

Glycoconjugate Vaccines for Prevention of *Haemophilus influenzae* Type b Diseases

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Abstract—This review summarizes the experience in laboratory- and industrial-scale syntheses of glycoconjugate vaccines used for prevention of infectious diseases caused by *Haemophilus influenzae* type b bacteria based on the linear capsular polysaccharide poly-3- β -D-riboseyl-(1 \rightarrow 1)-D-ribitol-5-phosphate (PRP) or related synthetic oligosaccharide ligands. The methods for preparation of related oligosaccharide derivatives and results of the studies evaluating effect of their length on immunogenic properties of the conjugates with protein carriers are overviewed.

Keywords: glycoconjugate vaccines, conjugation, synthesis, poly-3- β -D-riboseyl-(1 \rightarrow 1)-D-ribitol-5-phosphate, PRP, *Haemophilus influenzae* type b, Hib

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INTRODUCTION

Synthetic carbohydrate vaccines based on conjugates of adjuvant protein carriers with bacterial polysaccharides (so-called second generation carbohydrate vaccines [1, 2]) or synthetic oligosaccharides structurally related to immunodominant antigenic fragments of polysaccharides (known as third-generation carbohydrate vaccines) are now increasingly used for prevention of bacterial infections [3–9]. This type of preparations includes one of the most important vaccines in the public health history, the vaccine

against the dangerous bacterial pathogen *Haemophilus influenzae* type b (Hib). The Hib disease is the leading cause of bacterial meningitis and pneumonia in young children. To combat Hib infections, several vaccines have been created. Integration of these vaccines in the routine vaccination schedule allowed almost complete elimination of the diseases caused by these bacteria from the statistics on death and disability. The WHO estimates that the Hib conjugate vaccines are among the safest and most effective, preventing up to 90% of invasive Hib infections.

In Russia, vaccination against Hib infections has been included in the national immunization schedule since 2011. For children at risk, this vaccine is administered as a 3 dose schedule at 3, 4, and 5 months and revaccination at the age of 18 months [10]. This is due to the threat this pathogen poses to young children causing invasive infectious such as purulent meningitis (up to 55% of all invasive forms), epiglottitis, pneumonia, bacteremia, and sepsis [11]. Purulent meningitis is the most severe disease caused by Hib. The global average mortality rate related to this disease reaches 43% [12], and 14.5% of children who have recovered have longterm neurological complications, for example, mental disorders (up to 13%), motor disorders (up to 8%), and deafness (up to 8%). According to the WHO, the global and European incidence of purulent meningitis caused by Hib before the vaccine was introduced was 38–40% among all cases of purulent meningitis of established etiology in children under 5 years old; the annual disease rate in Europe was 11–40 cases per 100 thousand children under the age of one year [14]. To date, the world community has managed to

Abbreviations: ADH, adipic dihydrazide; BCR, B-cell receptor; BSA, bovine serum albumin; CDAP, 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate; CDI, carbonyl diimidazole; CRM197, recombinant diphtheria toxin that contains glycine instead of glutamine at position 52; D, *Corynebacterium diphtheriae* toxin; DSP, dithiobis(succinimidyl propionate); DT, diphtheria toxoid produced by formaldehyde inactivation of *Corynebacterium diphtheriae* toxin; DTT, dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HBV, hepatitis B virus vaccine; Hib, *Haemophilus influenzae* type b; HSA, human serum albumin; IL-4, interleukin-4; IPV, inactivated poliomyelitis vaccine; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; MHC II, major histocompatibility complex expressed on professional antigen-presenting cells; OMPC, outer membrane protein complex of *Neisseria meningitidis* group B; PRP, poly-3- β -D-riboseyl-(1 \rightarrow 1)-D-ribitol-5-phosphate; PRP_{low}, low molecular weight PRP; SATA, *N*-succinimidyl-*S*-acetyl mercaptoacetate; SMP, *N*-hydroxysuccinimide ester; SPDP, *N*-succinimidyl 3-(2-pyridylthio)propionate; TI-2, T-independent immune response type 2; TT, tetanus toxoid, i.e. formaldehyde-inactivated *Clostridium tetani* toxin; DTaP, diphtheria, tetanus, and acellular pertussis vaccine adsorbed; ELISA, enzyme-linked immunosorbent assay; CPS, capsular polysaccharide; PEG, polyethylene glycol.

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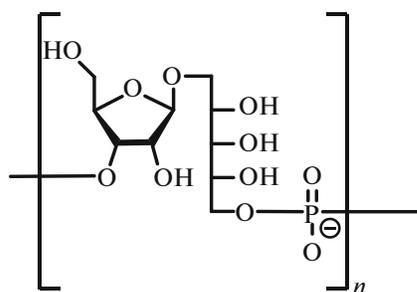


Fig. 1. Capsular polysaccharide of *Haemophilus influenzae* type b (PRP).

significantly reduce [12] the number of invasive Hib infections due to the widespread use of conjugate vaccines, which have been successfully used all over the world for more than 30 years. All countries that have included the Hib vaccine in the national routine vaccination schedule have experienced dramatic decline in the incidence of the invasive Hib diseases [12, 15].

Introduction of the routine Hib vaccination into the national immunization schedule started in 1986 in Canada; countries of South and North Americas had been covered with Hib vaccination by 2002; and by 2014, Hib vaccination had been introduced in most of Africa, as well as in countries of Western and Eastern Europe. By the end of 2018, more than 190 WHO Member States introduced Hib vaccination into the national immunization programs, with 72% of recipients being vaccinated with three doses [16]. The exceptions are two large territories on the Eurasian continent which are the Russian Federation and the People's Republic of China (PRC), in which the epidemiological situation for Hib differs significantly. In particular, annual mortality rate from Hib diseases in children under 5 years old in China is 10–25 cases per 100 thousand children, and as of 2018, only ~30% of young children received three doses of the Hib vaccine [17].

In Russia, annual mortality rate from the invasive Hib infections is on average less than 10 cases per 100 thousand children in this age group [18]. Microbiological analysis of 89 strains of *H. influenzae* isolated from the blood and cerebrospinal fluid of the patients with invasive hemophilic infection conducted by medical institutions in a number of Russian cities has shown that 95.5% of these cases were caused by Hib [19]. The annual incidence rate of meningitis caused by Hib in Russia in 2017 was 5.0–16.9 cases [20], and according to other data 3–20 cases per 100 thousand children under 5 years old [11, 21, 22]. In Moscow, this indicator in 2005 was 5.7 cases [23], and according to another source 6.5 cases per 100 thousand children under 5 years old [11]. These rates could be significantly reduced by introducing routine Hib vaccination in the national immunization schedule. The experience of foreign countries indicates that the incidence

rate of the invasive diseases caused by Hib can be reduced to 0.2 cases per 100 thousand children [14, 24].

Haemophilus bacteria are components of the nasopharynx microbiota in healthy adults and children that therefore is a natural reservoir of these bacteria capable of prolonged persistence in the human body. In Russia, carriage of *H. influenzae* type b in children is around 1–10%; it rises to 40% in overcrowded conditions and in primary school [11]. The carriage in adult population can reach 10% [25]. In the cases of concomitant viral infections or reduced immunity, these bacteria cause acute respiratory infections. The airborne transmission pathway which is typical for diseases of this type promotes their wide spread. Analysis of the pharyngeal carriage of *H. influenzae* among the preschool children diagnosed with adenoid hypertrophy, chronic tonsillitis, and pharyngitis showed that it constitutes 11–12% of the oropharynx microflora [26].

ASPECTS OF IMMUNE RESPONSE TO IMMUNIZATION WITH CAPSULAR POLYSACCHARIDE (CPS) OF Hib AND RELEVANT CONJUGATE VACCINES

The main virulence factor of Hib is the polysaccharide capsule that affects phagocytosis [12]. Its key component is the linear capsular polysaccharide PRP (poly-3-β-D-riboseyl-(1→1)-D-ribitol-5-phosphate), see structure of the repeating unit in Fig. 1. In the human body, PRP induces mainly the T-independent type 2 immune response (TI-2) as with CPSs of other human pathogens (such as *Neisseria meningitidis* or *Streptococcus pneumoniae*). This type of immune response does not involve presentation of peptides by the major histocompatibility complex (MHC-II) proteins and it stimulates production of protective antibodies without the involvement of T helpers.

The main function of the TI-2 response is recognition of the CPS in the bloodstream, which indicates the onset of bacteremia, and rapid production of the protective antibodies of the IgM isotype. This type of immune response is mediated by the marginal-zone B cells of the spleen, on the surface of which special BCR receptors are expressed, including the membrane-bound antibodies of the IgM isotype. In the bloodstream, CPS form complexes with an element of the innate immune system so called protein factor C3d. The key step in the uptake of the CPS by BCR receptor is interaction of the factor C3d with the CR2 coreceptor, which is expressed in large quantities on the spleen B cells in adults. At the same time, this coreceptor, which is the key participant in detection of bacterial CPSs, is poorly expressed in children under 2 years of age [27], thus reducing the efficiency of the TI-2 type immune response and weakening protection against the invasions of bacteria carrying polysaccharide capsules. High level of maternal antibodies, which protect children in the first two months of life, decreases by three months of age, and the TI-2 immu-

nity reaches the adult level only by the age of 4–5 years providing protective level of antibodies to CPS. Thus, the target population for Hib vaccination are children between two months and two years of age, who are most susceptible to the invasive diseases caused by this agent.

The T cell-dependent immune response (TD) in young children develops faster [28] than the TI-2, and the objective of prophylactic vaccines against the diseases caused by bacteria with polysaccharide capsule is to direct the immune response to the T cell-dependent pathway [6]. This objective was achieved by the development of conjugate vaccines which consist of CPS or their fragments covalently linked to an adjuvant protein carrier [29].

Initiation of the T cell-dependent immune response is a complex process of sequential and parallel events [30], and in the current state of immunology, specific details of this process cannot be unambiguously predicted from physicochemical properties of the conjugate vaccine preparations. However, key factors which determine immune response to the conjugate vaccines have already been investigated [31, 32].

After the injection, a monovalent conjugate vaccine which is dissolved (as recommended) in an isotonic solution [33–36], basically enter the bloodstream and lymphatic. The resident B cells in spleen and lymph nodes engulf the carbohydrate–protein conjugate, cleave the protein carrier into peptides, and present them by means of MHC-II to $\alpha\beta$ -TCR receptors of the CD4⁺ protein complex on the surface of T helper cells [37]. The thereby stimulated T cells produce IL-4, which accelerates maturation of the B cells that have processed the antigen.

Another way of initiation of immune response upon immunization with the conjugate vaccines engages dendritic cells. Hib vaccines are injected into the quadriceps muscle of the thigh, and in older children into the deltoid muscle of the shoulder, which are skeletal muscles, where three types of dendritic cells are present: immature dendritic cells of monocyte-derived (Mo-DC) and two subtypes of common dendritic cells derived from the same monocytic precursor Gr1⁺Ly-6C^{high}. In the course of microbial infection, these monocytes are recruited in large numbers to the site of inflammation, where they are transformed into macrophages with phenotypes CD11b⁺CD11c⁻2MH-CII⁺ and CD11b⁺CD11c⁺MHCII⁺ [31]. The same process is initiated at the site of the glycan–protein conjugate injection. Macrophages capture protein antigens, depolymerize them with preservation of glycan–peptide bonds [38], and deliver them to lymph nodes for presentation to the CD4⁺ T cells. Next, the primed T cells initiate maturation of the B cells that have absorbed the antigen, followed by proliferation and differentiation [39]. This stage is vital for development of the antigen-specific immune response and formation of immune memory. At the second stage,

one part of the mature B cells is transformed into the plasma cells with highly developed endoplasmic reticulum and Golgi apparatus, which start intensive synthesis of immunoglobulins of the IgG1 and IgE isotypes, the other part gets activated into the memory B cells [39]. Thus, the additional benefit of immunization with glycan–protein conjugate vaccines is the possibility to evaluate the efficiency of the development of immunological memory on the basis of postimmunization concentration of IgG antibodies in the blood serum.

Recruitment of the Gr1⁺Ly-6C^{high} dendritic cell precursors into muscles is greatly enhanced by the use of adjuvants [40]. Most modern multivalent conjugate vaccines with the Hib component are used as a mixture with insoluble adjuvants: aluminum hydroxide or phosphates [41]. Being introduced into the body in the form of suspension, adjuvants act as carriers of the adsorbed vaccine and activate the innate immune system with the attendant stimulation the immune response with participation of the T_H2 helper cells. At the same time, adsorption of conjugate Hib vaccines on aluminum salts is not a necessary condition for effective immunization including the induction of IgG antibodies and development of immune memory [42].

INDUCTION OF PRP-SPECIFIC ANTIBODIES AS A CRITERION OF EFFICIENCY OF Hib VACCINATION

Clinical trials carried out in Finland in 1977 [43, 44] demonstrated positive correlation between the increased levels of antibodies to PRP and the protective effect of the conjugate Hib vaccine. Numerous studies of the efficacy of vaccination with conjugate Hib vaccines demonstrate the importance of high postimmunization titers of the anti-PRP antibodies in young children [45–47]. A good example is the 8-year experience of Hib immunization in the UK, where the mandatory three-dose vaccination of children aged two, three, and four months was introduced in 1992. After the sharp decline in the incidence of invasive Hib disease by 1994, it began to rise in 1999, wherein 85% of the affected children were vaccinated. That vaccine failure was due to the use of a less immunogenic combination vaccine since 1996. It included not only the Hib component, but also the DTaP vaccine with the pertussis acellular component [48, 49], and induced low-avidity antibodies [50, 51]. This problem was resolved by additional immunization with the Hib monovaccine, which significantly increased concentration of the Hib-protective antibodies [52].

At present, the efficacy of Hib vaccines is evaluated by the proportion of patients with low (less than 0.15 $\mu\text{g}/\text{mL}$, do not provide protection against infection), medium (0.15–1.00 $\mu\text{g}/\text{mL}$, provide incomplete protection), and high (more than 1.0 mg/mL , provide reliable protection against infection) blood concentra-

tion of the PRP-specific antibodies one month after the first round of the three-dose immunization and one month after revaccination [45, 53].

Investigation of the efficiency of formation of the PRP-specific antibodies in children [54] vaccinated with the PRP–CRM197 conjugate (the synthesis is discussed below) showed that at least two immunizations with the conjugate vaccine are required for the formation of immune memory. The routine immunization schedule for prevention of Hib disease includes three doses of conjugate vaccine at three, four, and five months and, in some cases, a booster immunization at 18 months. Time points for triple immunization are chosen to synchronize Hib immunization with the DTaP vaccination and revaccination schedule. In order to provide effective vaccination immunization with the conjugate Hib preparations should be performed before immunization with a protein carrier, as the reverse order can result in 2–3-fold reduction of the concentration of anti-PRP antibodies [55].

Efficiency of the abovementioned immunization scheme has been confirmed in the course of clinical trials. For example, clinical trials conducted by GSK (manufacturer of the Hiberix® Hib vaccine), which included triple immunizations and a booster dose at 15–18 months, showed that in 95–100% of infants, the PRP-specific antibody titer was 0.15 µg/mL one month after three immunizations. One month after booster immunization, the antibody titer of 0.15 µg/mL was detected in 100% of children, and a titer of 1 µg/mL – in 94.7% of children [35]. These data clearly demonstrate that this vaccine provides reliable protection against Hib infection. Vaccination schedule that includes booster dose is more expensive, but significantly more effective than the three-dose vaccination. For example, use of this immunization schedule since 2002 in the United States has led to 99% reduction in the incidence of invasive Hib diseases in children under 5 years of age [24].

HISTORY OF Hib VACCINE DEVELOPMENT

The rapid development of vaccines for prevention of bacterial diseases started in 1930s, and after a period of stagnation caused by the onset of antibiotic era, proceeded in 1960–1970s. Among its first achievements was the creation of the vaccine against Hib infection [56]. In 1977, an extensive clinical trial was carried out in Finland [43, 44], with 50000 children aged three months to five years immunized with the purified PRP polysaccharide and another 50000 children followed up as a control group. All vaccinated children over 18 months of age developed antibodies and received protective immunity against Hib, while 11 children in the control group got a Hib disease. At the same time, this vaccination did not provide protection in children under 18 months of age (which is the age group most susceptible to dangerous conditions). These results evidenced, that PRP was a weak immunogen for these

children. Moreover, the PRP polysaccharide did not provide a booster effect [57] and did not affect the nasopharynx microflora [58]. The conclusions about low immunogenicity of PRP in children under 18 months of age were confirmed by 4-year use of the polysaccharide vaccine licensed in 1985 in the United States [59, 60]. Thus, prevention of diseases caused by Hib with the 1st generation vaccine, which consisted of PRP CPS, turned out to be ineffective [61].

The pioneering works conducted in Goebel and Landsteiner laboratories in the 1920s and 1930s [62–64], logically led to the replacement of the PRP polysaccharide for its conjugate with an adjuvant protein carrier in order to enhance immunogenicity of the preparation. According to this logic, a series of covalently bound PRP conjugates with proteins BSA, HSA, hemocyanin of horseshoe crab *Limulus polyphemus*, and diphtheria toxin (T) were synthesized in the Robbins laboratory [65]. Immunization with formulations of this type induced formation of bactericidal antibodies specific for Hib that possessed activity. The researchers did not compare effectiveness of the immunostimulating ability of these proteins. A similar product was created by Anderson et al. [66], who in 1985 published the results of successful immunization of infants with the conjugate Hib vaccine consisting of PRP and diphtheria toxoid. Clinical trials of the subsequent conjugate vaccine on a cohort of 61,080 infants in 1988 and 1990 in the US state of North Carolina confirmed 100% efficiency of the vaccine, which was administered as a two- or more dose series [61]. These pioneering works actually marked the beginning of a new era in the vaccine-mediated control of infectious diseases, which is the era of conjugate vaccines consisting of bacterial polysaccharides or synthetic oligosaccharides, structurally related to immunodominant fragments of polysaccharides, covalently linked to adjuvant protein carriers.

Since 1980s, the number of publications [3, 4, 37, 67, 68] describing synthesis and immunogenicity of the conjugate Hib vaccines dramatically increased; the industrial-scale production of the most effective ones began [5, 69]. However, one of the first commercial monovalent Hib conjugate vaccines that included diphtheria toxoid (DT) as a carrier (ProHIBiT®, PRP–DT, Connaught Laboratories Inc.) registered in the United States in 1987 proved to be inefficient. It was replaced by the HibTITER® vaccine (PRP–CRM197, Wyeth Pharmaceuticals Inc.) based on the CRM197 protein carrier and low-molecular-weight polysaccharide PRP. HibTITER® was approved for use by the US Food and Drug Administration (FDA) in 1990 and was discontinued only in 2007. In 1989, the PedvaxHIB® monovalent vaccine (PRP–OMP, MSD), which contained the outer membrane protein of meningococcus group B as an adjuvant carrier also conjugated with low-molecular-weight PRP has been registered (its production has also been stopped).

Later the monovalent vaccine PRP-TT (Pasteur Merieux) with tetanus toxoid (TT) as a protein carrier [70] registered in the United States in 1993 as ActHIB[®] (Sanofi), and the OmniHIB[®] vaccine (SmithKline Beecham Pharmaceuticals), registered in 1996, were created; the latter one is currently out of production.

A significant event in the field of carbohydrate conjugate vaccine research was the development of a conjugate vaccine based on TT and oligomeric mixture of the spacer-armed oligosaccharides corresponding to the fragments of PRP polysaccharide by the Cuban-Canadian team of researchers led by V. Verez-Bencomo and R. Roy [71], which is actually one of the first examples of the third-generation carbohydrate vaccines [2] (synthesis of this vaccine is discussed below, see Scheme 13). This product was registered in Cuba in 2003 under the trade name Quimi-Hib[®] (Heber Biotec S.A.) and is still used in several countries around the world today.

COMMERCIAL CONJUGATE Hib VACCINES BASED ON PRP

Currently, both mono- and polyvalent Hib vaccines are used to prevent Hib infection. The following monovalent Hib conjugate vaccines are on the market: VaxemHIB[®] (GSK, a conjugate of a low-molecular-weight PRP and CRM197), Hiberix[®] (PRP-TT, GSK, registered in 2009), Sii HibPRO[®] (PRP-TT, Serum Institute of India Ltd.), and PedvaxHIB[®] (PRP-OMPC, MSD). They are supplied as solutions or lyophilized powder that must be dissolved in an isotonic solution or suspension of other vaccine preparations before use.

Combination vaccines that include Hib conjugates, are becoming more common, primarily for economic reasons. In addition to the Hib component, they contain a DTaP complex, as well as one or two regionally targeted vaccine components (HBV or IPV). Chemical and immunological properties of the Hib conjugates have to be considered when combination vaccine are formulated. PRP conjugates are unstable in aqueous solutions and in the presence of aluminum-based adjuvants; therefore, in most cases, the freeze-dried Hib component of the multivalent vaccine is packaged separately from other components, which comprise an aqueous suspension of aluminum hydroxide or phosphate with protein immunogens adsorbed on them. In addition, researchers have repeatedly observed [72] that inclusion of the conjugate Hib vaccine in the composition of multivalent vaccines containing DTaP with the acellular pertussis component, leads to the induction of low-avidity antibodies to PRP when administered in one syringe; therefore, it is recommended to inject DTaP preparations and Hib vaccines in different parts of the body [33, 34].

Typically, the Hib conjugate vaccines in combination vaccines are supplied as a Hib lyophilized powder mixed with excipients. The PRP-TT lyophilized powder is included in the following combination vaccine preparations: TETRAct-HIB[®] (DTaP-Hib, Sanofi), ComBE Five[®] (DTaP-HBV-Hib, Biological E Ltd.), Quadrovax[®] (DTaP-Hib, Serum Institute of India Ltd.), Pentaxim[®] (DTaP-IPV-Hib, Sanofi) [73], Pentacel[®] (DTaP-IPV-Hib, Sanofi) [74], Pentavac[®]SD/PFS (DTaP-HBV-Hib, Serum Institute of India Ltd.), Tritanrix HB-Hib[®] (DTaP-HBV-Hib, GSK), EasySix[®] (DTP-IPV-Hib, Panacea Biotec), Infanrix-IPV/Hib[®] (DTaP-IPV-Hib, GSK), and Infanrix Hexa[®] (DTaP-IPV-HBV-Hib, GSK).

The convenience of multivalent vaccines for mass vaccination stimulates further research to develop safer ingredients, convenient formulations, and more efficient methods of administration. Thus, the completely liquid pentavalent vaccine Pediacel[®] (DTaP-IPV-PRP-TT, Sanofi) [75, 76], EasyFour[®] (DTaP-PRP-TT, Panacea Biotec), EasyFive-TT[®] (DTaP-IPV-PRP-TT, Panacea Biotec), Quinvaxem[®] (DTaP-HBV-PRP-CRM197, GSK) [77], and Hexaxim[®] hexavalent vaccine (DTaP-IPV-HBV-PRP-TT, GSK) were developed.

Immunogenicity and safety of the liquid pentavalent vaccine Pediacel[®] (Sanofi) are analogous to other commercial multivalent formulations, such as Quadracel[®] + ActHIB[®] (DTaP-IPV-PRP-TT) [75] and Infanrix-IPV/Hib[®] (GSK), while the completely liquid formulation significantly simplifies vaccination [76, 78].

Convenience of using multivalent vaccine preparations for immunization against Hib has been confirmed in numerous studies on application of respective vaccines in overcrowded urban areas with low standard of living. In particular, the number of cases of Hib-meningitis decreased by 79% upon 69% vaccination coverage among children under 2 years old [81] in Chennai (Tamil Nadu, India) after only three years (2012–2014) of the use of the Pentavac[®] PFS (Serum Institute of India) pentavalent vaccine that includes Hib component [79], as compared to 19 cases per 100 thousand of the population in 2008 [80]. Similarly, the use of the tetravalent DTaP-Hib vaccine, which began in 1998 in Johannesburg (South Africa), reduced the number of cases of Hib meningitis in children under one year old by 65% over six years, while in 1994, 170 cases of this disease per 100 thousand children on average were registered [82].

Efficiency effectiveness of the Hib component of the pentavalent DTaP-HepB-Hib vaccine was confirmed [83] by its use in Bamako (~2 million inhabitants), capital of one of the poorest countries in the world, Mali, where more than 200 cases of invasive Hib infections per 100 thousand of the population per

year were registered among young children during the pre-vaccination period. Vaccination coverage of the children under two years of age, which began in 2002, reached 94% of the population by 2006 and led to 80% reduction in the incidence of invasive Hib diseases.

Two conjugated monovaccines against *Haemophilus* infections are registered in Russia, (see Table). These are *Haemophilus influenzae* type b conjugate vaccine[®] (FGUN Rostov Research Institute of Microbiology and Parasitology, Rospotrebnadzor, Russia) and Hiberix[®] (GSK). The domestic *Haemophilus* type b conjugate vaccine, registered in 2011, is produced in amounts of 200 thousand doses per year, which is sufficient to vaccinate no more than 5% of Russian infants. Some time ago, the ActHIB[®] vaccine (Aventis Pasteur) was widely used, but now the state registration in Russia for this preparation has been canceled.

Combination vaccines Pentaxim[®] (DTaP–IPV–Hib; Sanofi) [73] and Infanrix Hexa[®] (GSK) (table) are also on the pharmaceutical Russian market. Recently, a local pentavalent combination vaccine DTaP–HepB–Hib[®] was registered in Russia [84, 85] (Perm NPO Biomed, which is a part of the National Immunobiological Company JSC, subdivision of the State Corporation Rostech), which includes the Quimi-Hib[®] vaccine (see above) as the Hib component [84]. Efficiency of this preparation was confirmed in clinical trials [86].

COMPOSITION OF COMMERCIAL CONJUGATE Hib VACCINES BASED ON PRP

Commercial Hib conjugate vaccines differ by the size of poly- or oligomeric PRP chains, protein carrier, conjugation method, and spacer presence and structure. Structural characteristics of Hib vaccines are included in the European Pharmacopoeia, governing document for manufacture of pharmaceutical products in the European Union. The following proteins are recommended for use as protein carriers: diphtheria toxoid (DT), tetanus toxoid (TT), diphtheria recombinant protein CRM197 [87], and OMPC protein.

Comparative analysis of the efficacy of conjugate vaccines consisting of PRPs with different protein carriers performed in 1992 by Decker et al. [88] showed that vaccination with the DT-based conjugate led to formation of protective antibodies specific to PRP in only 39% of children, while conjugates including TT, CRM197, or OMPC showed significantly higher immunogenicity. The protein carriers TT, CRM197 [89], and OMPC [90] are the proteins most widely used nowadays in commercial vaccines, including Hib vaccines.

Considering that the effectiveness of Hib vaccine is determined not only by the nature of protein carrier, but also by the type of conjugation and processing used for vaccine production, numerous studies on the effi-

ciency of these proteins as carriers are somewhat ambiguous. Comparison of immunogenicity of the commercial vaccines PRP–CRM197 (HibTITER[®]) and PRP–OMPC (PedvaxHIB[®]) in an animal model (rhesus monkey) revealed that the PRP–OMPC conjugate exhibited a significantly higher immunogenicity compared to the PRP–CRM197 conjugate [91]. In this model, PRP–CRM197 induced PRP-specific antibodies only when injected simultaneously with DT. Comparison of the PRP conjugates with TT and CRM197 protein carriers in clinical trials showed no significant differences in immunogenicity of these products [92, 93]. At the same time, the recently obtained data favored the PRP–CRM197 conjugates to the PRP-TT conjugates [94].

PRODUCTION METHODS OF Hib CONJUGATE VACCINES BASED ON PRP

For the production of vaccines based on PRP, both high-molecular-weight polysaccharide obtained by biotechnological processing, the products of their medium-to-high degree depolymerization, as well as synthetic oligosaccharides, structurally related to PRP fragments, are used. At present, a significant number of conjugation methods have been developed [95] allowing efficient binding of these oligo- and polysaccharides to protein carriers.

Preparation of the conjugates based on the native PRP or partially hydrolyzed PRP_{lmw} is based on introduction of functional groups into the polysaccharide molecule that allow condensation with the carboxyl or amino groups of the protein carrier. One of the first approaches of this type was implemented in the works of Robbins et al. [65, 96–98] that involved PRP activation with cyanogen bromide followed by conjugation with a protein carrier.

The activated derivative of PRP (**1**) is formed in the course of reaction of PRP with cyanogen bromide creating polysaccharide with cyanate groups randomly distributed along the polysaccharide chain (Scheme 1, conditions a). Next, the product (**1**) interacts with ADH and isourea (**2**) is formed. Conjugation of the latter with a protein carrier is performed in the presence of a water-soluble condensing agent EDAC via formation of amide bonds between the hydrazide residues and carboxyl groups, which leads to formation of the cross-linked high molecular weight product (**3**) with molecular weight of up to 5 MDa [99]. This type of process is used in the production of the Hib conjugate vaccines based on DT (ProHIBiT[®]) and TT (ActHIB[®], OmniHIB[®]) [100, 101].

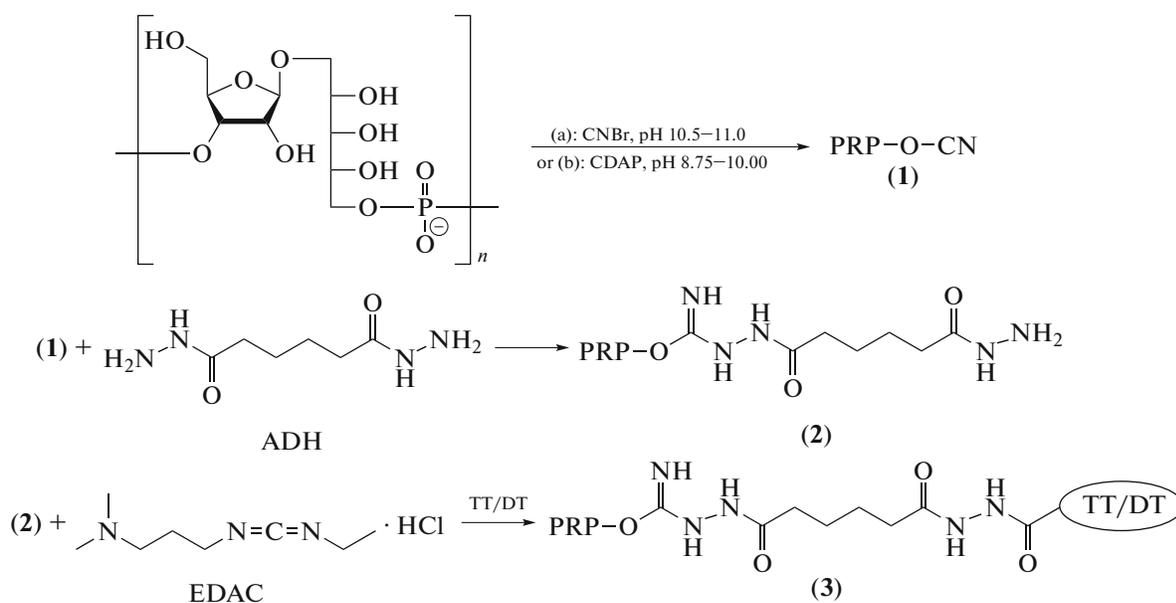
6-Aminocaproic acid can be used instead of ADH [102, 103]. In this case EDAC is also used for protein conjugation. This type of process is used in the production of another vaccine of the PRP-TT type, Hiberix[®] (GSK) [5].

Table 1. *Haemophilus influenzae* vaccines registered in the Russian Federation

Vaccine	Hib substance and dosage form	CPS content in the active ingredient, µg	Content of the carrier protein in the Hib substance, µg	Excipients (mg per dose)	Registration in Russia; number of registration ID
<i>Haemophilus influenzae</i> type b conjugate vaccine® (FBUN Rostov Research Institute of Microbiology and Parasitology, Rospotrebnadzor, Russia)	PRP-TT, freeze-dried powder to prepare solution*	9.5–14.3	19.0–28.6	Sucrose (20–30)	LP-000499 of 23.03.11, active
Hiberix® (GSK)	PRP-TT, freeze-dried powder to prepare solution**	10	~30	Lactose (10.08)	P N015829/01 of 18.05.09, active, perpetual
Act-Hib® (Aventis Pasteur)	PRP-TT, freeze-dried powder to prepare solution**	10	18–30	Sucrose (42.5) Trometamol (0.6)	P N013850/01 of 11.12.08, cancelled
Pentaxim® (Sanofi)	PRP-TT, freeze-dried powder to prepare suspension*, administered as mixture with DTaP-IPV	10	Not determined	Sucrose (42.5) Trometamol (0.6)	LSR-005121/08 of 01.07.08, active, perpetual; renewal date, 09.09.15
Infanrix Hexa® (GSK)	PRP-TT, freeze-dried powder to prepare suspension*, administered as mixture with DTaP-IPV-HBV	10	25	Lactose (12.6) Aluminum phosphate (0.12)	LP-000877 of 18.10.11, active
DTaP-HBV + Hib® (Perm NPO Biomed, National Immunobiological Company JSC, State Corporation Rostech)	PRP-TT, freeze-dried powder to prepare suspension*, administered as mixture with DTaP-HBV	10	21–31	Sucrose (42.5) Sodium dihydrophosphate (0.16) Disodium hydrophosphate (0.5)	LP-005412 of 20.03.19, active

* Intramuscular.

** Intramuscular and subcutaneous.



Scheme 1. PRP–protein conjugation with PRP preactivation.

Technological disadvantages of the cyanogen bromide method include basic conditions (pH 10.5–11.0) required for the effective functionalization of PRP, which causes uncontrolled disruption of phosphodiester bonds of the polysaccharide. Moreover, cyanogen bromide is poorly soluble in aqueous media, which requires the use of organic solvents, in which, however, PRP is insoluble. In addition, cyanogen bromide is highly toxic and is readily hydrolyzed under the used reaction conditions. Due to uncontrolled destruction and polymerization, the structure of the PRP–protein conjugate obtained by this method cannot be reliably characterized by physicochemical methods and, hence, cannot be standardized.

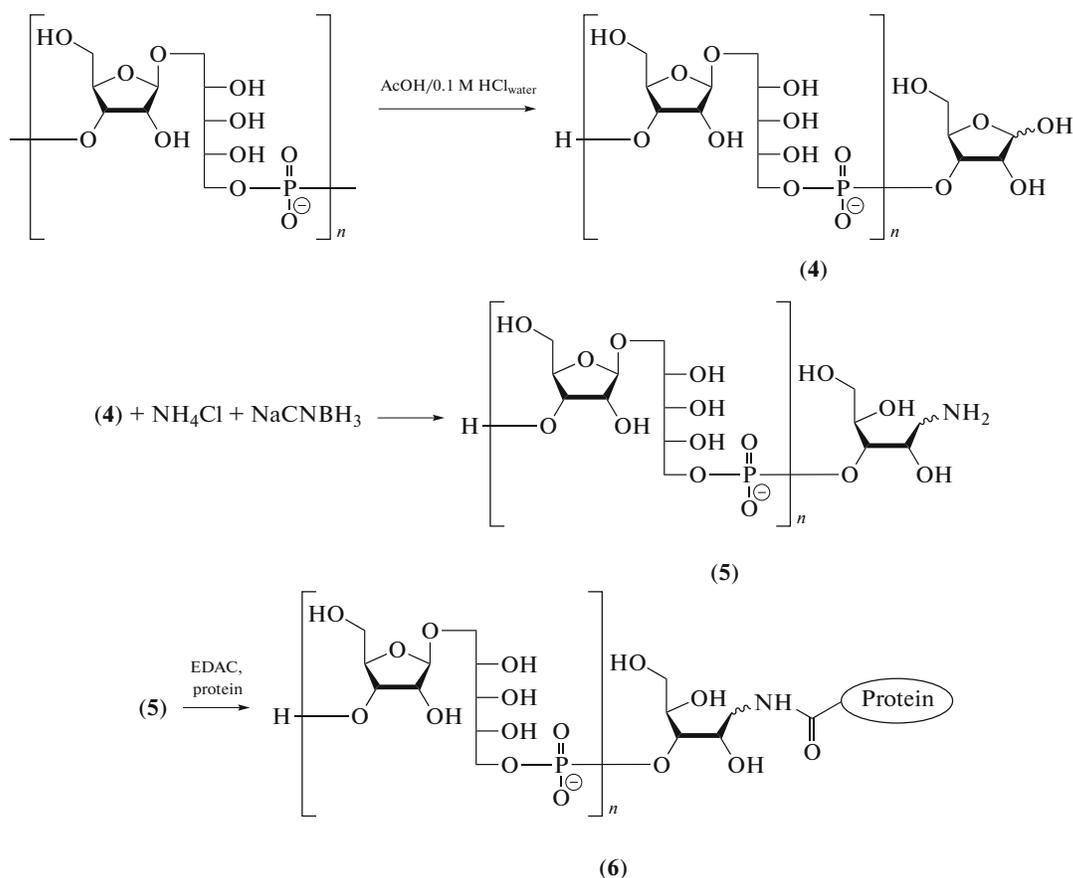
PRP content in the dose of the conjugate of high-molecular-weight PRP with DT obtained by the cyanogen bromide method was 25 μg , and that of protein was 18 μg [5]. An obvious advantage of this method is high ratio of PRP in the conjugate, up to 58%. However, this vaccine exhibited a relatively low immunogenicity, and hence it should be used at a higher dose (25 μg of PRP) than the modern vaccines containing only 10 μg of PRP.

The water-soluble CDAP reagent (Scheme 1, conditions b) proposed by Lees et al. in 1996 [105] could be used as an alternative to cyanogen bromide [104]. However, this method is not used in the industrial production of vaccines due to insufficient activity of the PRP conjugates produced via cyanidation process (see above).

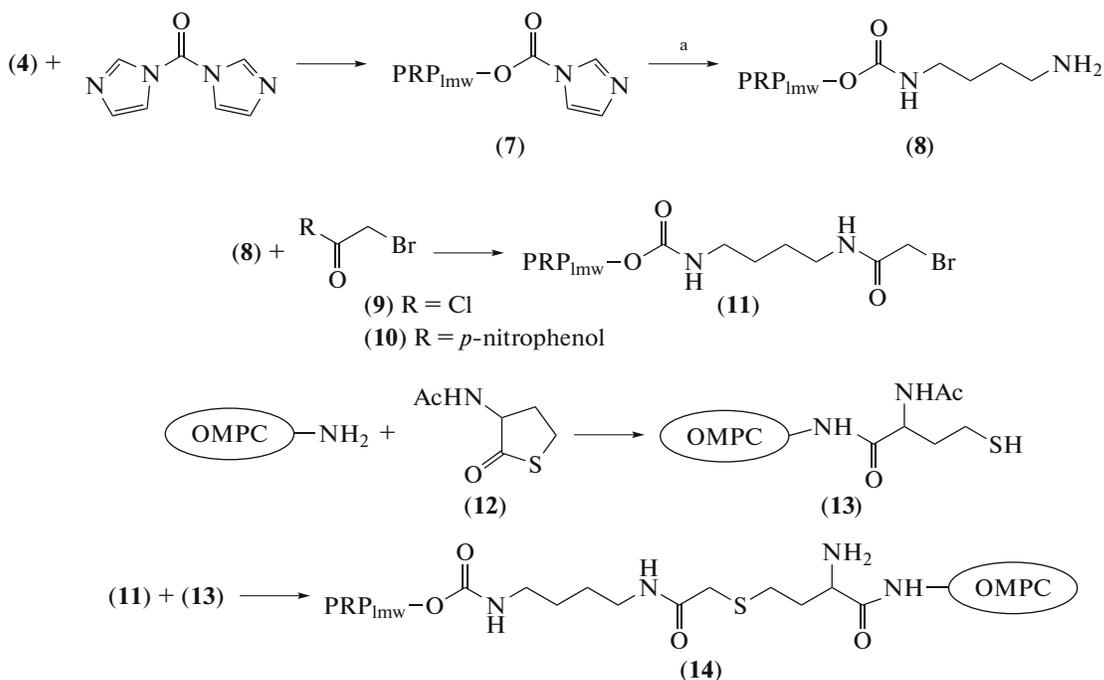
The presence of the nonconjugated PRP can reduce the efficiency of immunization with the PRP–protein conjugates. To simplify purification of vaccine conjugates, it was suggested in 1985 by Anderson et al. [106] to use low-molecular-weight PRP, PRP_{lmw} (4) (Scheme 2), which consists of 3–10 repeating units of 3- β -D-ribose-(1 \rightarrow 1)-D-ribitol-5-phosphate with free ribose residue at the reducing end formed during

partial hydrolysis of the native polysaccharide with 0.1 M aqueous HCl solution. A similar mixture of oligomers can be obtained by partial hydrolysis of PRP in the presence of aqueous AcOH [107]. Conjugation of the oligomers (4) is carried out after preliminary reductive amination by NaCNBH₃ in the presence of NH₄Cl [95] (shown in Scheme 2) or ethylenediamine [101]. In this case, glycosylamines (5) (or *N*-substituted glycosylamines) are formed, which are directly introduced into the conjugation reaction with D, DT, or CRM197 in the presence of EDAC, which in turn leads to formation of the conjugates with general formula (6) [106]. This type of process is used in the production of the VaxemHIB[®] (GSK) vaccine containing CRM197 as a protein carrier [5, 95].

The partially hydrolyzed PRP_{lmw} was also used in the conjugate with another adjuvant protein carrier, OMPC (4) (Scheme 3). First PRP_{lmw} is converted into the tetrabutylammonium salt to improve its solubility in DMF or DMSO, followed by the reaction with CDD, which transforms some of the OH groups of PRP_{lmw} into oligo-imidazolylurethane (7) [108], as suggested by Marburg et al. in 1986 (Scheme 3). Next, the oligo-imidazolylurethane (8) is converted into an amine in the reaction with excess of 1,4-butanediamine, which is then treated with bromoacetyl chloride (9) or *p*-nitrophenyl bromoacetate (10) to obtain bromoacetyl derivative (11). OMPC, which consists of 3–7 subunits with an average weight of 40000 Da each, is treated with a suitable thioylating agent, such as *N*-acetyl homocysteine thiolactone (12), to obtain thioylated derivative (13). Subsequent conjugation occurs in the reaction of the bromoacetyl groups in the modified derivative (11) with thio groups in the thioylated protein (13) to form conjugate (14). This type of the process is used by MSD to produce the Pedvax-HIB[®] vaccine [101].



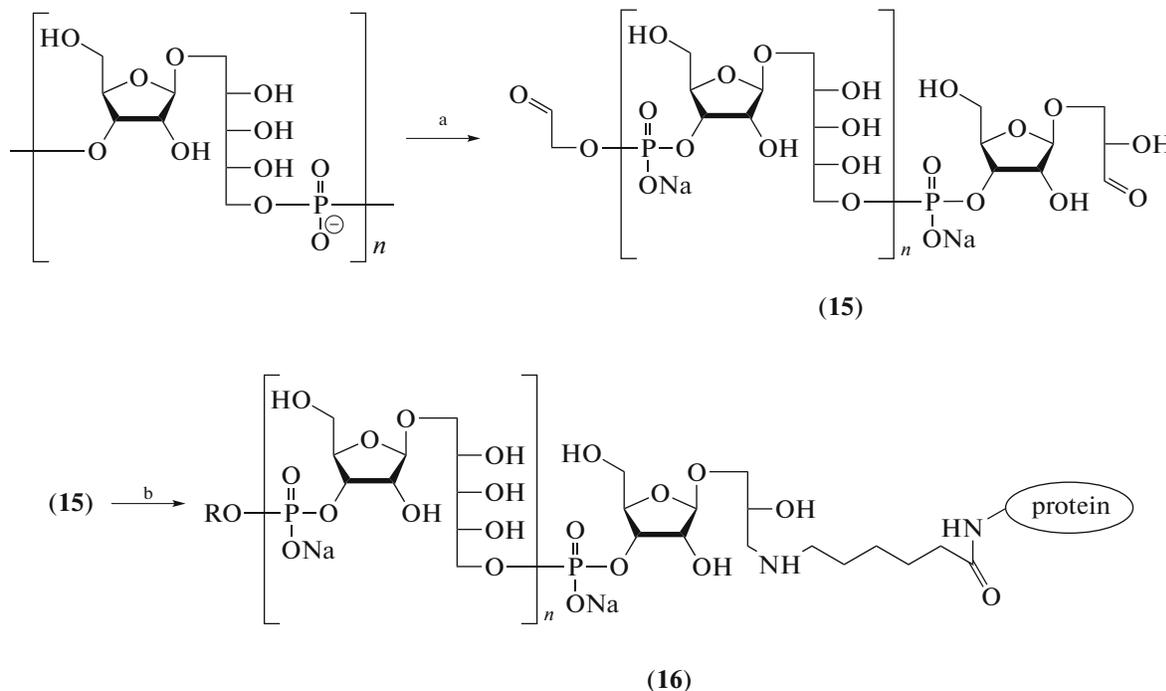
Scheme 2. Preparation of low-molecular-weight PRP (4) ($n = 3-8$) and its conjugates with protein carriers (6) using reductive amination.



Scheme 3. Synthesis of OMPC conjugate with low-molecular-weight PRP (14).
Reagents: a—1,4-butanediamine.

Another method for production of functionalized derivatives of the low-molecular-weight PRP_{lmw} for conjugation with proteins was proposed in 1986 by P. Anderson et al. [109]. Instead of hydrolytic cleavage of the polysaccharide (see above in Scheme 2), it involves periodate-mediated oxidative cleavage (Scheme 4). The resulting aldehyde products of general formula (15) are then subjected to condensation with the protein carrier by the reductive amination, which involves the amino groups of amino acids (for example, lysine) in the protein carrier. In this case,

Schiff bases are formed, which are reduced with sodium cyanoborohydride to form conjugates of general formula (16). For example, when CRM197 is used as a protein carrier, a conjugate is formed in which 6–20 oligosaccharide ligands on average are attached to each protein molecule [110]. This method has been successfully used by Pfizer for production of the Hib-TITER[®] vaccine, in which Hib oligosaccharides are conjugated to CRM197 directly, without a spacer (Scheme 4) [95].



Scheme 4. Preparation of a Hib vaccine using PRP_{lmw} formed by periodate cleavage of native PRP. Reagents: a—NaIO₄; b—protein, NaCNBH₃.

In order to accelerate the formation of Schiff bases this method was further improved by introducing an additional stage of conversion of protein carboxyl groups into hydrazides, which are more reactive than the ε-amino groups of lysine residues [111].

The use of low-molecular-weight PRP_{lmw} significantly simplified purification, sterile filtration, and assessment of physicochemical characteristics of the glycan–protein conjugate. This is important for testing of vaccine identity in industrial production. At the same time, structural changes may occur during the cleavage of PRP to reduce its molecular weight, as well as during isolation and purification [112], which complicates control and standardization of both initial PRP and its conjugates.

PREPARATION OF CONJUGATE Hib VACCINES BASED ON SYNTHETIC FRAGMENTS OF PRP

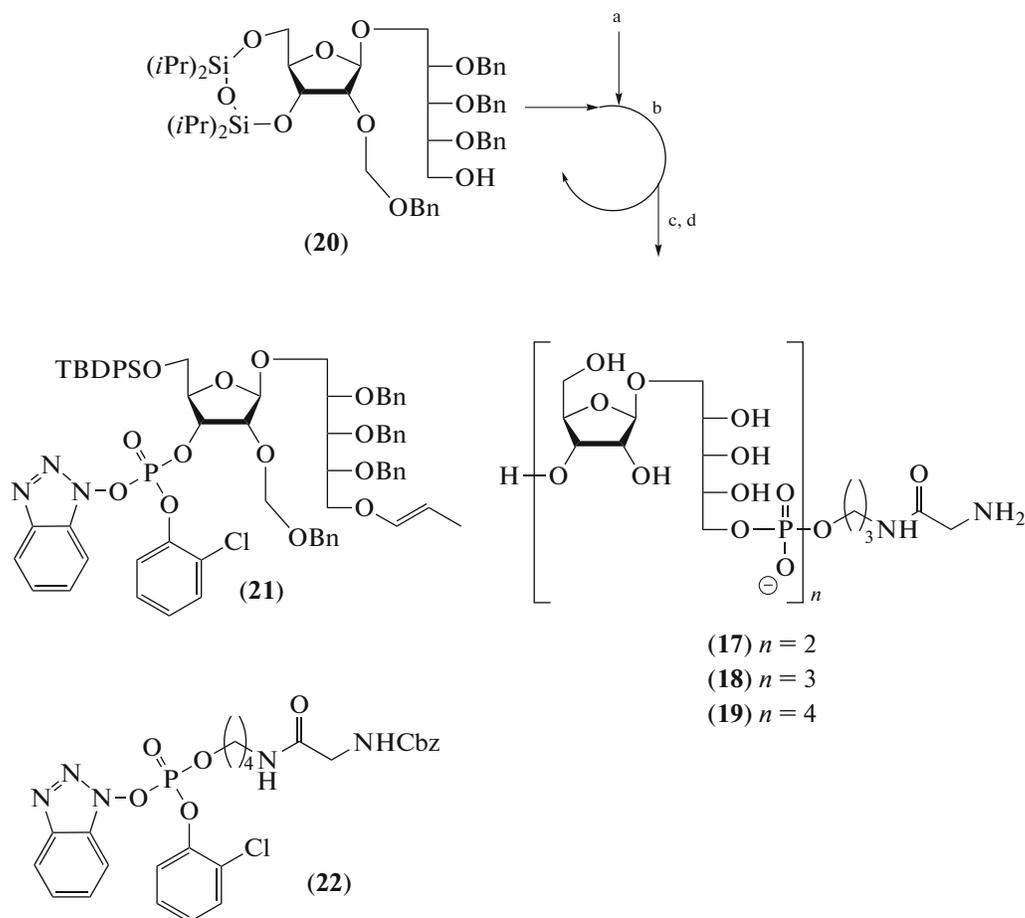
The development of a synthetic approach for preparation of spacer-armed oligosaccharides structurally related to immunodominant PRP fragments has been initiated, which was aimed to tackle challenges associated with processing of native PRP and its low-molecular weight fractions. Optimal spacer groups in the required positions should be mentioned as an advantage of using synthetic analogs of PRP. This is an important issue that facilitates efficient conjugation to the protein carrier and allows reliable verification of the structure and purity of the final conjugates and intermediates by carbohydrate analysis, NMR spectroscopy, and mass spectrometry, which, in turn, provides the higher quality control standards.

Moreover, synthesis of the vaccine oligosaccharide ligands eliminates the need for culturing pathogenic strains of *H. influenzae*, laborious isolation of PRP, and separation of bacterial impurities, such as toxic lipooligosaccharides.

The first examples of the synthesis of spaced oligosaccharides related to PRP and intended for conjugation with protein carriers were published in 1987–1989. In particular, three basic schemes were developed for the synthesis of spacer-armed oligosaccharides consisting of repeating disaccharide fragments of 3- β -D-ribose-(1 \rightarrow 1)-D-ribitol-5-phosphate. Each of them included the three stages: (1) chain elongation in the liquid-phase process with isolation of intermediate oligomers; (2) sequential chain build-up on a solid (polymer or glass) substrate; and (3) polycondensation to obtain a mixture of oligomers. Obvious technological and immunological advantages of the synthetic vaccine ligands over the native PRP and its low-molecular-weight derivatives stimulated significant simultaneous efforts of several leading scientific groups to find convenient and economical methods

for the synthesis of spaced PRP-related oligosaccharides.

The first series of PRP-related spacer-armed oligosaccharides was synthesized by van Boom et al. who carried out assembly of the oligosaccharide chains via sequential extension with phosphorylation as a key step. Thus, the dimer (**17**), trimer (**18**) [113, 114], and tetramer (**19**) [115] containing the *N*-glycyl-tetramethyleneamine spacer, were obtained by linear block synthesis from the selectively protected disaccharide (**20**) (Scheme 5). The disaccharide block (**21**) was used to extend the chain, which was attached at the free hydroxyl group of the ribitol residue in the presence of *N*-methylimidazole. The trimer and tetramer were obtained by repeating the sequence of reactions, including removal of the propenyl protective group and addition of the new unit; the spacer residue was introduced at the end of synthetic schemes in the reaction with the derivative (**22**). After removal of all protective groups, spaced oligomers (**17–19**) were obtained.

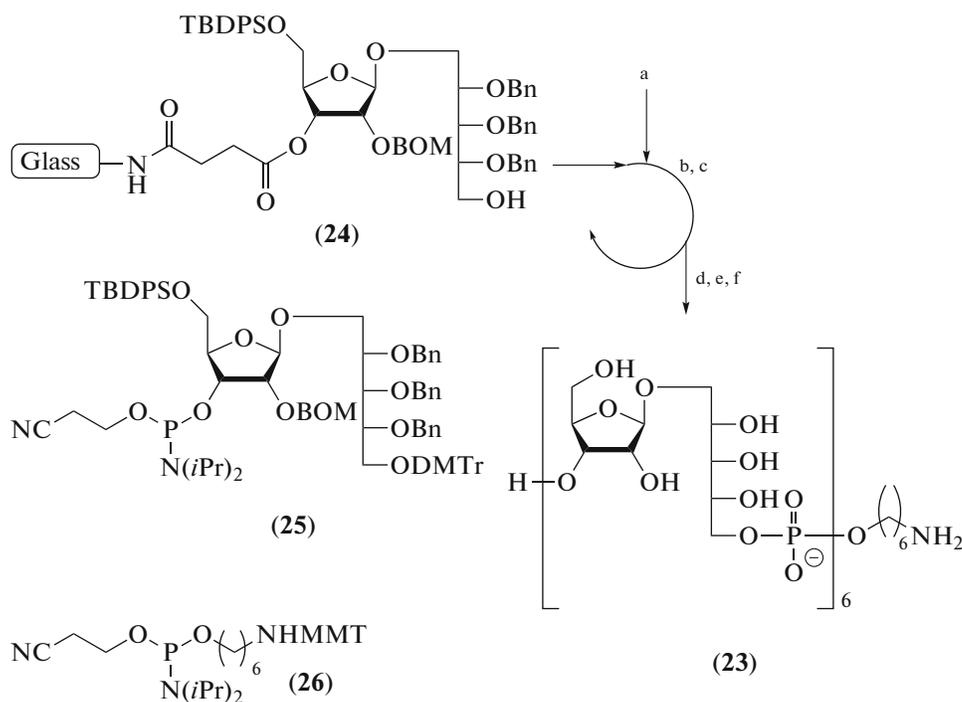


Scheme 5. Key synthetic blocks for the synthesis of spaced PRP oligomers (**17–19**).

Reagents: a—*N*-methylimidazole/pyridine, (**21**), yield 72%; b— HgO , $HgCl_2$, acetone, water, yield 82%; c—*N*-methylimidazole, pyridine, (**22**), yield 50%; d—removal of protective groups.

One of the most important factors limiting efficiency of scaling up the synthesis of the spaced PRP-related oligosaccharides is the need for chromatographic isolation of intermediates. In order to reduce the number of steps of chromatographic purification of intermediate products, van Boom developed a protocol for solid phase synthesis [116]. In the course of this work, the tactics of using protective groups was optimized and a more convenient method for formation of the phosphodiester bond was developed to improve the synthesis protocol suggested previously in this laboratory [113–115]. The first step of the synthesis of hexamer (23) [117] with hexamethyleneamine

spacer at the reducing end involved preparation of the selectively protected disaccharide (24) immobilized on aminated porous glass (Scheme 6). Next, phosphoramidite (25) was attached in the presence of 1*H*-tetrazole [116, 117], phosphite was oxidized to phosphate, and the dimethoxytrityl group was removed. The sequence of these reactions was repeated four more times, and then a spacer group was introduced by treatment with 2-cyanoethyl-6-[4-(monomethoxytrityl)-amino]hexyl-*N,N*-diisopropylphosphoramidite (compound (26)). Removal from the substrate and unblocking resulted in the formation of hexamer (23).



Scheme 6. Key synthetic blocks for the synthesis of the spaced PRP hexamer (23) [117].

Reagents: a—1*H*-tetrazole/acetonitrile, (25); b—0.02 M I₂, acetonitrile, water, 2,4,6-trimethylpyridine; c—2% CCl₃COOH in CH₂Cl₂; d—1*H*-tetrazole, acetonitrile, (26), 0.02 M I₂, acetonitrile, water, 2,4,6-trimethylpyridine; 2% CCl₃COOH in CH₂Cl₂; e—NH₃, H₂O, dioxane, 12 h, 50°C; f—0.5 M (*n*-Bu)₄NF, dioxane, 10% Pd/C.

A series of four homologous spacer-armed oligomers (27–30) with alternative location of the spacer fragment (at the nonreducing end) was obtained by Chan and Just [118] using not only monomeric (31), but also dimeric (32) phosphoramidite blocks (Fig. 2). A terminal ribose residue with unprotected OH group at C(1) was used as a spacer.

Low capacity of the glass substrate significantly limited the possibility of scaling up this process [117]. Candil et al. [119, 120] (Scheme 7) suggested a soluble polymer support as an alternative, which was based on polyethylene glycol (PEG) that could be precipitated

from the reaction mixtures by adding diethyl ether. The properly protected block (33) was immobilized on a PEG support with a succinic acid linker and extended with disaccharide phosphoramidite (34) with the following oxidation of the phosphite with *t*-butyl hydroperoxide and detritylation. These steps were repeated the necessary number of times, and the last cycle of chain extension was carried out with addition of the spacer reagent (35), followed by the removal of the protective groups and the support. The dimer (36), trimer (37), pentamer (38), and hexamer (39) were synthesized using this procedure.

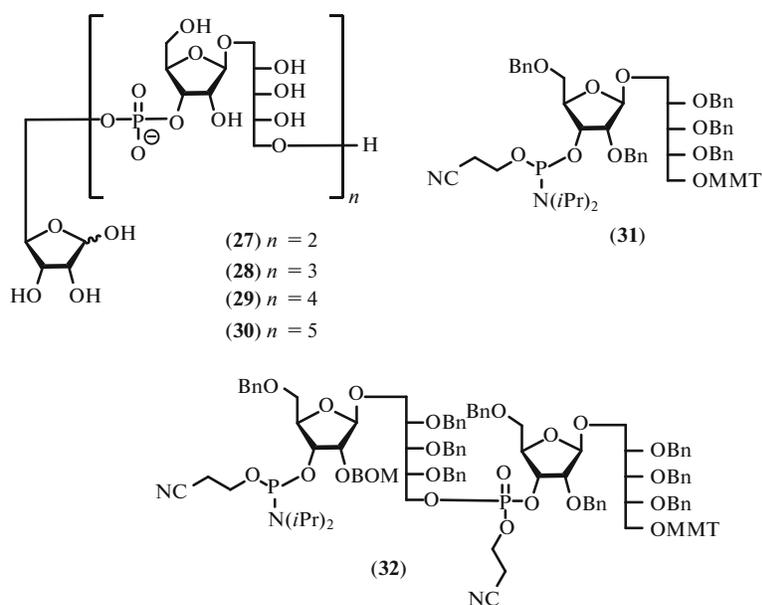
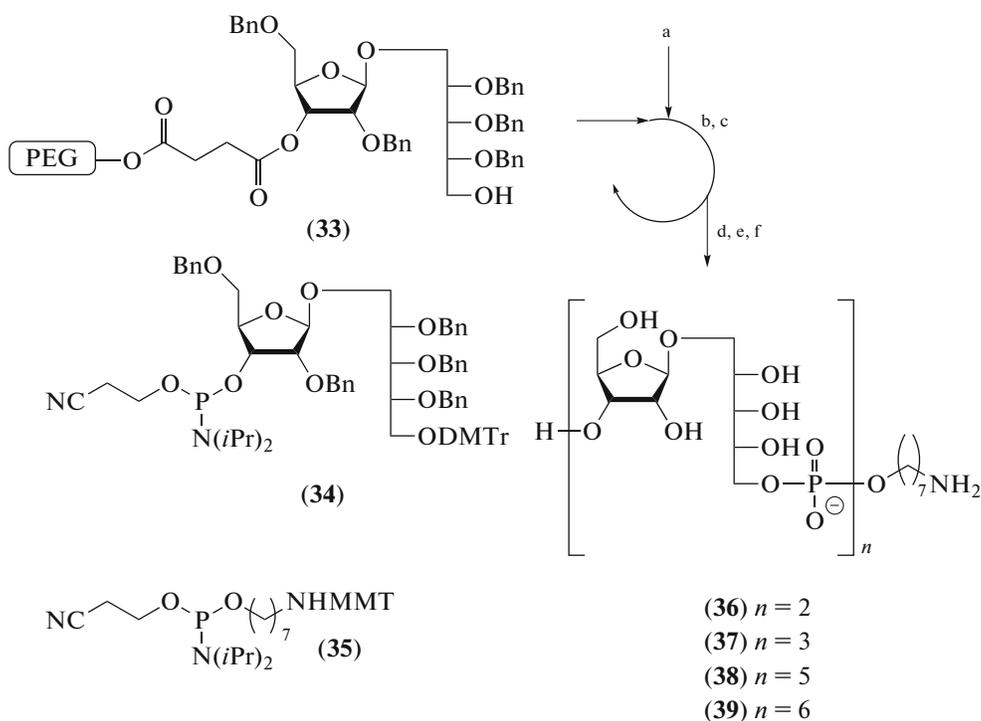


Fig. 2. Oligomers of PRP (27–30) and key synthetic blocks (31) and (32) for synthesis thereof [118].

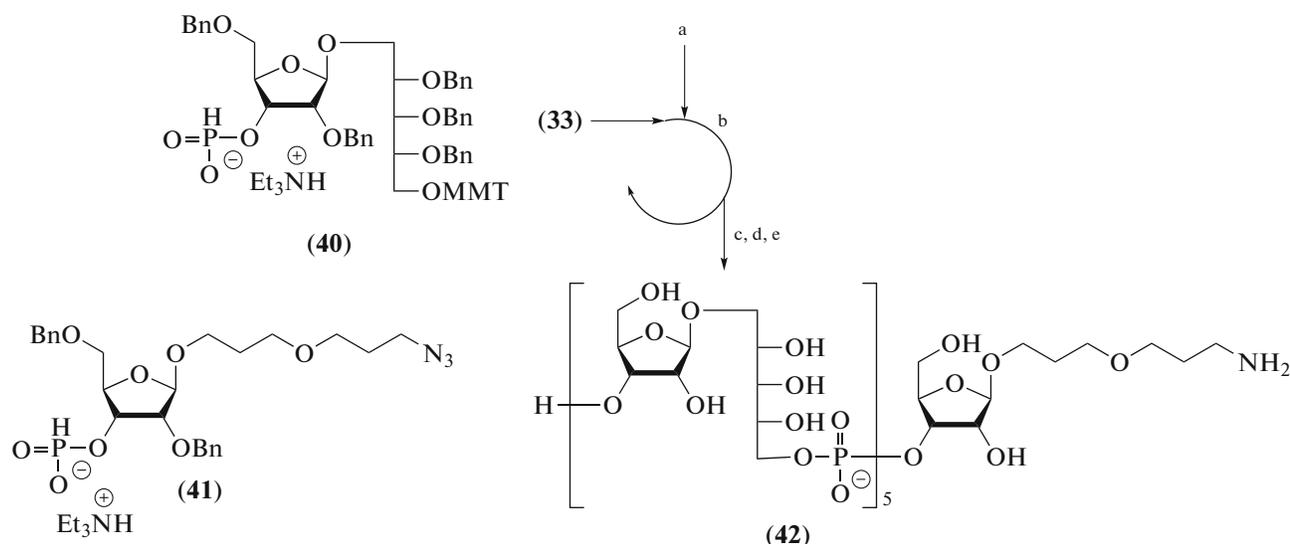


Scheme 7. Key synthetic blocks used in the synthesis of spaced oligomers (36–39) [119].

Reagents: a—1*H*-tetrazole, acetonitrile (34); b—*t*-BuOOH; c—pTsOH; d—1*H*-tetrazole, acetonitrile, (35), *t*-BuOOH; e—NH₄OH, THF; f—10% Pd/C.

Another variant of the solid-phase synthesis of spaced PRP-related oligosaccharides was proposed by Nilsson et al. [121] (Scheme 8). In this work, block (40) was attached to disaccharide (33) (Scheme 7) bound to a polystyrene support through a succinate linker in the presence of pivaloyl chloride, which was followed by

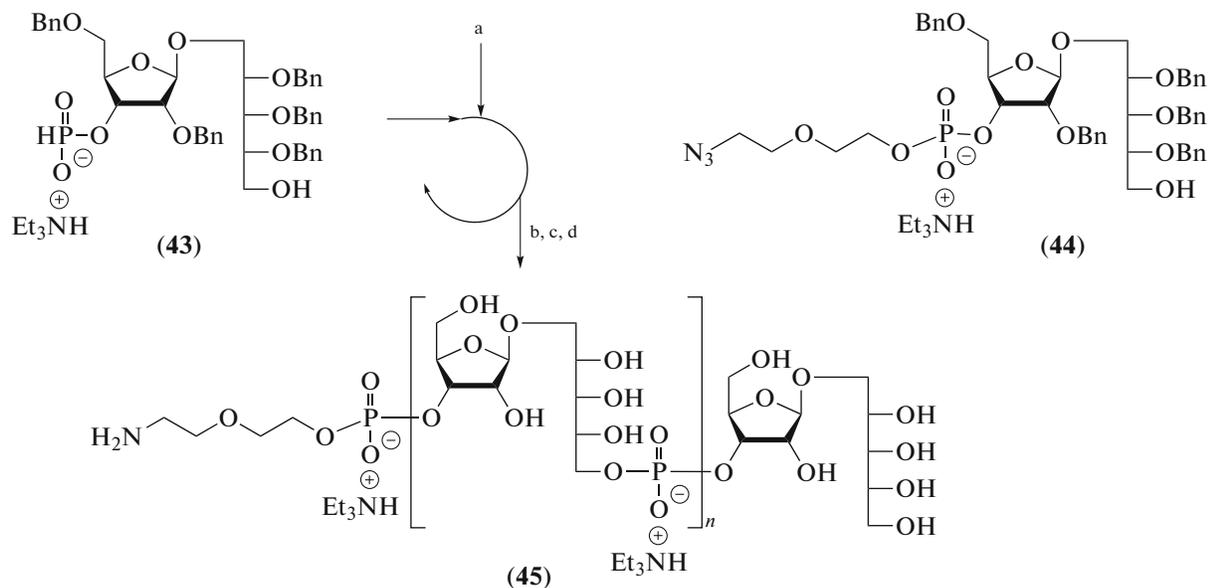
detritylation in the reaction with 0.5% trifluoroacetic acid in dichloromethane. After four cycles, the phosphonate (41) containing a prespacer group was attached at the last stage. After oxidation of the phosphonate to phosphate, reduction of the azide group, and removal of protective groups, the pentamer (42) was obtained.



Scheme 8. Key synthetic blocks for pentamer (**42**) synthesis [121]. Reagents: a—PivCl, CH₂Cl₂, pyridine (**40**); b—0.5% CF₃COOH in CH₂Cl₂; c—PivCl, CH₂Cl₂, pyridine (**41**); d—1% I₂, pyridine, water, NaOMe, MeOH, dioxane; e—10% Pd/C.

As follows from the above examples, optimization of the oligosaccharide synthesis was accompanied by the improvements in synthetic techniques and reduction in the number of chromatographic steps, but the degree of convergence, one of the most important parameters in a successful multistep synthesis, remained almost unchanged. A totally different highly convergent approach to the synthesis of the considered compounds based on polycondensation of the hetero-bifunctional monomer (**43**) (Scheme 9) containing free OH group at ribitol O(5) and phosphonate group at ribose O(3), was developed under the leadership of Verez-Bencomo and Roy [71, 122–124]. In the sug-

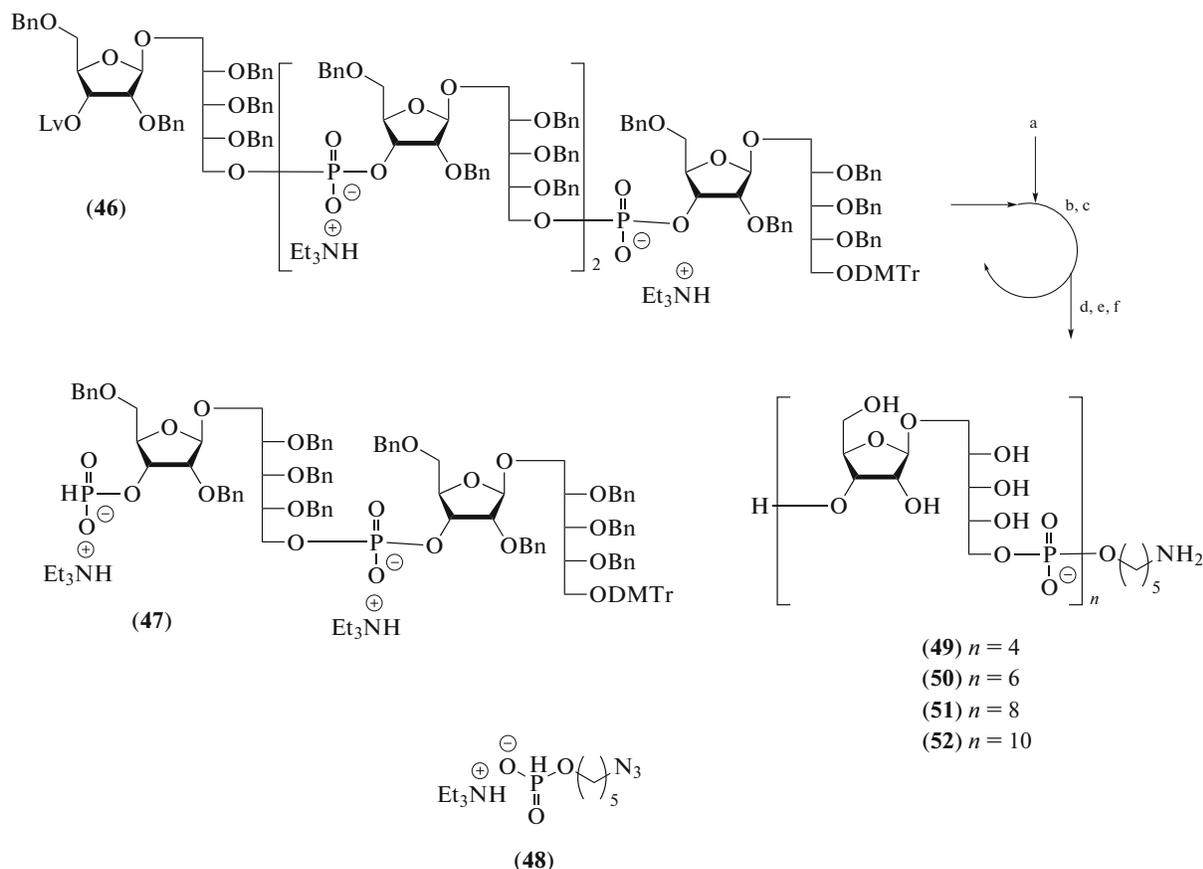
gested scheme, the process of monomer (**43**) polycondensation catalyzed by pivaloyl chloride is terminated by the addition of monomer (**44**), to which a diethylene glycol spacer is attached by phosphodiester bond. Oxidation of phosphite to phosphate, reduction of the azido group, and deblocking lead to formation of the mixture of oligomers (**45**) containing, on average, 8–11 repeating units. The optimized one-step synthesis of the spaced oligomers from monomeric precursors has been scaled up to commercial production and the resulting conjugate is included in the Quimi-Hib[®] vaccine (Heber Biotec S.A.).



Scheme 9. Key synthetic blocks for the synthesis of oligomers (**45**), $n = 8–11$ [71]. Reagents: a—PivCl, pyridine, (**45**); b—PivCl, pyridine, (**44**); c—I₂, pyridine; d—Pd/C.

One of the latest approaches to the synthesis of spaced PRP-related oligosaccharides (Scheme 10) was proposed by Seeberger et al. [125]. The implemented scheme is characterized by high degree of convergence and the selectively protected tetramer (46) with orthogonal protecting groups (a levulinoyl group at O(3) of the ribose residue at the nonreducing end

and a 4,4'-dimethoxytrityl group at O(5) of the ribitol unit at the reducing end) is used as a main starting compound. The chain was extended by attaching dimer blocks (47), then the chain was terminated with a spacer (48). After removal of the protecting groups, the spacer-armed derivatives of tetra- (49), hexa- (50), octa- (51), and decamer (52) were obtained.



Scheme 10. Key synthetic blocks for the synthesis of oligomers (49–52) [125].

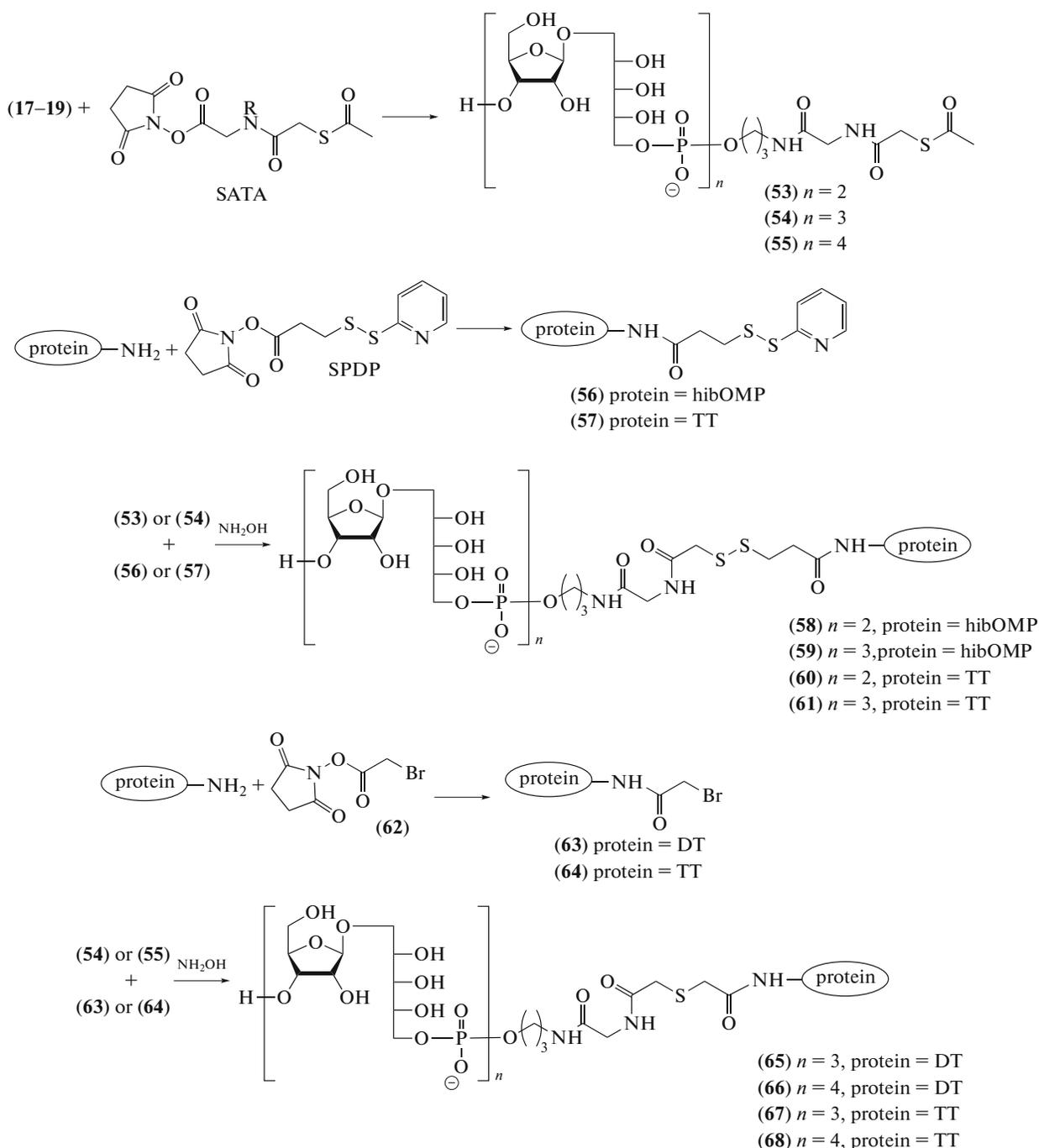
Reagents: a—PivCl, pyridine (47), 0°C; b—I₂, pyridine, H₂O, 20°C; c—CCl₃COOH/CH₂Cl₂, 0°C; d—PivCl, pyridine, (48); e—hydrazine acetate, CH₂Cl₂, 20°C; f—Pd/C, H₂, EtOAc, MeOH, AcOH, water.

SYNTHESIS OF PRP-RELATED SPACER-ARMED OLIGOSACCHARIDE CONJUGATES AS CANDIDATE VACCINES

The first conjugates of the synthetic PRP-related spacer-armed oligosaccharides (17–19) with the outer membrane protein of Hib (hibOMP) [126], TT [126], and CRM197 [115] were obtained in 1992 (Scheme 11). The glycine residue in the spacer was acylated by SATA to obtain S-acetylated products (53–55), and the proteins carrier hibOMP and TT were activated by SPDP. The activated proteins (56) and (57) reacted with the thiols obtained in situ during deacetylation of

compounds (53) and (54). The PRP : hibOMP molar ratio in the conjugates (58) and (59) was 5 : 1, and the PRP : TT ratio was 20 : 1 for the conjugate (60) and 13 : 1 for the conjugate (61).

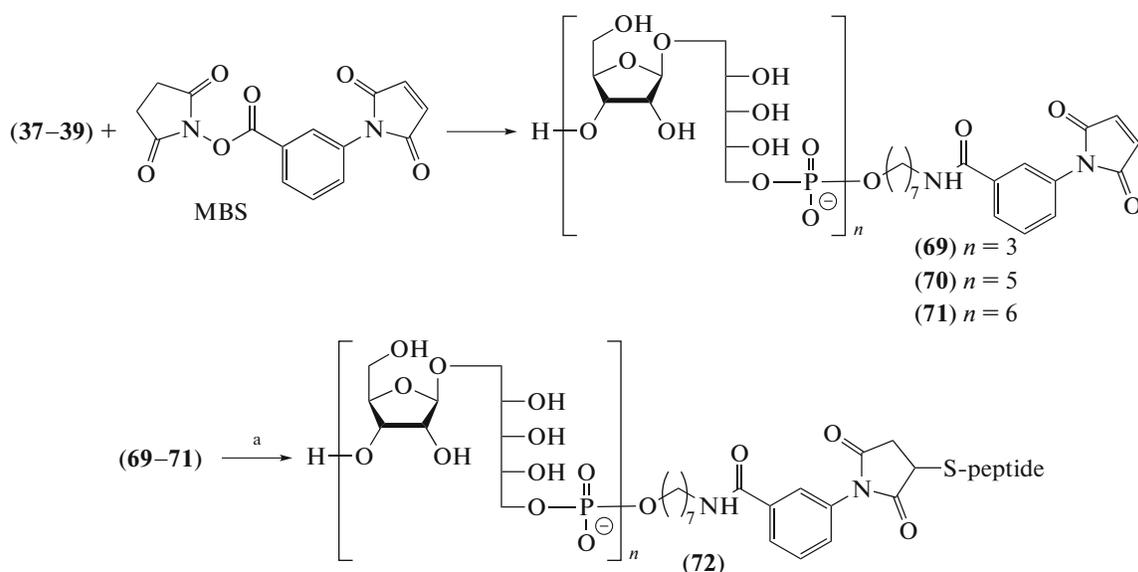
The toxoids TT and DT were bromoacetylated with *N*-succinimidyl bromoacetate (62) prior to attachment to oligosaccharides (54) and (55) [115]. The activated protein derivatives (63) and (64) were conjugated with oligosaccharides (54) and (55) in the presence of hydroxylamine. The oligosaccharide : protein molar ratio for conjugates (65), (66), (67), and (68) was 9.9 : 1; 6.5 : 1; 21 : 1, and 5.3 : 1, respectively.



Scheme 11. Synthesis of conjugates of oligosaccharides (17–19) with hibOMP, TT, and DT protein carriers.

The conjugates of oligosaccharide ligands (17) and (18) with TT were also obtained using glutaraldehyde as a homobifunctional cross-linking agent [126]. As a result, in addition to the attachment of oligosaccharide ligands, a uncontrollable cross-linking of the protein carrier also occurred, with formation of products with wide range of molecular weights (from 160 to 1000 kDa), which had very low content of oligosaccharide ligands comprising 0.5–4.0% w/w.

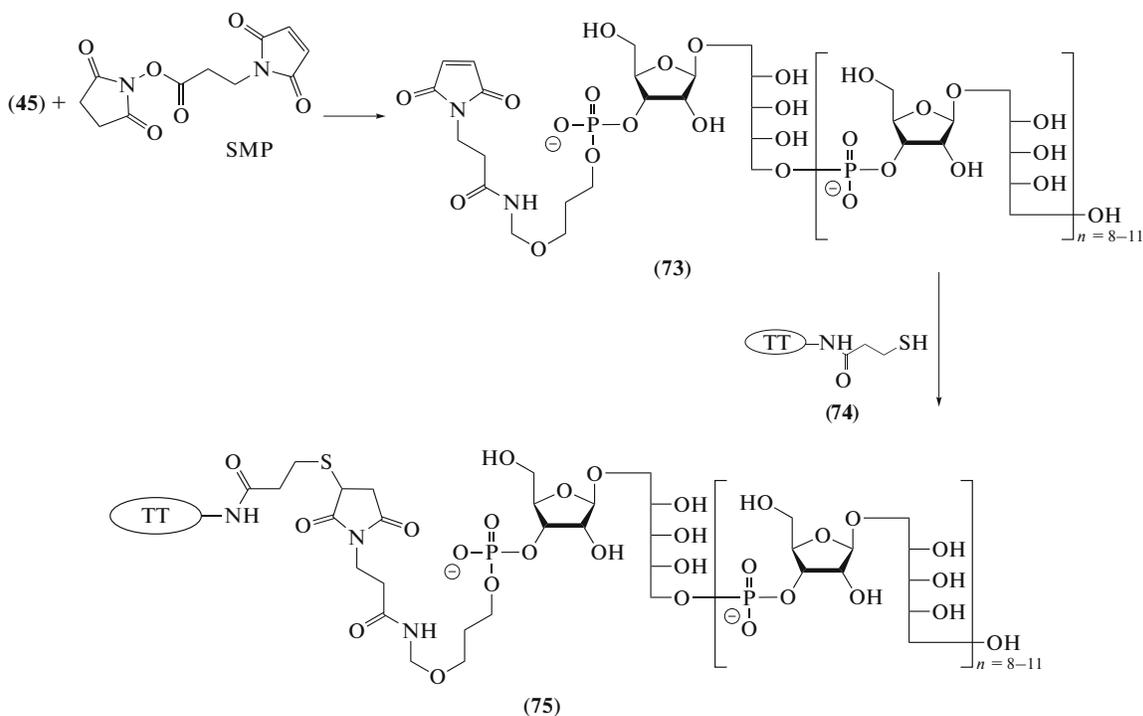
Other chemical methods were used for conjugation of proteins with the oligosaccharide ligands (37–39) containing heptamethyleneamine spacer (Scheme 12). First, these compounds were acylated with MBS to form maleimide derivatives (69–71) (Scheme 12) [119, 120]. The latter were next conjugated with three synthetic oligopeptides related to the proteins P1, P2, and P6 of the outer membrane of Hib that contained terminal cysteine residue; as a result the conjugates of general formula (72) were produced (Scheme 12) [127].



Scheme 12. Synthesis of conjugates of spaced oligosaccharides (37–39) with synthetic peptides. Reagents: (a) synthetic peptides P1, P2, and P6 of the Hib outer membrane with a terminal cysteine residue [127].

A different scheme of two-stage carbohydrate–protein conjugation was implemented to combine a mixture of the spacer-armed oligosaccharides (45) (Scheme 9) with TT [71]. First, the mixture (45) was converted into maleimide derivatives of general formula (73) in the reaction with SMP (Scheme 13). Conjugation of this product with the thiolated TT derivative (74) by the Michael reaction led to forma-

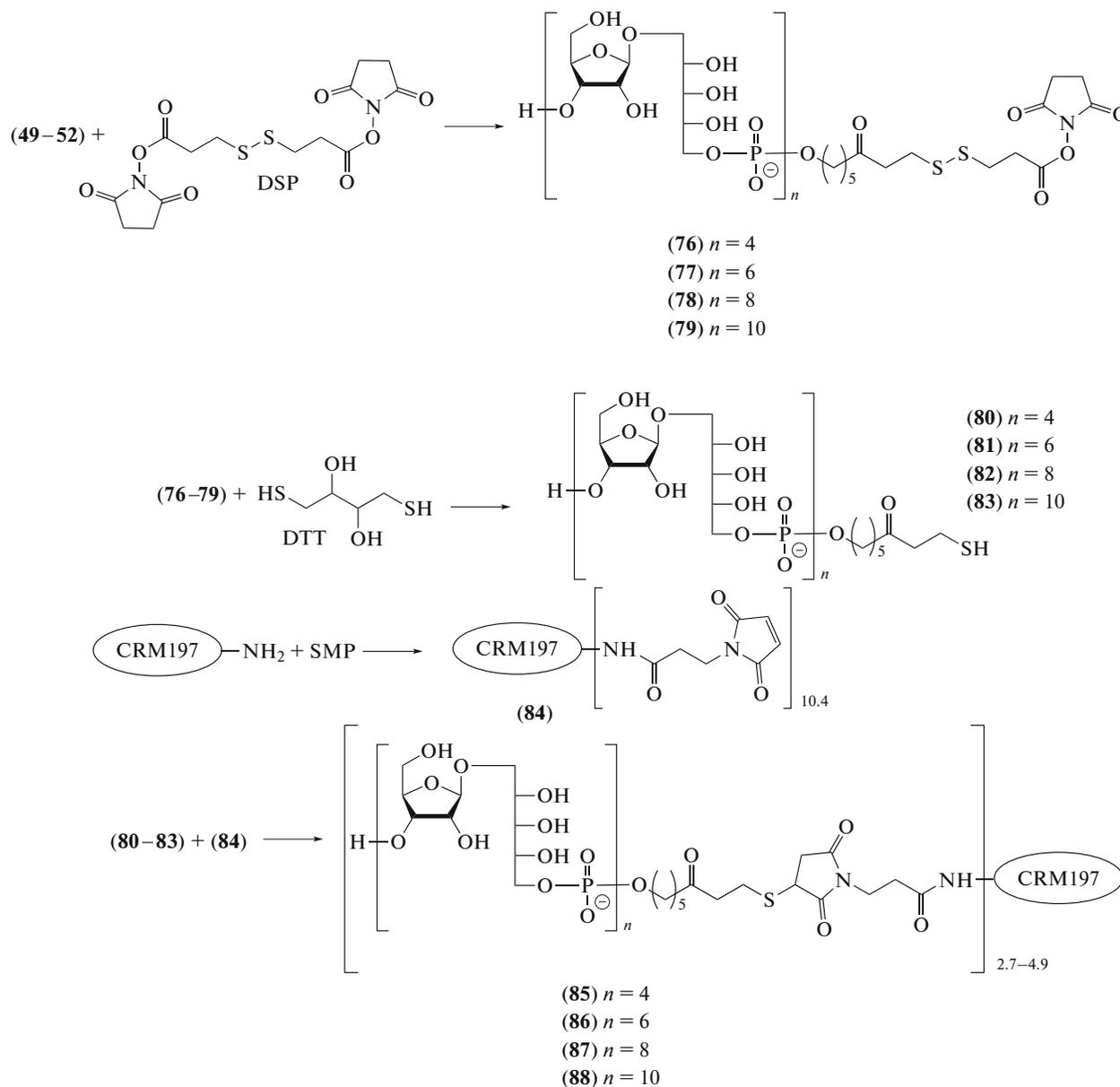
tion of the mixture of conjugates of general formula (75), in which oligosaccharide residues of different lengths were attached to a single protein unit. This product was further used as the main ingredient of the first commercial Hib vaccine Quimi-Hib® (Heber Biotec S.A.) with fully synthetic carbohydrate component.



Scheme 13. General scheme for the synthesis of the conjugate (75), the active substance of the vaccine Quimi-Hib® (Heber Biotec S.A.) [71].

A similar process for the addition of thiol to the double bond of maleimide was used by Seeberger et al. for preparation of the conjugates of individual oligosaccharides (**49–52**) [125]. They were first converted into derivatives of thiopropionic acid (**76–79**) in the reaction with DSP, then reduced to thiols (**80–83**)

using DTT, as shown in Scheme 14, and finally conjugated with the activated protein (**84**), which was obtained by *N*-acylation of the adjuvant protein carrier CRM197 using SMP. The content of oligosaccharide ligands in the resulting conjugates (**85**), (**86**), (**87**), and (**88**) was 4.9, 4.0, 3.1, and 2.7, respectively.



Scheme 14. Synthesis of conjugates (**85–88**) of spaced oligosaccharides (**49–52**) and CRM197.

EFFECT OF THE SIZE OF PRP-RELATED OLIGOSACCHARIDES ON THEIR IMMUNOLOGICAL PROPERTIES

The length of oligosaccharide components [109, 128, 129], the degree of conjugation, and the type of protein carrier are the main structural factors which

have an impact on immunogenicity of the conjugate vaccines based on PRP-related oligosaccharide ligands and protective properties of the induced antibodies. Unfortunately, due to the high complexity and relatively low availability of oligosaccharide derivatives, only fragmentary data on their immunological properties are available, which limits the possibilities

for systematic studies and assumptions about the relationship between the structures and biological properties of the discussed compounds. Nevertheless, the data known today allow us to draw some conclusions.

The length of B-epitope, i.e., the minimum length of the oligosaccharide chain required for binding to PRP-specific antibodies, can be determined by competitive ELISA. In this experiment, hyperimmune serum obtained by immunization with the protein conjugate of the PRP polysaccharide or by infection of animals with Hib is incubated with synthetic glycoconjugates containing PRP-related oligosaccharides of known length and structure, or directly with oligosaccharides. At the same time, the ability of the serum immunoglobulins to bind to the PRP-antigens immobilized on the surface of polystyrene microplate wells is being investigated. In particular, comparison of the results of inhibition of binding of the human hyperimmune serum antibodies to immobilized PRP antigen in the presence of oligosaccharide derivatives related to trimer (**18**) and tetramer (**19**) of the repeating RRP unit, as well as their conjugates with the TT protein carrier (compounds (**67**, **68**)) in the study by C. C. Peetres et al., showed that the tetramer (**19**) and its conjugate (**68**), but not the derivatives of trimer (**18**) and (**67**), effectively interfered with the interaction of human hyperimmune serum if PRP polysaccharide used as a coating antigen [115].

In these experiments, inhibition of the binding of the PRP polysaccharide (immobilized on an ELISA plate) with the PRP-specific poly- and monoclonal human antibodies using oligomers (**15**) (Scheme 4) of the PRP repeating unit with an average degree of polymerization of 1, 2, 7, 21, 47, 80, and 262, as expected [130], showed that the degree of inhibition increased with the length of the oligosaccharide haptens. However, these studies did not yield a consistent conclusion about the intensity of the potential immunogenicity of oligomers, the type and protective properties of induced antibodies, and the possibility of the immunological memory formation. To determine optimal hapten size for induction of antibodies specific to PRP, clinical experiments on immunization with glycan–protein conjugates with oligosaccharide chains of different lengths were carried out. To conduct these studies, Anderson et al. obtained conjugates of DT with oligosaccharide fractions of PRP (**5**) with an average number of repeating units of 8 and 20 [109]. The degree of conjugation for the conjugate with the PRP octamer was 3.3 mol of oligosaccharide per mol of DT, and for the conjugate with the 20-mer – 2.1 mol/mol of DT. Clinical trials of these preparations showed that the conjugate with the octameric ligand was a weak immunogen in children 9–15 months old, while the use of the vaccine containing conjugates of low-molecular-weight PRP with a degree of polymerization of ~20 induced high titers of the PRP-specific antibodies after the second immuni-

zation. At the same time, both vaccines induced an intense immune response in adults.

A similar study was carried out with another set of synthetic immunogens based on the oligosaccharide PRP fragments with ribitol residue at the nonreducing end [128] and average degree of polymerization of 4, 6, and 12. Their conjugates with CRM197 with a low hapten content were used to study immunogenicity. Clinical trials, conducted with 1 year old children, showed that all these formulations induced formation of immune memory to PRP, but did not reveal dependence of immunogenicity on the length of oligosaccharide ligands. To study the effect of other structural factors on immunogenicity, similar conjugates with PRP oligosaccharides were obtained that had phosphate or ribose residue at the nonreducing end, average degree of polymerization of seven, and higher content of oligosaccharide haptens. Immunogenicity of these compounds was 20 times higher than for the conjugates with low hapten content but did not depend on modification of the residue at nonreducing end.

The advantage of longer ligands was confirmed by the data obtained using conjugates of the synthetic oligosaccharide ligands with the same low degree of conjugation. Immunization of laboratory animals with the conjugates of the TT protein carrier with synthetic dimer (**36**) and trimer (**37**) (Scheme 7), in which the PRP : TT molar ratio was 2 : 1, obtained using 0.1% glutaraldehyde, showed that the immune response to vaccination with the trimer conjugate (**37**) was up to 200 fold higher than for the dimer conjugate (**36**) [131].

One of the recent published works on the development of a third-generation vaccine against Hib [125] summarizes the results of studies of conjugates (**85**–**88**) based on the CRM197 protein carrier containing tetra- (**49**), hexa- (**50**), octa- (**51**), and decamer (**52**) of the PRP repeating unit as carbohydrate ligands. The laboratory animals immunized with the conjugates (**85**) and (**87**) showed a significantly higher level of antibodies to PRP than in the case of conjugates (**86**) and (**88**). Based on these data, the authors suggested that the tetramer is the minimum PRP fragment containing the B-epitope of anti-PRP antibodies, which can only be taken as a qualitative result, since the studied conjugates had different degrees of ligand conjugation, and their immunogenicity can therefore be compared only conditionally.

CONCLUSIONS

The works reviewed above clearly demonstrate that Hib vaccines are an important component of the immunization schedule. Despite the presence of a number of effective vaccines based on the PRP polysaccharide on the market, which are used both individually and, more often, as part of combination vaccines, the development of third-generation vaccines based on synthetic oligosaccharide ligands structurally

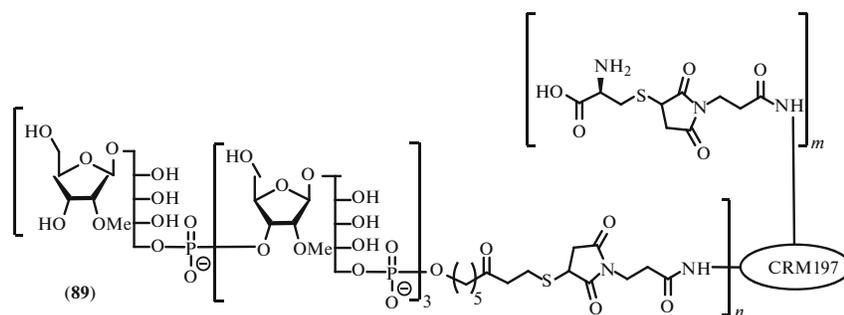


Fig. 3. Structure of the conjugate of CRM197 and tetrameric ligand with 2-*O*-methylated ribose units [132].

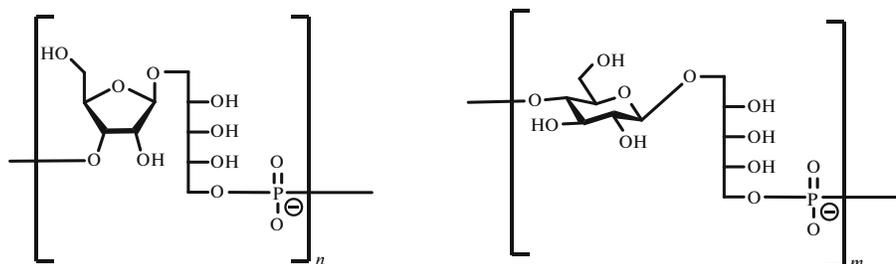


Fig. 4. Structure of the CPS of *H. influenzae* type b (left) and *H. influenzae* type a (right).

related to the PRP polysaccharide fragments is an urgent task. These studies are being actively carried out by researchers in specialized laboratories worldwide, including the authors of this review. The use of synthetic oligosaccharide ligands for preparation of vaccines against Hib eliminates the presence of biogenic impurities in the final products, which are formed during biotechnological production of the PRP polysaccharide. Moreover, the use of synthetic oligosaccharide ligands in production of conjugated vaccines with predetermined structural characteristics that meet the current high-quality GMP standards of pharmaceutical production makes this process more accurate and controllable.

To date, the only commercial vaccine against Hib that can be arbitrarily assigned to the third generation of vaccines, Quimi-Hib[®], contains not one type of oligosaccharide ligand, but a set of homologs with different numbers of repeating units. Because of this, the Quimi-Hib[®] vaccine does not have the important advantage of the products of this class – strictly defined structure of the vaccine ligand. However, the pioneering research conducted during development of the Quimi-Hib[®] vaccine, and other works discussed above, showed that the optimal oligomer length is in the region of low degrees of polymerization. The use of short PRP oligomers (such as tetrameric and even smaller) for the manufacture of conjugate vaccines against Hib could significantly reduce the costs of their industrial production.

Thus, the currently available effective synthetic approaches to the preparation of conjugates of synthetic oligosaccharides corresponding to the fragments of the PRP polysaccharide, and the results of optimization of the structure of the carbohydrate vaccine ligand have created the basis for the development of third-generation conjugate Hib vaccines with predetermined structural characteristics, in which there are almost no impurities of biogenic origin. In addition, synthetic nature of the antigenic ligand in the composition of such conjugated preparations makes it possible to optimize the structure of the ligand by introducing structural changes aimed at improving physicochemical characteristics and immunological properties. One of the first examples of such approach was reported by Seeberger et al., who suggested to introduce methyl group at the O2 of ribose residues to increase structural stability of the vaccine ligand, as in the conjugate (89) (Fig. 3) [132].

Another advantage of the synthetic approach to creation of vaccines against Hib is the possibility to vary the structure of the oligosaccharide ligand, which is inherent to chemical schemes, but not possible in microbiological processes, strictly limited by the CPS-producing strain. This means that only chemical schemes for vaccine production allow cost-effective replacement of key blocks in the ligand structure in order to optimize the vaccine ligand. Such need may arise in connection with the possible changes in epidemiologically significant strains of *Haemophilus influenzae*. This phenomenon is already being observed in

a number of countries in which Hib vaccines are widely used. For example, over the past 30 years, the *H. influenzae* type a (Hia) pathogen has increasingly been the cause of invasive infections in Canada, the United States, Australia, and Brazil [133, 134]. In this regard, there is a need in development of the Hia vaccine based on the corresponding CPS [134, 135], which, in turn, will require creation of the resource-intensive microbiological technology for production of this pathogen. Considering that the CPS of the *H. influenzae* type a pathogen has a structure of $\rightarrow 4$ - β -D-Glcp-(1 \rightarrow 1)-D-ribitol-5-OPO₂HO \rightarrow , which differs from the structure Hib CPS only by the presence of β -D-glucose residue instead of ribose (Fig. 4) [136], there is a possibility to develop universal approach to the synthesis of Hib and Hia oligosaccharides, which will enable preparation of the conjugate vaccine against two strains of *Haemophilus influenzae* within the same technological process.

The Hib vaccine was the first commercial conjugate carbohydrate vaccine and has good prospect to become the first conjugate vaccine, which includes a synthetic oligosaccharide ligand with a pre-determined structure. The efficacy of this vaccine could promote the development of the third generation vaccines as a new prospective direction in vaccinology. To date, many studies have been published devoted to synthesis of antigenic oligosaccharides and development of vaccines on their basis for prevention of dangerous human infectious diseases caused by bacteria *Streptococcus pneumoniae* [1–5, 8, 14, 137–141], *Enterococcus faecalis* [142, 143], *Shigella flexneri* serotype 2a [144, 145], *Neisseria meningitidis* serotype X [145], *Klebsiella pneumoniae* [146–148], *Clostridium difficile* [149], *Staphylococcus aureus* [150–153], as well as fungi *Aspergillus fumigatus* (based on oligosaccharide ligands related to α -glucan [154–157], galactomannan [158–162], and α -(1 \rightarrow 4)-galactosaminogalactan [163–165]), *Candida albicans* [166–170], and other pathogens [171–173].

In Russian Federation, the *Haemophilus influenzae* vaccine is currently included in the national immunization schedule only for children at risk, and these needs are met mainly through imported preparations (Table). This situation is likely due to the fact that compared to many other countries, the level of invasive Hib infections in Russia is low (see above). However, economic studies show that expanding the coverage of immunization programs against *H. influenzae* to total vaccination of newborns is cost-effective even if the vaccine is imported, although more costly than using the domestically-produced vaccine, because the need for this vaccine is already very high today. Assessment of socio-economic losses from five vaccine-preventable infections (pertussis, diphtheria, tetanus, poliomyelitis, and diseases caused by invasive forms of Hib infection) in Russia using the retrospective simulation model and the cost of vaccines with varying proportion (28–100%) of the DTaP–IPV–Hib vaccine

with acellular pertussis component in the total volume of DTaP–poliomyelitis vaccination, showed a 15-fold decrease in such key economic indicators of the vaccination effectiveness as annual losses of years of life including in monetary equivalents [174]. This study confirms the need to introduce Hib vaccine, for example, as part of the DTaP-based polyvaccine, in the mandatory vaccination schedule for the entire population, not just the at-risk groups.

Economic efficiency of the vaccination programs with modern vaccines against Hib can be significantly increased by replacing the currently imported Hib component in combination vaccines used in Russia with a domestic preparation produced using a full cycle technology. Combined efforts of the research organizations that have the required arsenal of methods and technologies used in modern carbohydrate chemistry and Russian biotechnological companies with the necessary production potential can undoubtedly lead to arrival of the third-generation domestically-produced vaccine against Hib. Development of manufacturing technology will allow creating technological base for the production of other carbohydrate conjugate vaccines required today to address national security and immunization problems.

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COMPLIANCE WITH ETHICAL STANDARDS

The article contains no description of studies on humans or animals performed by any of the authors.

Conflict of interest

The authors declare no conflict of interests.

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