date.[16–21] Earlier we have described the synthesis of β-(1-6)-linked *N*-acetyl-glucosamine oligosaccharides series with phenylthio aglycone (from disaccharide to pentasaccharide) and their application to investigate the substrate specificity of Dispersin B enzyme. For the synthesis of oligomers, 1+2, 2+2, 1+4 block syntheses were applied, using phenyl 1-thioglycosides as glycosyl acceptors and bromo-sugars as glycosyl donors.[22]

A similar approach was used to prepare hexasaccharide **4** and octasaccharide **7**. The formation of 1,2-*trans* interglycosidic bond has been ensured by 2-phtalimido protecting group; during the synthesis chloroacetyl group was used to protect temporarily the 6-hydroxy group and acetate esters were applied as permanent protecting groups. After bromination of disaccharide **1**[22] with bromine at room temperature the obtained bromo sugar **2** was coupled without purification with tetrasaccharide acceptor **3**[22] using AgOTf promotion to give the hexasaccharide **4** in 49% yield. (Scheme 1).

Tetrasaccharide **5**[22] was also converted into glycosyl bromide whose coupling with tetrasaccharide acceptor **3** afforded octasaccharide **7** in 39% yield. (Scheme 2).

Subsequently, the synthesized phenyl 1-thioglycosides **1**, **5**, **4** and **7** were reacted with 7-(1,3-dioxane-2-yl)-heptane-1-ol (**8**)[23] to yield the corresponding spacer-containing oligosaccharides **9**, **10**, **11** and **12** (Scheme 3).

Based on previous experiments, deblocking of the fully protected oligosaccharides **9**–**11** was performed by simultaneous dephthaloylation and deacetylation with ethylenediamine in ethanol, then the obtained amines were acetylated with acetic anhydride and pyridine to give **13**, **14** and **15** in 84%, 66% and 46% overall yields, respectively. We performed peracetylation after the removal of the phthaloyl groups since dephthaloylation resulted in a rather complex reaction mixture and isolation of the fully acetylated derivatives was easier by silica gel column chromatography than the selectively N-acetylated derivatives. Unfortunately, in the case of octasaccharide **12** the removal of the phthaloyl groups was unsuccessful. Finally, de-O-acetylation of **13**, **14** and **15** by Zemplén transesterification gave spacer-containing β(1-6)-linked GlcNAc oligosaccharides **16**, **17** and **18** (Scheme 3).

Before the conjugation step, the acetal protecting group of the aglycone was removed with acid hydrolysis, then aldehydes **19**, **20** and **21** were linked to the lysine ε-amino groups of BSA to form the corresponding Schiff bases, which were subsequently reduced with sodium cyanoborohydride to give the stable synthetic oligosaccharide conjugates **22a**, **22b**, **23a**, **23b** and **24a** (Scheme 4). Molecular mass determination of the synthesized glycoproteins was achieved by MALDI-TOF mass spectrometry.

To reach different densities of oligosaccharides on the protein two different molar ratios of oligosaccharide to BSA were applied in the case of disaccharide **19** and tetrasaccharide **20**. Hence, five oligosaccharide conjugates were synthesized (**22a**, **22b**, **23a**, **23b** and **24**) by means of the conjugation methods, possessing different number of sugar residues as shown in Table 1.

In conclusion, we have synthesized di-, tetra- and hexasaccharide fragments of PNAG with formyl-heptyl aglycone (**16**, **17** and **18**) and they have been covalently attached to BSA by reductive amination. Five oligosaccharide conjugates have been synthesized (**22a**, **22b**, **23a**, **23b** and **24**) which makes possible the investigation of the influence of oligosaccharide chain length and oligosaccharide density on the protein for the immune response.

3. Experimental

Optical rotations were measured at room temperature with a Perkin–Elmer 241 automatic polarimeter in $CHCl_3$. TLC was performed on Kieselgel 60 F254 (Merck) with detection by charring with 50% aqueous sulphuric acid. Column chromatography was performed on Silica gel 60 (Merck 63–200 mesh). The ^1H (360 MHz and 400 MHz) and ^{13}C NMR (90.54 MHz and 128 MHz) spectra were recorded with Bruker AM-360 and Bruker DRX-400 spectrometers. Internal references: TMS (0.000 ppm for ^1H), $CDCl_3$ (77.00 ppm for ^{13}C for organic solutions). The ^1H and ^{13}C NMR assignments have been established from 1D NMR spectra and the proton-signal assignments were supported by analysis of two-dimensional ^1H–^1H correlation spectra (COSY), as well as the carbon-signal assignments by two-dimensional ^{13}C–^1H correlation maps (HETCOR). MALDI-TOF MS analyses of oligosaccharides were carried out in the positive reflectron mode using a BIFLEX III mass spectrometer with saturated 2,4,6-trihydroxy-acetofenon in acetonitrile as matrix. MALDI-TOF MS analyses of the glycoproteins were carried out in positive linear mode using a BIFLEX III mass spectrometer (Bruker) with delayed-ion extraction. External calibration was applied using bovine serum albumin (6–8 mg/mL in 0.1% aq trifluoroacetic acid, TFA). TA solution was prepared by dissolving 0.1% TFA in a mixture of 2:1 acetonitrile–water. 10 μL sample solution (6–8 mg/mL in 0.1% aq TFA), 25 μL matrix solution (saturated 3,5-dimethoxy-4-hydroxycinnamic acid in TA) and 15 μL TA solution were mixed and 0.5 μL was applied to the target plate and allowed to dry at room temperature before analysis.

3.1. Typical procedure A for glycosylation reaction I

To a solution of starting material (0.29 mmol) in dry CH_2Cl_2 (10 mL) bromine (19 μL, 0.35 mmol) was added at room temperature. After stirring for 2 h the reaction mixture was concentrated. Dry toluene was added to and evaporated from the residue. To a solution of crude bromo sugar (0.29 mmol) and acceptor (0.24 mmol) in dry CH_2Cl_2 (10 mL) were added collidine (46 μL, 0.35 mmol) and 4 Å molecular sieves. After stirring for 30 min at