Russian Chemical Reviews **84** (11) 1100 – 1113 (2015) © 2015 Russian Academy of Sciences and Turpion Ltd

DOI 10.1070/RCR4574

Development of approaches to a third-generation carbohydrate-conjugate vaccine against *Streptococcus pneumoniae*: the search for optimal oligosaccharide ligands

M L Gening,[a] E A Kurbatova,[b] Yu E Tsvetkov,[a] N E Nifantiev [a]

[a] *N D Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky prosp. 47, 119991 Moscow, Russian Federation*
[b] *I I Mechnikov Research Institute for Vaccines and Sera, Malyi Kazennyi per. 5a, 105064 Moscow, Russian Federation*

The review addresses the application of synthetic oligosaccharides related to fragments of capsular polysaccharides from different serotypes of the bacterium *Streptococcus pneumoniae* for the design of third-generation pneumococcal conjugate vaccines. Special focus is given to characteristic features of the chemical structures of oligosaccharides required for the induction of the protective immune response when using synthetic glycoconjugate vaccines based on oligosaccharide ligands and carrier proteins.
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I. Introduction

Homo- and heteropolysaccharides are major components of the bacterial cell wall and capsule. Most of them belong to lipopolysaccharides (LPS) and capsular polysaccharides. Biopolymers of the latter type can be produced by both Gram-negative bacteria, such as *Neisseria meningitidis*, *Haemophilus influenzae* and *Salmonella typhi*, and Gram-positive bacteria, for example bacteria belonging to the *Streptococcus* and *Staphylococcus* genera. Polysaccharides not only provide the bacterial cell surface with mechanical protection but also can act as protective antigens and virulence factors. Carbohydrate structures located on the bacterial cell surface usually differ from those on the host cell surface. Consequently, the recognition of carbohydrates located on the bacterial cell surface by cells of the immune system is essential for the host, resulting in the elimination of bacteria from the host organism. It should be noted that some bacteria bear carbohydrates structurally similar to surface components of mammalian and human tissues, thus hindering their recognition by the immune system in infected cells.[1]

The immune response specific for surface bacterial polysaccharides can protect the organism from related bacterial pathogens.[1] In the 1970s, studies in the field of immunology showed that T lymphocytes play a role in the creation of immunological memory. These data, along with structural studies of surface polysaccharides, stimulated the development of vaccines based on these antigenic biopolymers.[1]

Streptococcus pneumoniae is one of the major bacterial pathogens and a cause of a large number of serious diseases in children and adults, including otitis media, pneumonia and meningitis. More than 90 serotypes of this bacterium were identified. The main virulence factors of the bacterium are capsular polysaccharides. However, out of the large number of serotypes of this bacteria, all of which have a characteristic capsular polysaccharide, only a rather small

M L Gening Candidate of Chemical Sciences, Senior Researcher at the ZIOC RAS. Telephone: +7(499)135 – 6433, e-mail: marina@mcl.ru
Current research interests: carbohydrate chemistry.
E A Kurbatova Doctor of Medical Sciences, Professor, Head of the Laboratory of Therapeutic Vaccines of the MRI for Vaccines and Sera. Telephone: +7(495)917 – 5774, e-mail: kurbatova6162@yandex.ru
Current research interests: immunology, vaccinology.
Yu E Tsvetkov Candidate of Chemical Sciences, Senior Researcher at the ZIOC RAS.
Telephone: +7(499)135 – 6433, e-mail: tsvetkov@ioc.ac.ru
Current research interests: carbohydrate chemistry.
N E Nifantiev Corresponding Member of the Russian Academy of Sciences, Doctor of Chemical Sciences, Professor, Head of the Laboratory of Chemistry of Glycoconjugates, ZIOC RAS.
Telephone: +7(499)135 – 6433, e-mail: nen@ioc.ac.ru
Current research interests: carbohydrate chemistry.

Received 1 June 2015
Uspekhi Khimii **84** (11) 1100 – 1113 (2015); translated by T N Safonova

group of pathogens (~20 serotypes) cause pneumococcal diseases. The predominant serotypes of this group can substantially differ in different geographical locations, as well as in different age groups of population.

First-generation polysaccharide vaccines consist of purified capsular polysaccharides. Thus, the first pneumococcal polysaccharide vaccine produced in 1945[2] was a tetravalent vaccine that included polysaccharides of serotypes 1, 2, 5 and 7. This vaccine rather effectively prevented human diseases caused by pneumococci of these four serotypes.

For historical reasons, *e.g.* due to the discovery of antibiotics, studies aimed at developing new pneumococcal carbohydrate vaccines were terminated for several decades. The next, 14-valent, pneumococcal vaccine was approved in 1977 (under the trademarks Pneumovax™, Pnu-Imune™ and Imovax Pneumo 14™). This vaccine contained polysaccharides of serotypes 1, 2, 3, 4, 5, 6A, 7F, 8, 9N, 12F, 18C, 19F, 23F and 25F, which covered ~70%–80% of the serotypes epidemiologically important in the United States at that time.[3]

Combined efforts of several research centres culminated in 1983 in the development of the 23-valent pneumococcal polysaccharide vaccine (Pneumovax™23, Pnu-Imune™23, Pneumo 23™), which covered up to 87% of causative agents of invasive pneumococcal diseases in the United States.[4] However, since polysaccharides are T-independent antigens, the immunization with these vaccines does not induce immunological memory. Besides, first-generation polysaccharide vaccines were found to be not immunogenic in children younger than two years of age and elderly persons, as well as in patients with immunodeficiency.[5–7]

The problems of low immunogenicity and the absence of immunological memory in response to polysaccharides were solved by creating second-generation carbohydrate vaccines — the related capsular polysaccharides conjugated to carrier proteins. These conjugates can initiate the T-cell immune response against carbohydrate antigens. The involvement of T cells in the immune process leads to generation of immunological memory and the formation of IgG antibodies capable of activating the complement. This immune response pathway makes carbohydrate conjugate vaccines immunogenic for infants as well.[8–10]

The first pneumococcal conjugate vaccine was licensed in 2000 (under the trademarks Prevnar™ and Prevenar™) and consisted of conjugated polysaccharides from bacteria of seven serotypes — 4, 6B, 9V, 14, 18C, 19F and 23F.[11] This product has proven to be highly effective for the immunization of children under 2 years of age. However, the extensive use of this vaccine, for example, in the United States [12–14] resulted in a sharp rise in pneumococcal serotypes, the capsular polysaccharides of which were not included in this conjugate vaccine. Therefore, the 10-valent conjugate vaccine (Synflorix™) containing conjugated polysaccharides of types 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F received market authorization in 2009 to substitute the 7-valent vaccine.[3] The 13-valent conjugate vaccine (Prevnar™ 13 or Prevenar™ 13) containing polysaccharides of pneumococcal types 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F was licensed in 2010.[3]

Despite the efficacy of the above-considered first- and second-generation pneumococcal vaccines, they have serious disadvantages limiting their use. Thus, the isolation of bacterial polysaccharides from live bacterial cultures and their purification, as well as the control of the purity of the resulting vaccines, are rather complicated and labourious procedures. The control of the purity is particularly essential because the presence of even insignificant bacterial impurities can lead to vaccine side effects. The high-molecular-weight of polysaccharide ligands also interferes with their conjugation to carrier proteins and makes it essentially impossible to strictly control the composition of the final conjugate. The high cost of these vaccines due, in particular, to the complexity of their production is another substantial barrier to their application. Besides, as mentioned above, the wide use of polysaccharide vaccines leads to a change in the prevalent pneumococcal serotypes and does not prevent the activation of serotypes of this causative agent, including antibiotic-resistant strains, which are not covered by the protection of the vaccine used. Moreover, the approved pneumococcal vaccines not fully take into account the regional differences in the pneumococcal serotype distribution. Therefore, the development of new pneumococcal carbohydrate vaccines remains a topical problems and requires new efficient approaches, which would address shortcomings of first- and second-generation vaccines.

Due to considerable progress in carbohydrate chemistry, procedures were developed for the synthesis of rather complex oligosaccharides, which enables researchers to focus on the creation of third-generation conjugate vaccines bearing synthetic oligosaccharide ligands in the optimal configuration. For this purpose, targeted synthesis of a representative group of oligosaccharides related to capsular polysaccharide fragments is performed. Oligosaccharide ligands thus obtained are conjugated to vaccine carrier proteins. Modern physicochemical methods allow researchers to monitor the course of conjugation and determine the number of ligands involved in the vaccine conjugate. The conjugates are tested by a combination of immunological techiques in order to determine the mimimal active oligosaccharide ligand, the inclusion of which in the vaccine conjugate induces the required immune response. Besides, it is possible to efficiently determine the structure of the epitope in the oligosaccharide ligand essential for the induction of protective antibodies using modern methods of investigation of carbohydrate–protein interactions, including studies of the binding of monoclonal antibodies to series of oligosaccharide ligands related to different fragments of capsular polysaccharide chains.[15]

Vaccine conjugates based on synthetic oligosaccharides have some advantages over conjugated polysaccharides produced from bacterial biomass. Synthetic ligands are not only free of microbial components but also have a strictly defined structure and high purity. In addition, they contain special spacer groups at known sites, thus making it possible to perform the controlled conjugation to a carrier protein. Besides, this facilitates the standardization of the vaccine production stages. It should be emphasized that the design of vaccine oligosaccharide ligands involves the optimization of the structural parameters that influence the specificity and efficacy of the immune response, particularly, the optimization of the size of the oligosaccharide ligand and the sequence of monosaccharide units in the ligand. The application of vaccines containing exclusively optimal oligosaccharide ligands ensures the desired efficacy of immunization and reduces undesirable side effects, which are more difficult to control when using polysaccharides as vaccine components.[16]

The first example of carbohydrate conjugate vaccines based on a synthetic ligand was the vaccine against *Haemophilus influenzae*.[17] In many countries, specialized laboratories are currently performing works on the development of carbohydrate conjugate vaccines based on synthetic oligosaccharide ligands to prevent various bacterial, fungal and viral diseases (see reviews [15, 18, 19]), including conjugates that induce the protective effect against pathogens of different taxonomic groups.[20, 21]

In the present review, we discuss the data on the structures and activity of synthetic oligosaccharide ligands related to the structure of pneumococcal capsular polysaccharides of particular serotypes, which were examined as vaccine components. We consider only publications that include the results of immunological studies of the corresponding carbohydrate–protein conjugates, which can be used to draw conclusions about the influence of the structure of the oligosaccharide ligand on the antigenic and immunological properties of both the ligands and their protein conjugates. In particular, serogroup 19 is beyond the scope of the present review because, although the synthesis of capsular polysaccharide fragments of this serogroup has been considered in sufficient detail,[22–25] the results of immunological assays of synthetic oligosaccharides are lacking in the literature. We do not consider the influence of such essential parameters as the type of the carrier protein and methods of conjugation of oligosaccharide ligands to carrier proteins on immunological properties. However, in particularly important cases, the ligand-to-protein ratios for some vaccine conjugates and the influence of this ratio on the efficacy of synthetic immunogens are given.

II. Search for oligosaccharide ligands for the design of a third-generation pneumococcal glycoconjugate vaccine

II.1. *Streptococcus pneumoniae* type 3

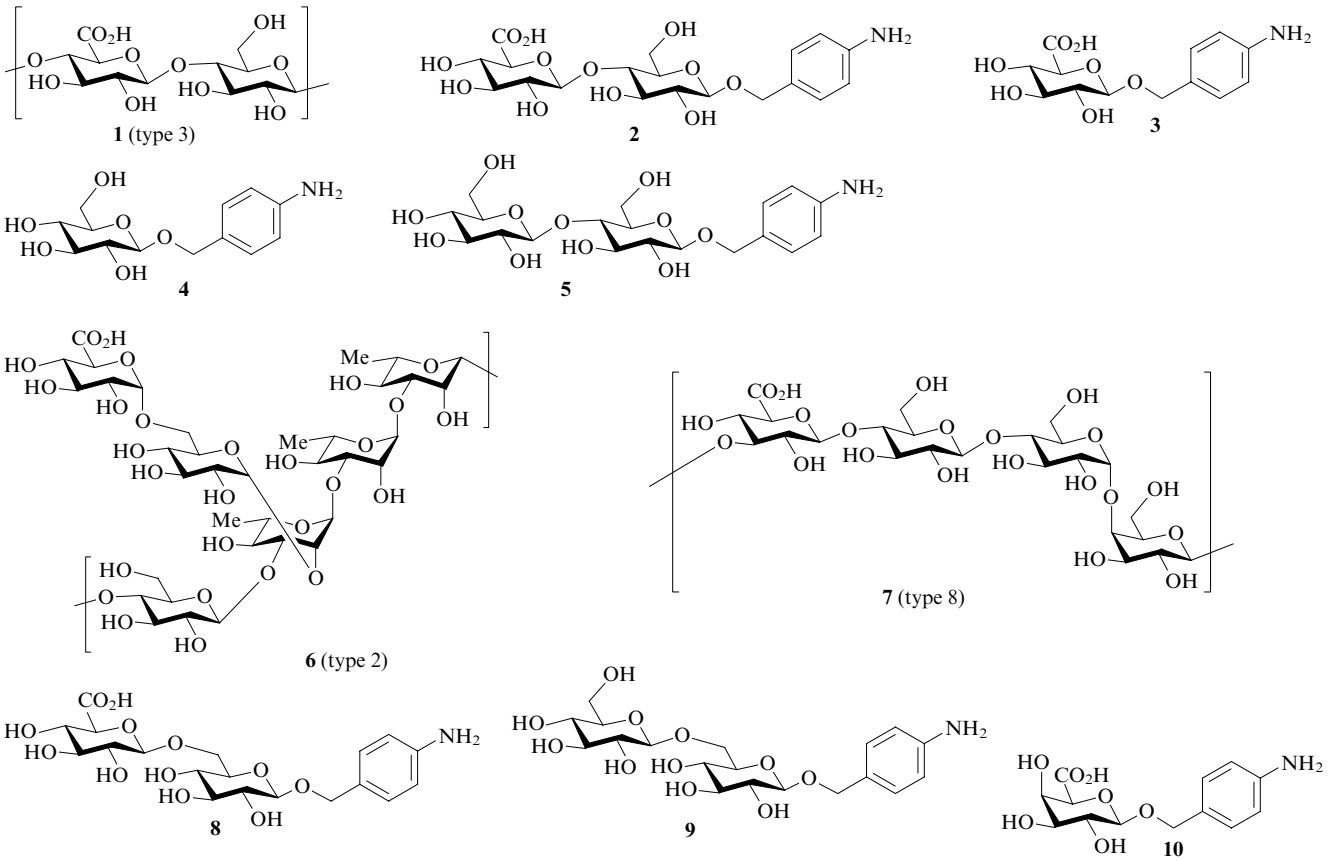
The very first experiments on the construction of oligosaccharide–protein conjugates related to *S. pneumoniae* type 3 capsular polysaccharide with repeating unit **1** † and the evaluation of their immunological activity were performed in Goebel's laboratory [26, 27] more than 80 years ago. The sequence of immunological tests and the logic of the selection of oligosaccharide antigens in these experiments served as the reference line for many subsequent studies on the development of oligosaccharide conjugate vaccines.

The structure of the repeating unit of this polysaccharide derived from the disaccharide cellobiuronic acid was reliably determined at the time the studies discussed herein were made. Initially, capsular polysaccharide conjugated to horse serum globulin was produced.[26] This conjugate, when injected in rabbits, induced antibodies specific for *S. pneumoniae* type 3 polysaccharide and produced active immunity against virulent strains of these bacteria.[27]

Somewhat later, this research group demonstrated that it is necessary to use protein conjugates of small synthetic oligosaccharides related to *S. pneumoniae* type 3 capsular polysaccharide fragments with a strictly defined structure

† Hereinafter, the repeating unit for natural polysaccharides is given in square brackets; the index denoting the number of repeating units is omitted because this value is not discussed.

Structures 1–10



for the detailed investigation of the factors responsible for immunological specificity of bacterial polysaccharides.[28] For this purpose, aminobenzyl glycoside of cellobiuronic acid (**2**), as well as related derivatives of glucuronic acid (**3**), glucose (**4**) and cellobiose (**5**), were conjugated to horse serum globulin.[28]

All four conjugates induced the formation of immunoglobulins specific for homologous oligosaccharide antigens. This was established by the precipitation reaction of conjugate antisera with ligands **2**–**5** conjugated to chicken globulin in order to prevent the reaction with antibodies specific for horse globulin, which was used as the carrier protein. It was found that only the antiserum to conjugated cellobiuronic acid **2** can recognize bacterial capsular polysaccharide **1** (as well as its protein conjugate).[28] Moreover, a conjugate of ligand **2** was also able to kill virulent strains of *S. pneumoniae* type 3 after the active immunization of rabbits.[29]

Attempts to induce immunity against *S. pneumoniae* type 3 infection in mice, rabbits, goats and horses using conjugated derivatives of glucuronic acid **3**, glucose **4** and cellobiose **5** failed.[29] Meanwhile, the antiserum to the conjugate of cellobiuronic acid **2** exhibited protective properties not only against *S. pneumoniae* type 3 but also against pneumococcal types 2 and 8 in passively immunized rabbits. It should be noted that the structures of capsular polysaccharides of type 2 (**6**) and type 8 (**7**) were not precisely known at the time these experiments were performed; it has been only shown that type 8 polysaccharide also contains a cellobiuronic acid fragment.[30] This structural identity was responsible for cross-specificity of the conjugate based on ligand **2**.

The experimental results suggested that the oligosaccharide ligand should contain both the glucose and glucuronic acid residues to induce the immune response against *S. pneumoniae* type 3. Out of all the ligands **2**–**5** studied at that time, both these conditions are met only for cellobiuronic acid **2**. In order to verify the role of the direction of the glycosidic linkage between monosaccharide units in the vaccine ligand, the isomeric ligand was synthesized based on gentiobiuronic acid (**8**) that contain the β-(1 → 6)- rather than β-(1 → 4)-glycosidic linkage, as in ligand **2**. In addition, gentiobioside ligand **9** containing no carboxy groups was tested.[31]

Horse serum globulin was conjugated to ligands **8** and **9** and galacturonic acid derivative **10**. The immunization of rabbits with the conjugates based on ligands **8**–**10** produced antibodies specific for homologous antigens; however, cross-reactive antibodies were also detected. Thus, antibodies against conjugated cellobiuronide **2** recognize gentiobiuronide **8** and *vice versa*. It was hypothesized that this cross-reactivity can be due to the presence of the glucuronic acid residue in both ligands. This hypothesis was confirmed also by the fact that the antiserum to cellobiuronic acid **2** did not react with conjugated gentiobioside **9**, which did not contain the glucuronic acid residue. However, tests on the inhibition of the precipitation reaction showed that the reaction of antisera with homologous antigens is most specific.[31] In all cases, the homologous reaction was inhibited exclusively by homologous glycoside.

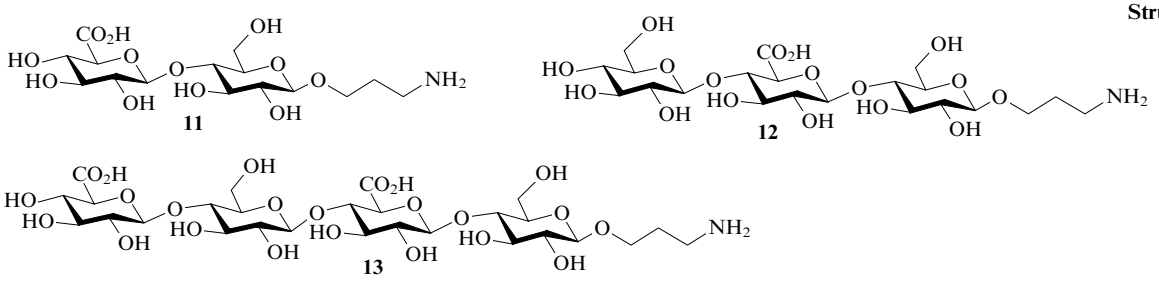
The reaction of oligosaccharide conjugates of horse antisera to type 2, 3 and 8 pneumococci produced after horse immunization with whole-cell preparations of these bacteria was also studied. It was found that the conjugated derivatives of cellobiuronic acid **2**, glucuronic acid **3** and gentiobiuronic acid **8** give a precipitation reaction with antisera to all the above-mentioned three serotypes.[31] It was hypothesized that in all cases the reaction occurs because these sera contain antibodies against glucuronic acid induced by bacterial antigens, which also contain a glucuronic acid fragment. The latter, in the authors' opinion, should be involved in bacterial polysaccharides. The experiments on the inhibition of the precipitation reaction showed that only the reaction of conjugated cellobiuronic acid with antisera to serotypes 3 and 8 is specific, which is consistent with the current data on the structures of the related polysaccharides (**1**, **6** and **7**).

Unlike the antiserum to conjugated cellobiuronic acid **2**, which exhibited protective activity against *S. pneumoniae* types 2, 3 and 8 in passively immunized mice, the antiserum to conjugated gentiobiuronic acid **8** had protective properties only against serotype 2.[31] It was also shown that protective antibodies that are present in the antiserum to gentiobiuronic acid **8** are specifically adsorbed onto the encapsulated form of *S. pneumoniae* type 2 (bacterial cells devoid of a capsule served as the control). The antiserum to gentiobioside **9** did not have protective properties. This also provided evidence that the glucuronic residue plays an essential role in the ligand.

At the moment of the investigation, the fact that antisera to conjugates of disaccharides **2** and **8** have protective properties against serotype 2, whereas only the serum to a conjugate of acid **2** was active against serotype 3 seemed to be a paradox [31] because the structure of type 2 polysaccharide was unknown. It was suggested that antibodies against the glucuronic acid residue that is present in both ligands play the major role in the reaction with serotype 2, whereas the full structure of cellobiuronic acid is required for the reaction with serotype 3. In order to verify this hypothesis, mice infected with *S. pneumoniae* type 2 were passively immunized with antisera to conjugates of the simplest monosaccharide ligands based on glucuronic (**3**) and galacturonic (**10**) acids, as well as glucose (**4**). Of these three antisera, only the antiserum to conjugated glucuronic acid was able to protect mice infected with 100 000 minimum lethal doses of serotype 2, but it was not effective against serotype 3. In this case, protective antibodies also specifically bound to serotype 2 capsular polysaccharide.[31] In this study, long before the complete determination of the structure of *S. pneumoniae* type 2 capsular polysaccharide (**6**) by immunological methods, the presence of the glucuronic acid residue in **6** was predicted. It was mentioned for the first time that even monosaccharide can act as a hapten in the protein conjugate and induce the formation of protective antibodies.[31] It should be noted that the rabbit antiserum that was produced after immunization with a conjugate based on gentiobiuronic acid **8** possessed passive protective activity in mice infected with type 2 pneumococci despite the difference in the anomeric configuration of the glucuronic acid residue in ligand **8** and natural capsular polysaccharide **6**.

More than 60 years after the pioneering studies of Goebel and co-workers,[26–31] researchers resumed investigations aimed at determining the minimal oligosaccharide structure required for the induction of protective immunity against *S. pneumoniae* type 3. The commonly used vaccine carrier protein CRM197 (cross-reactive material — a recombinant nontoxic mutant of diphtheria toxin) was

Structures 11 – 13



conjugated to oligosaccharide fragments of type 3 pneumococcal capsular polysaccharide having various chain lengths: disaccharide **11**, trisaccharide **12** and tetrasaccharide **13**.[32–34] The activity of these conjugates was tested in actively immunized mice. The immunization of all animals with conjugates of tri- (**12**) and tetrasaccharides (**13**) elicited equally high titers of antibodies that recognize type 3 polysaccharide (**1**). In the case of conjugated disaccharide ligand **11**, comparable titers of specific antibodies were observed in 75% of mice. All animals, the serum of which contained antibodies against capsular polysaccharide **1**, were alive after exposure to the lethal dose of *S. pneumoniae* type 3.[32] These results confirm that disaccharide fragment **11** is sufficient for the induction of protective immunity against type 3 pneumococci in mice.

The authors of the present review synthesized also oligosaccharide ligands **11** – **13** (the results will be published elsewhere) and prepared their bovine serum albumin (BSA) conjugates by dialkyl squarate chemistry (the average number of carbohydrate ligands linked to one protein molecule was 16 – 19). All conjugates inhibited the related specific antibodies in the antimicrobial rabbit serum. No considerable difference was observed in the inhibitory activity of the three conjugates under consideration. The mice immunized with the BSA-**11** conjugate produced specific antibodies against *S. pneumoniae* type 3 capsular polysaccharide (**1**). The blood serum of mice immunized with this conjugate in the presence of aluminium hydroxide enhanced opsonophagocytosis of acetone-inactivated microbial cells of pneumococcal serotype 3 with mouse peripheral blood neutrophils and monocytes. The active immunization of mice with the BSA-**11** conjugate combined with aluminium hydroxide (used as the adjuvant) resulted in the protection of mice from infection with *S. pneumoniae* type 3. It was shown for the first time that the single immunization with the BSA-**11** conjugate in the absence of the adjuvant led to an increase in the number of T lymphocytes with the surface molecules CD3, CD4, CD8 and γδT, B lymphocytes (CD5), NK and NKT lymphocytes, as well as marker-containing activated (CD4 + CD25 + T) lymphocytes in the mouse spleen.

Based on the available data, it can be hypothesized that disaccharide **11**, which is the single repeating unit of *S. pneumoniae* serotype 3 polysaccharide, contains a key protective epitope that is present also in capsular polysaccharide and, therefore, can be used to produce a conjugate vaccine that protects against infection with this serotype.[35]

II.2. *Streptococcus pneumoniae* type 6

Pneumococcal serotype 6, along with types 14, 19F and 23F (so-called pediatric serotypes), is especially frequent in small children, the immune response to type 6 being developed most slowly compared to all known *S. pneumoniae* serotypes.[36] The *S. pneumoniae* serogroup 6, which was discovered as early as in the 1920s, has long been divided into subtypes, 6A and 6B. The structures of their capsular polysaccharides composed of repeating units **14** and **15**, respectively, were determined in 1960.[37] The tetrasaccharide repeating units of these polysaccharides differ in the direction of the bond in the rhamnosyl-ribitol block: for type 6A, the (1 → 3) bond; for type 6B, the (1 → 4) bond. In 2007 subtype 6C was identified;[38] in 2010, subtype 6D.[39] Capsular polysaccharides of these subtypes are composed of repeating units **16** and **17**, respectively.

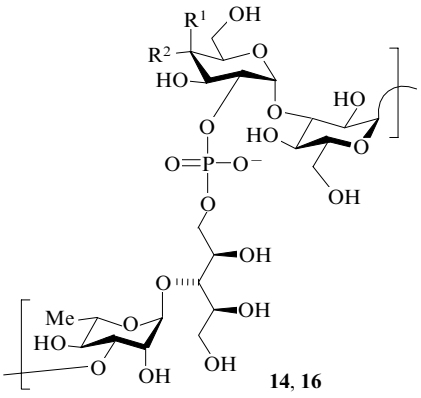
The synthesis of di- (**18**), tri- (**19**)[40] and tetrasaccharide (**20**)[41,42] ligands, which are related to fragments of type 6B polysaccharide (**15**) and contain the 3-aminopropyl spacer, was reported in the late 1990s. These ligands were conjugated to the carrier protein KLH (keyhole limpet hemocyanin; hemocyanin isolated from the marine gastropod *Megathura crenulata*), and the conjugates were used to immunize mice and rabbits.

Enzyme immunoassay (EIA) showed that the immunization of rabbits with conjugated tri- (**19**) and tetrasaccharides (**20**) induced cross-reactive antibodies, which were capable of binding to both type 6B (**15**) and type 6A (**14**) polysaccharides, whereas conjugated disaccharide **18** induced antibodies that selectively recognized exclusively type 6B polysaccharide (**15**). The results of studies of the phagocytic activity fully correlate with the enzyme immunoassay data. Thus, all three antisera were able to promote phagocytosis of type 6B pneumococci, whereas the antisera to three- and tetrasaccharide conjugates can promote phagocytosis of type 6A pneumococci. In experiments in mice, only the conjugate of tetrasaccharide **20** produced sustained levels of anti-6B antibodies in all immunized animals, whereas conjugated di- (**18**) and trisaccharide (**19**) ligands were less immunogenic.[43]

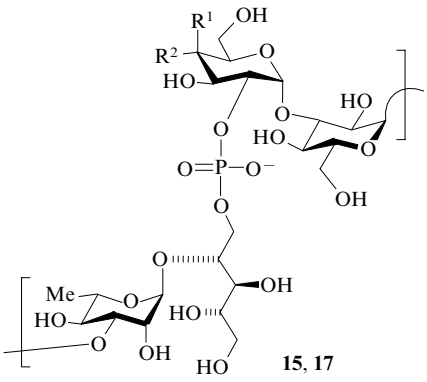
It should be noted that the titers of anti-6B antibodies, which were produced after immunization with conjugated tetrasaccharide **20**, were higher even than the titers of antibodies against type 6B capsular polysaccharide (**15**) conjugated to KLH, which was used as the control. In these experiments, the specificity of antibodies in mice differs from that in rabbits. Thus, mouse antibodies against conjugated di- and trisaccharides (**18** and **19**) equally well recognize type 6A and 6B polysaccharides (**14** and **15**), whereas mouse antibodies against tetrasaccharide **20** are much better bound to type 6B polysaccharide (**15**). As expected, only the antiserum to tetrasaccharide **20** exhibited phagocytic activity and was selective for type 6B pneumococci.[43]

The recognition of synthetic oligosaccharides in conjugates using sera from persons immunized with polyvalent polysaccharide vaccines was examined. Two commercially available vaccines, namely, 23-valent polysaccharide vaccine (Pneumovax) and 7-valent conjugate vaccine (Pn,

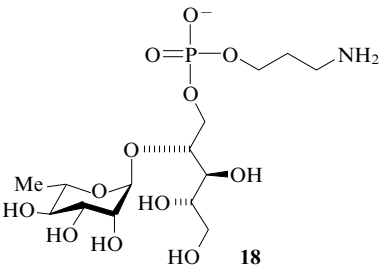
Structures 14–20



14, **16**



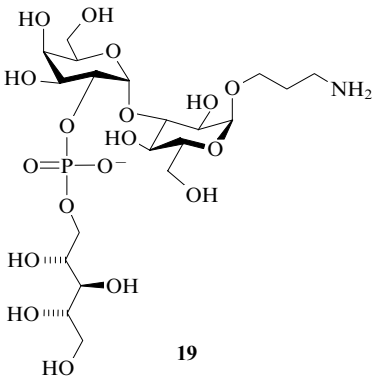
15, **17**



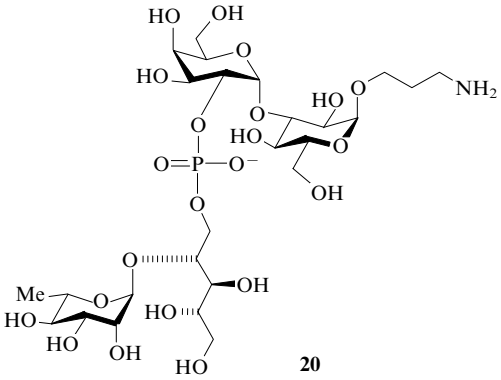
18

14 (type 6A): R^1 = OH, R^2 = H (D-Gal);
16 (type 6C): R^1 = H, R^2 = OH (D-Glc)

15 (type 6B): R^1 = OH, R^2 = H (D-Gal);
17 (type 6D): R^1 = H, R^2 = OH (D-Glc)



19



20

Wyeth-Lederle Vaccines and Pediatrics), which include 6B polysaccharide, were used. The reaction was observed only between the serum to 7-valent vaccine and conjugated tetrasaccharide **20**, and this reaction was inhibited by both type 6A polysaccharide and type 6B polysaccharide (KLH and type 14 and 17F polysaccharides were not inhibitors).[43]

In mice passively immunized with the rabbit serum to conjugated oligosaccharides **18**–**20**, all three antisera were able to protect mice from infection with type 6B pneumococci, while the antisera to conjugated tri- (**19**) and tetrasaccharides (**20**) provided protection against type 6A pneumococci as well. The active immunization was effective only in the case of the conjugate of tetrasaccharide **20**. This led to an important conclusion that disaccharide **18** and tetrasaccharide **20** are the minimal fragments capable of inducing protective antibodies in rabbits and mice, respectively.[43] The observed difference in the immunogenicity between mice and rabbits is probably attributed to the known fact that rabbits, unlike mice, can produce anticarbohydrate antibodies of two types: antibodies with a groove-like antigen-binding region, which can accommodate one or two monosaccharide units, and antibodies with an antigen-binding cleft capable of recognizing longer epitopes containing up to 7 monosaccharide units.[44]

The fact that human serum antibodies that were produced after immunization with pneumococcal polysaccharide vaccine were able to recognize the tetrasaccharide conjugate may indicate that tetrasaccharide **20** contains an epitope for the induction of anti-6B antibodies in humans. These results attest to the importance of the selection of an adequate animal model for immunological tests and additionally demonstrate that even small synthetic oligosaccharides involved in conjugates, which are composed of a required amount of oligosaccharide ligands conjugated to optimal carrier proteins, can elicit higher titers of the produced specific antibodies compared with bacterial polysaccharide conjugates.[45,46]

Since 2000, the polysaccharide conjugate vaccine (Prevnar and Prevenar) containing capsular polysaccharides of seven *S. pneumoniae* serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) was widely used in the United States. The application of this vaccine substantially decreased the number of cases of pneumococcal diseases; however, the emergence of serotypes not included in the vaccine has been a concern because these serotypes became more widespread in recent years.[12] As shown earlier, antibodies produced against type 6B polysaccharide are effective also against serotype 6A. Actually, the rates of serotype 6A decreased after the introduction of the 7-valent conjugate vaccine. However, a new serotype, 6C, was identified and an increase in the rate of this serotype was observed. It should be noted that the structure of *S. pneumoniae* type 6C capsular polysaccharide (**16**) substantially differs from that of type 6B polysaccharide (**15**) included in the vaccine, which accounts for the absence of antibodies with cross-specificity capable of inducing opsonization of *S. pneumoniae* type 6C.[38]

In order to find effective oligosaccharide ligands for a third-generation pneumococcal vaccine, it was suggested to examine their recognition by antibodies isolated from the human serum and specific for the related types of capsular polysaccharides. This would help to avoid any ambiguity in the interpretation of the results of immunization in different animal models.[47] This study was performed using the monoclonal antibody Dob1 specific for *S. pneumoniae* type

6B polysaccharide (**15**), which was isolated from the human serum after immunization with 23-valent polysaccharide vaccine,[48] combined with a series of BSA conjugates of oligosaccharide ligands, which are fragments of repeating units of capsular polysaccharides of types 6A (**21**–**23**) and 6B (**24**–**26**) serving as coating antigens for EIA.[49, 50]

The enzyme immunoassay showed that the monoclonal antibodies Dob1 recognize trisaccharide ligands **22** and **25**, as well as tetrasaccharide analogues **23** and **26**, but do not recognize disaccharide ligands **21** and **24**. Relying on these data, the authors hypothesized that Dob1 recognizes the disaccharide fragment α-D-Glc*p*(1 → 3)α-L-Rha*p* common to ligands **22**, **23**, **25** and **26**. To test this hypothesis, comparative studies were carried out using EIA (the direct binding and inhibition of the reaction with type 6B polysaccharide) for a series of pneumococcal capsular polysaccharides, including type 6A, 6B, 6C and 19A polysaccharides, which contain the disaccharide fragment α-D-Glc*p*(1 → 3)α-L-Rha*p*, as well as type 2, 14 and 19F polysaccharides lacking this fragment. As expected, the Dob1 antibodies react exclusively with polysaccharides containing the fragment α-D-Glc*p*(1 → 3)α-L-Rha*p*, *i.e.*, with polysaccharides of types 6A, 6B, 6C and 19A.

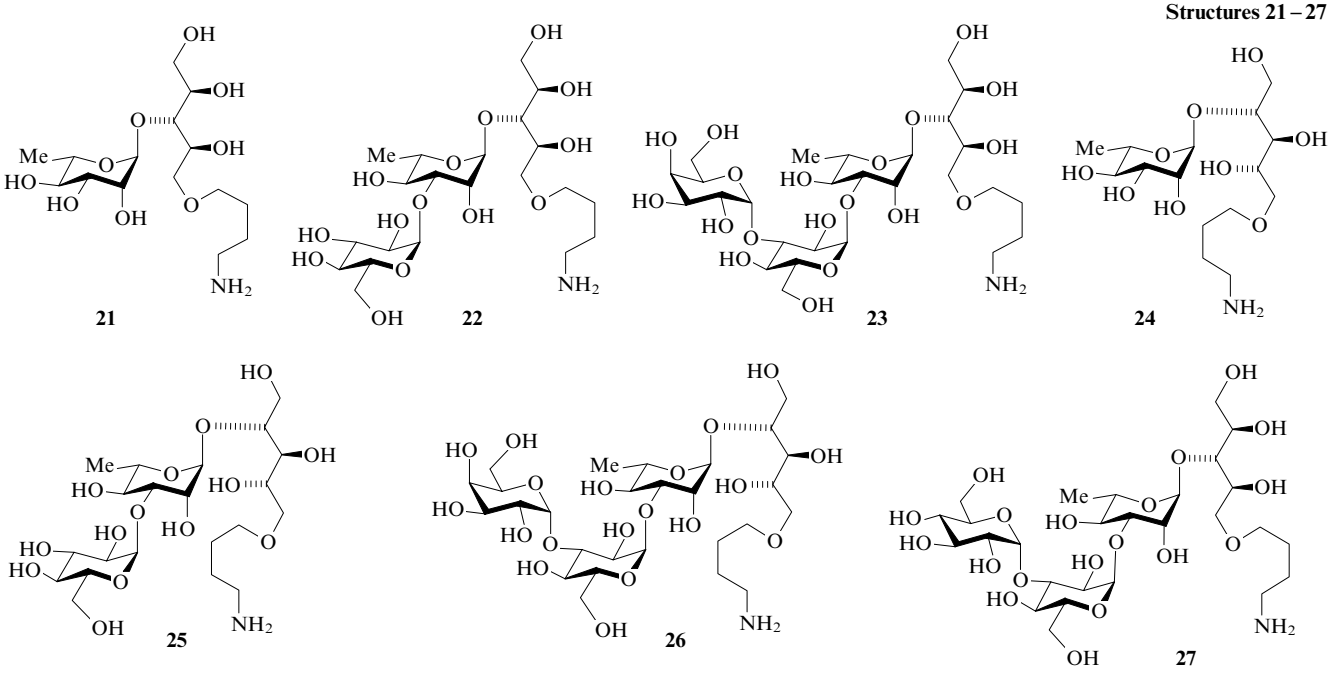
As the extension of studies, this research team synthesized for the first time[51] tetrasaccharide **27**, the repeating unit of which has a structure of type 6C polysaccharide (**16**) but without a phosphate group. For EIA tests, this tetrasaccharide was conjugated to BSA. Conjugates of oligosaccharides **21**–**26** and **27** were used as coating antigens in EIA to characterize the carbohydrate specificity of the rabbit antiserum Pool B, which contains antibodies against serotypes 6A, 6B and 6C and is used for serotyping pneumococci. It was found that the serum Pool B binds only to tetrasaccharide ligands **23**, **26** and **27**. Therefore, it was concluded that these tetrasaccharides include epitopes of serotypes 6A, 6B and 6C.

The results contradicting this conclusion were obtained in experiments on the inhibition of the reaction of the antibodies Pool B to conjugated tetrasaccharide ligands **23**, **26** and **27** with natural polysaccharides **14**–**16**. The reaction of the antibodies Pool B to conjugated ligands **23** and **27** was inhibited in the case of type 6A and 6C polysaccharides, whereas type 6B polysaccharide was unable to inhibit the reaction of rabbit antibodies to the conjugate of ligand **26**. This may indicate that tetrasaccharides **23** and **27** contain epitopes that are present in related polysaccharides **14** (type 6A) and **16** (type 6C), whereas epitopes that are present in type 6B polysaccharide (**15**) differ from those in tetrasaccharide **26**,[51] for example, due to the difference in the conformational state of tetrasaccharide **26** and 6B capsular polysaccharide (**15**) in solution.

The immunogenicity of BSA conjugates of tetrasaccharide ligands **23**, **26** and **27** was tested in experiments on mouse immunization. The raised sera were tested by EIA for the ability to bind to polysaccharides of types 6A, 6B and 6C and to capsular polysaccharides of other pneumococcal serogroups (serotypes 2 and 14). It was found that all mouse antibodies produced in response to immunization with the above-mentioned conjugates are polyreactive and are not specific only for capsular polysaccharides of types 6A, 6B and 6C. It remains unknown whether the raised antibodies have protective properties against pneumococcal infection, which is very interesting and important in the context of understanding the prospects of the design of third-generation pneumococcal conjugate vaccines. The authors noted that the polyreactivity of the raised sera may be associated not with immunization but with the existence of so-called natural antibodies, which can cross-react with many antigens not related to each other. They also suggested that antigens **23**, **26** and **27** adopt various conformations in solution, resulting in the production of antibodies with broad specificities for antigenic carbohydrate structures.[51]

The results of the study of the *S. pneumoniae* gene regions responsible for the production of type 6A, 6B and 6C polysaccharides suggest also the existence of serotype 6D (polysaccharide **17**), which was artificially created by the directed gene mutation in 2009.[52] This resulted in the

Structures 21 – 27



production of specific antibodies for the identification of serotype 6D, and the latter was found during the year in many countries, where it sometimes comprises a significant proportion of the total amount of serogroup 6 pneumococci.[53,54] It was found that antibodies raised after immunization with the 7-valent vaccine (contains only type 6B polysaccharide) also have protective activity against serotype 6D.[55] The recent studies provide evidence for the existence of gene mutations that can lead to the synthesis of several types of repeating units within one capsular polysaccharide. Thus, pneumococci capable of producing 'mixed' polysaccharide with properties of both serotypes 6A and 6B (type 6A/6B), and the directed gene mutations led to the induction of the synthesis of 'triple' polysaccharide of type 6A/6B/6D.[56] This additionally illustrates the diversity of natural capsular polysaccharides and holds promise for the use of such polysaccharides as components of polyvalent vaccines.

The 13-valent polysaccharide conjugate vaccine (apart from components of the 7-valent vaccine, this vaccine includes polysaccharides of serotypes 1, 3, 5, 6A, 7F and 19A) was licensed in the United States in 2010. Studies showed that this vaccine can prevent also infections caused by type 6C pneumococci.[57] The authors of the cited work attributed cross-specificity to insignificant structural differences between polysaccharides of serotypes 6A and 6C (**14** and **16**). However, the currently available statistical data are insufficient to draw a meaningful conclusion about the influence of the 13-valent conjugate vaccine on the overall distribution of serogroup 6 pneumococci.

Therefore, in order to create an effective conjugate vaccine against *S. pneumonia* type 6, it is necessary to use oligosaccharide ligands related to capsular polysaccharides of several (at least two) serotypes of this serogroup, including ligands with both (1 → 3)- and (1 → 4)-linked rhamnosyl-ribitol blocks.

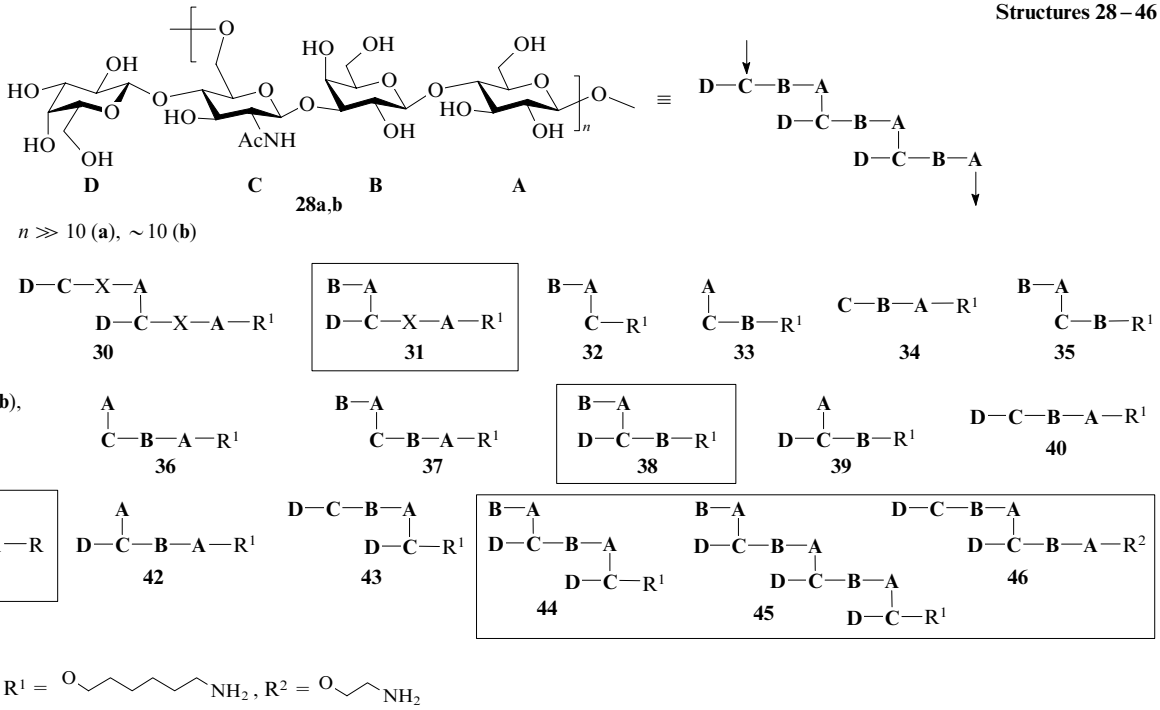
II.3. *Streptococcus pneumoniae* type 14

S. pneumoniae type 14 capsular polysaccharide is composed of branched tetrasaccharide repeating units **28a**.[58] Polysaccharides of this pneumococcal type are characterized by rather low immunogenicity compared with polysaccharides of other pneumococcal types.[59,60] This fact was attributed to the structural similarity between the repeating unit, which includes the lactose and lactosamine disaccharide fragments, and human antigenic oligosaccharides, for example, human milk oligosaccharides, specific carbohydrate blood-group determinants and many other. Oligosaccharide ligands related to pneumococcal type 14 capsular polysaccharide fragments or their isomers were searched for in order to prevent the formation of cross-reactive antibodies and production of autoimmune antibodies. These fragments were to be used to determine the structures of specific protective epitopes. For instance, a series of synthetic oligosaccharide derivatives **29a–c**, **30** and **31** was studied (see Refs 61 and 62).‡

Experiments on the inhibition of the reaction of pneumococcal type 14 capsular polysaccharide (**28a**) with the mouse serum, which was raised after immunization with the commercially available polysaccharide conjugated to the carrier protein CRM197, showed that tetrasaccharide **29a** and the mimetic of hexasaccharide **31** containing a trimethylene bridge instead of galactose unit **B** inhibited this reaction, while an analogous mimetic of octasaccharide **30** did not exhibit inhibitory activity.[60] Oligosaccharides **29a** and **30** conjugated to the carrier protein CRM197 were prepared for immunological studies.[60] After immunization of mice with these conjugates, the titer of antibodies that recognize type 14 capsular polysaccharide (**28a**) was much higher for the conjugate of tetrasaccharide **29a** than for the

‡ In the scheme that displays structures **28–46**, the structures that were immunogenic and induced the formation of antiboides specific for related polysaccharides are enclosed in boxes.

Structures 28–46



conjugated mimetic of octasaccharide **30**. These data suggest that tetrasaccharide **29a**, which is a single repeating unit of type 14 capsular polysaccharide, already contains a protective epitope.[60]

In continuation of this work, a large group of ligands, from tri- to dodecasaccharides (**32**–**45**), related to other fragments of *S. pneumoniae* type 14 capsular polysaccharide were synthesized.[63–66] In order to immunize mice, these ligands were conjugated to the carrier protein CRM197.[67]

It was shown that none of trisaccharides **32**–**34**, as well as neither tetra- nor pentasaccharides, which contain simultaneously the galactose side chain (**D**) at the main chain and the lactose unit (**B**–**A**) at the oligosaccharide nonreducing end (**32**–**37**, **39**, **40**, **42** and **43**), induce the formation of antibodies capable of binding to capsular polysaccharide **28a**.

As expected, conjugates of oligosaccharide ligands containing one, two or three total repeating units (oligosaccharides **29a**, **38**, **41a**, **44** and **45**) were immunogenic. These compounds produced the highest titers of antibodies specific for *S. pneumoniae* type 14 polysaccharide, the conjugate of tetrasaccharide **29a** being even somewhat more immunogenic than the conjugates of its di- and trimers (**44** and **45**).

It was found that the presence of the β-Gal-(1 → 4)-β-Glc fragment (**B**–**A**) at the nonreducing end of the oligosaccharide ligand plays a critical role. Thus, a conjugate of tetrasaccharide **39** that is isomeric with the repeating unit, as well as the conjugate of hexasaccharide **43** that contains an additional lactosamine fragment linked to the residue **B** of the repeating unit, produced lower (on average, fivefold) titers of specific antibodies compared with conjugates of oligosaccharides **29a**, **30**, **41a**, **44** and **45** containing the required terminal fragment **B**—**A**. The use of different adjuvants (MPL and Quil-A) led to an increase (on average, by a factor of 1.5–2) in the titers of antibodies specific for polysaccharide **28a**, but the adjuvants had no effect on the specificity of the raised antibodies. Thus, only conjugates of ligands **29a**, **38**, **41a**, **44** and **45** still induced the formation of antibodies capable of binding to natural polysaccharide **28a**. It was also shown that homologous antibodies specific for the oligosaccharide ligands used are raised for all conjugates, except for conjugates of oligosaccharides **35** and **43**.

The phagocytic activity of the raised antibodies directly depends on the titers of antibodies against type 14 capsular polysaccharide. Thus, the best results were obtained for conjugated tetrasaccharide ligand **29a** (related to the structure of the repeating unit of **28a**) and its analogue **38** containing an additional galactose residue at the reducing end. It was especially demonstrated that sera lacking antibodies specific for type 14 polysaccharide (**28a**) exhibited no phagocytic activity. These experiments confirmed that tetrasaccharide ligand **29a** contains epitopes for antibodies specific for *S. pneumoniae* type 14 capsular polysaccharide. It was also shown that the size and structure of this ligand are sufficient for the production of antibodies that enhance the phagocytic activity against this bacterium. If antibodies against the conjugate of tetrasaccharide **29a** exhibit also protective properties in animals, this conjugate can be considered as a promising candidate vaccine against *S. pneumoniae* type 14.[66]

In 1987, Kochetkov *et al.*[68] applied the polycondensation reaction and prepared for the first time synthetic polysaccharide **28b** composed, on average, of 10 tetrasaccharide repeating units of pneumococcal type 14 capsular polysaccharide. The enzyme immunoassay (competitive inhibition in solution) showed that this polysaccharide can efficiently bind to the antiserum to natural type 14 capsular polysaccharide (polysaccharides **28a** and **28b** have comparable values of inhibitory activities). In this experiment, the inhibitory activity of tetrasaccharide **29b** was found to be more than 100 times lower.

Recently, tetra- (**29c**), hexa- (**41b**) and octasaccharide (**46**) ligands with aminoethyl spacers, as well as their BSA conjugates (the average number of carbohydrate ligands linked to one protein molecule was 9–15) were synthesized by dialkyl squarate chemistry.[69,70] Competitive EIA showed that synthetic polysaccharide **28b** and hexasaccharide ligand **41b** conjugated to BSA (BSA-**41b**) inhibit equally well the reaction of the BSA-**41b** conjugate with the antibacterial serum to *S. pneumoniae* type 14, whereas the starting hexasaccharide **41b** is a much less active inhibitor.

It was also demonstrated that the immunization of mice with the BSA-**41b** conjugate results not only in the more efficient production of antibodies specific for hexasaccharide **41b** but also, in the case of the simultaneous use of the BSA-**41b** conjugate and the adjuvant (aluminium hydroxide), induces an antibacterial protective effect.[71,72] Kurbatova *et al.*[71,72] performed the detailed analysis of different immunological effects after immunization with the BSA-**41b** conjugate. Thus, the bactericidal activity of mouse peripheral blood leukocytes was shown to be enhanced 4 and 24 h after the first immunization with the BSA-**41b** glycoconjugate adsorbed on aluminium hydroxide. The adsorption of the glycoconjugate on aluminium hydroxide led to the modification of the immune process at the stage of activation of natural immunity followed by the enhancement of the adaptive immune response.[72]

Preliminary experimental data on the active immunization of mice with BSA conjugated to oligosaccharide ligands **29c**, **41b** and **46** (combined with aluminium hydroxide) provide evidence that all three conjugates induce the production of specific opsonizing antibodies and can protect mice from infection with high doses of *S. pneumoniae* type 14. The immune sera administered to mice were found to have protective properties. However, BSA conjugates of oligosaccharide ligands **29c** and **46** containing one and two repeating units, respectively, were shown to be immunologically more active than conjugated hexasaccharide **41b** in respect to the level of the formation of specific antibodies, their opsonizing activity and the mouse protection from infection. These data confirm the results obtained by other researchers who suggested that oligosaccharide **29c** is the minimal necessary structural motif for the production of opsonizing antibodies against the repeating unit of capsular polysaccharide.[67] In our studies,[72] this statement was confirmed for the first time using a model of the active mouse protection against infection with *S. pneumoniae* type 14.§ Therefore, tetrasaccharide **29c** can be considered as a promising ligand for the construction of a conjugate vaccine against infections caused by *S. pneumoniae* type 14.

§ E A Kurbatova, N K Akhmatova, E A Akhmatova, N B Egorova, E V Sukhova, D B Yashunskii, Yu E Tsvetkov, N E Nifantiev (unpublished data).

II.4. *Streptococcus pneumoniae* type 17F

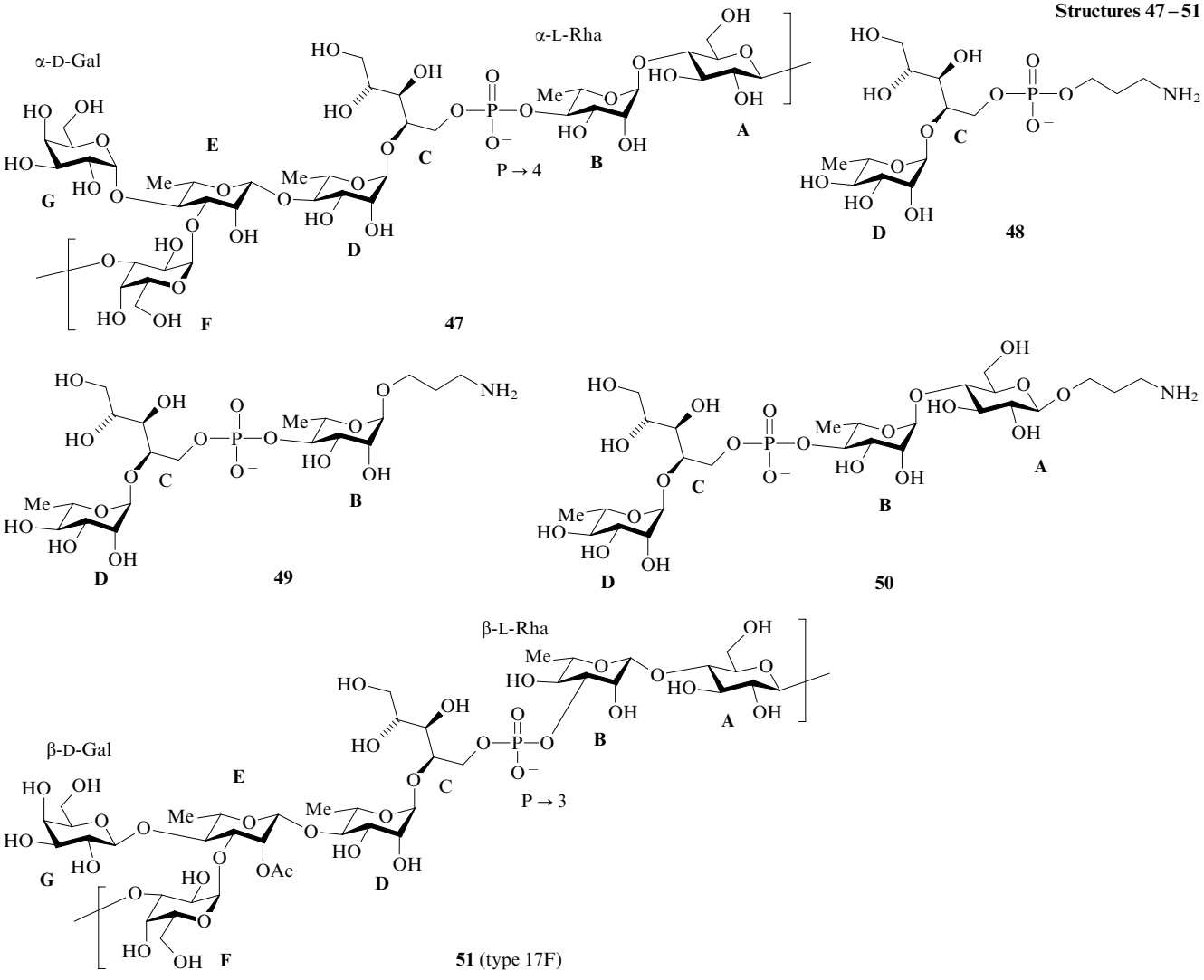
S. pneumoniae type 17F capsular polysaccharide is involved in the 23-valent polysaccharide vaccine (Pneumovax™ 23, Pnu-Imune™ 23, Pneumo 23™). Structure **47** was initially assigned to its heptasaccharide repeating unit (see the review[73] and publications cited therein). Spacer-modified di- (**48**), tri- (**49**) and tetrasaccharides (**50**) containing overlapping structural elements were synthesized[74] in order to examine the immunogenicity of small oligosaccharides (involved in carbohydrate–protein conjugates) related to fragments of this capsular polysaccharide. Oligosaccharides **48**–**50** were conjugated to the carrier protein KLH, and a broad range of their immunological studies were carried out.[75]

Experiments in mice showed that the conjugates of all three oligosaccharides **48**–**50** exhibit immunogenicity and induce the production of antibodies specific for homologous antigens. However, only conjugates of disaccharide **48** and trisaccharide **49**, but not of tetrasaccharide **50**, were found to be able to induce antibodies that recognize type 17F capsular polysaccharide. In addition, the active immunization with conjugated disaccharide **48** and trisaccharide **49** protected mice from infection with encapsulated strains of *S. pneumoniae* type 17F, the protective properties of conjugated trisaccharide **49** being somewhat higher compared with conjugated disaccharide **48**. As expected, the antibodies raised against conjugated tetrasaccharide ligand **50** did not bind to capsular polysaccharide and did not exhibit protective effects.[75]

In order to find out whether immunological properties of these conjugates have a common nature, Jansen *et al.*[76] performed tests in rabbits. The results of these tests did not contradict the results of experiments in mice. In these experiments also, only conjugates of disaccharide **48** and trisaccharide **49** were able to inhibit the reaction of the rabbit antiserum, which was produced in response to the infection with *S. pneumoniae* 17F type, with capsular polysaccharide of this serotype. Antibodies induced by conjugates of disaccharide **48** and trisaccharide **49** were also able to promote phagocytosis of type 17F pneumococci, as opposed to antibodies raised against conjugated tetrasaccharide **50**.

Relying on the results of all experiments, it was concluded that disaccharide **48** already includes a protective epitope that is present in *S. pneumoniae* type 17F polysaccharide. Moreover, unlike conjugated tetrasaccharide **50**, which should also contain this protective epitope, the conjugates of di- and trisaccharides (**48** and **49**) present this epitope in such a way that it is more efficiently recognized by antibodies against *S. pneumoniae* type 17F

Structures 47–51



capsular polysaccharide, thus providing the antibacterial protective effect.[76]

Jansen *et al.*[76] also hypothesized that the inability of conjugated tetrasaccharide **50** to induce the formation of antibodies against type 17F capsular polysaccharide (**47**) may be associated with the fact that the conformation of this tetrasaccharide as an individual compound and in the KLH-**47** conjugate substantially differs from the conformation of the related sequence in bacterial polysaccharide and the conformations of its parts in structurally related di- and trisaccharide ligands **48** and **49**.[75]

Later on, *S. pneumoniae* type 17F capsular polysaccharide was studied in detail, and the complete assignments of its signals in the ^{1}H, ^{13}C and ^{31}P NMR spectra were made. This led to the revision of the known structure of the repeating unit of **47**.[77] The phosphorylated rhamnose unit, which is present in trisaccharide **49** and tetrasaccharide **50**, was found to have the β-configuration (see the refined structure of the repeating unit of **51**) rather than the α configuration, as it is depicted in structure **47**. Besides, it was demonstrated that the phosphate bridge is linked to the O(3) atom rather than to O(4) of the same rhamnose residue **B**. The galactose residue **D** in the side chain of the refined structure has another anomeric configuration (β instead of α), and the rhamnose residue **E** contains an acetyl group at the O(2) atom.[77]

Taking into account the new data on the structure of *S. pneumoniae* type 17F capsular polysaccharide, Alonso de Velasco *et al.*[75] revised the results of their experiments.[78] The fact that the conjugate of trisaccharide **49**, despite the mistake in the structure, can induce protective antibodies that recognize capsular polysaccharide **51** was attributed to that the influence of the single erroneous bond is negligible and that the disaccharide fragment L-Rhaα-(1 → 2)-D-Ara-ol-(1 → P →) (**48**) makes the major contribution in this case. However, the double mistake in the structure of synthetic tetrasaccharide **50** was apparently critical for the production of antibodies specific for polysaccharide **51**.

Nevertheless, the refinement of the structure of *S. pneumoniae* type 17F capsular polysaccharide did not change the main conclusion that the disaccharide fragment related to ligand **48** plays a key role in the production of protective antibodies.[78]

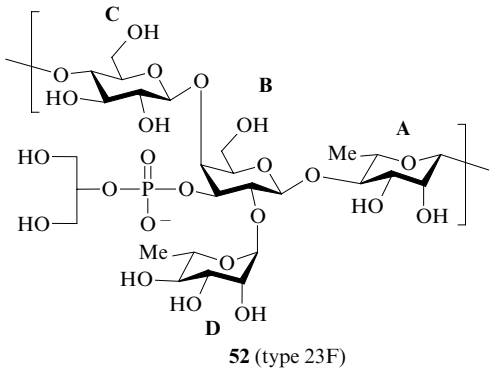
II.5. *Streptococcus pneumoniae* type 23F

Capsular polysaccharide of this serotype composed of tetrasaccharide repeating units **52** (Ref. 79) is also a component of the 23-valent pneumococcal polysaccharide vaccines (Pneumovax™ 23, Pnu-Imune™ 23, Pneumo 23™). In order to find the optimal oligosaccharide ligand for a vaccine candidate, the protein carrier KLH was conjugated to trisaccharide **53** and tetrasaccharide **54**, as well as to type 23F capsular polysaccharide (**52**), and the immunogenicity of the conjugates was tested.[80, 81]

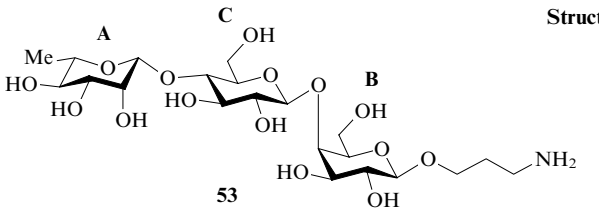
Rabbit immunization with conjugated tetrasaccharide **54** induced the formation of antibodies specific for type 23F capsular polysaccharide, whereas the same conjugate showed no immunogenicity in mice.[44] Conjugated trisaccharide **53** was weakly immunogenic in rabbits, which can be attributed to the absence of the rhamnose side chain (**D**) and glycerol phosphate in its structure. The immunological significance of this residue was confirmed by a special experiment. The reaction of capsular polysaccharide **52** to antibodies raised after rabbit immunization with this KLH-conjugated polysaccharide was completely inhibited by polysaccharide **52**, as well as by conjugated tetrasaccharide **54** containing the rhamnose residue **D**, but was not inhibited by conjugated trisaccharide **53** lacking this rhamnose residue **D**. The binding of the antiserum to conjugates of tetrasaccharide **54** and polysaccharide **52** was also completely inhibited only by polysaccharide **52** and conjugated tetrasaccharide **54**, which attest to the absence of essential difference in the epitope specificity of antibodies induced by the conjugates of capsular polysaccharide **52** and tetrasaccharide ligand **54** related to its repeating unit.[44]

Essential differences were found in the epitope specificity of rabbit and human sera to pneumococcal type 23F polysaccharide (**52**). Sera of human recipients immunized with the commercial 23-valent pneumococcal polysaccharide vaccine were used for tests. These sera were characterized by high titers of antibodies against serotype 23F polysaccharide. However, as opposed to the serum of rabbits immunized with conjugated polysaccharide **52**, the reaction of bacterial type 23F polysaccharide (**52**) with the human serum produced in response to the introduction of this polysaccharide can be only slightly inhibited by the conjugates of oligosaccharides **53** and **54**. *In vitro* assays of opsonizing activity of the human serum showed that this

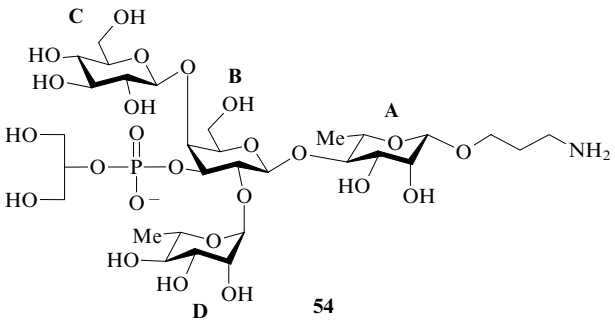
Structures 52 – 54



52 (type 23F)



53



54

serum can stimulate the phagocytosis of type 23F pneumococci five times more efficiently than the rabbit serum to conjugated polysaccharide **52**. The phagocytic activity of the antiserum to conjugated tetrasaccharide **54** was only two times lower than that of the antiserum to polysaccharide **52**, while the serum raised after immunization with conjugated trisaccharide **53** was unable to promote phagocytosis in the broad concentration range under study.[44]

These results led to the conclusion that human IgG have a larger size of the binding epitope compared to rabbit IgG. In the other words, human anti-polysaccharide antibodies can recognize a conformational epitope on a large oligosaccharide or polysaccharide antigen, but not in small oligosaccharides. The cited study, which demonstrated the difference in the epitope specificity of rabbit and human antibodies raised against the same polysaccharide immunogen, showed that oligosaccharide vaccines should be tested not only in laboratory animals but also in people.[44]

III. Conclusion

The choice of the optimal synthetic oligosaccharide ligand is an important step in the construction of antibacterial conjugate vaccines, since this ligand is largely responsible for antigen specificity of the immune response to the conjugate. However, the determination of the minimal size of the ligand, which is capable of efficiently inducing specific antibodies that recognize the target bacterial polysaccharide, is a difficult problem with no standard solution. It can be only said with certainty that in order to find the optimal carbohydrate ligand, it is necessary to examine a rather large and representative series of synthetic oligosaccharide ligands related to different fragments of bacterial polysaccharide and to use them to obtain data on the topology of binding of bacterial polysaccharide to antibacterial sera. The three-dimensional structures of both synthetic oligosaccharides and bacterial polysaccharide should be taken into account for the adequate modelling on the reaction of oligosaccharide ligands with antibodies.

The studies discussed in the present review and devoted to the search for the structures of potential ligands, which could serve as a basis for a combined third-generation pneumococcal vaccine, suggest that the rational design of a series of immunological tests can simplify the search for appropriate vaccine oligosaccharide ligands. It would be expected that novel methods for the synthesis of oligosaccharide libraries and screening of their biological characteristics would ensure considerable progress in the identification of potential vaccine ligands.[15] The subsequent selection of such ligands should be performed after investigation of conjugated forms of the ligands in *in vitro* and *in vivo* experiments. Taking into account the state-of-the-art technology and advances in carbohydrate chemistry and biochemistry, immunology and bacteriology, as well as growing requirements for the standards of quality for the production of conjugate vaccines, it can reasonably be expected that three-generation pneumococcal vaccines based on synthetic oligosaccharide ligands will be created in the foreseeable future. Quite recently, this problem has seemed to be elusive because of the complex structures of the repeating units in the majority of *S. pneumoniae* capsular polysaccharides. Currently, this challenging task is achievable and commercially reasonable owing to the state-of-the-art fine organic synthesis, including oligosaccharide synthesis relying on the advanced chemical, enzymatic and instrumental methods. It is expected that 'mixed' vaccines containing both synthetic oligosaccharides and bacterial polysaccharides or oligosaccharide products of their cleavage as ligands will be created.

Carbohydrate-conjugate vaccines belong to complex biomolecular systems.[82] The development of such systems should not be based solely on methods of carbohydrate chemistry because non-carbohydrate linker elements that connect an oligosaccharide ligand to the carrier protein should be produced in high yields, provide the optimal spatial presentation of the carbohydrate ligand, exclude nonspecific binding and, in addition, improve the ability to recognize the ligand, for example, due to hydrophobic properties, which is well-known from investigations of the activity of lectins, carbohydrate-binding proteins.[83] New methods of chemistry of heterocyclic compounds,[84–86] metal-complex,[87–89] enzyme[89] and other types of catalysis,[90] the design of hybrid[91–95] and dendrite[96–99] systems, as well as the targeted modification of carrier proteins,[15, 16, 19, 100, 101] allow researchers to efficiently solve the above-listed problems.

This review has been written with the financial support of the Russian Science Foundation (Project No. 14-50-00126).

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