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Development in the Concept of Bacterial Polysaccharide Repeating Unit-Based Antibacterial Conjugate Vaccines

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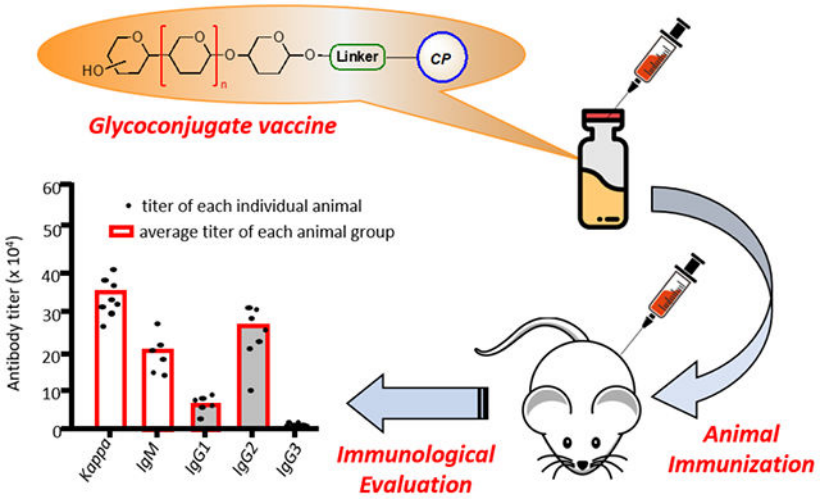
Abstract

The surface of cells is coated with a dense layer of glycans, known as the cell glycocalyx. The complex glycans in the glycocalyx are involved in various biological events, such as bacterial pathogenesis, protection of bacteria from environmental stresses, etc. Polysaccharides on the bacterial cell surface are the highly conserved and accessible molecules, and thus are excellent immunological targets. Consequently, bacterial polysaccharides and their repeating units have been extensively studied as antigens for the development of antibacterial vaccines. This review surveys the recent development in the synthetic and immunological investigations of bacterial polysaccharide repeating unit-based conjugate vaccines against several human pathogenic bacteria. The major challenges associated with the development of functional carbohydrate-based antibacterial conjugate vaccines are also considered.

Graphical abstract



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Keywords

Polysaccharide; Oligosaccharide; Glycoconjugate; Immunogenicity; Conjugate Vaccine; Antibacterial Vaccine

1. Introduction

Pathogens, such as bacteria, viruses, parasites, fungi, etc., are the biggest threat to human life.[1] The recent COVID-19 pandemic is a clear example. Currently, there are two main strategies to address pathogenic infections. One strategy is to treat patients with antibiotics or antiviral and antiparasitic drugs. The other strategy is to immunize the human population against pathogens via vaccination. Of the two strategies, vaccination is not only effective but also economical and long lasting, and has helped control and even eliminate many infectious diseases.[2, 3] Moreover, the rapid emergence of drug resistance to various antibiotics in the past few decades has made the vaccination strategy even more attractive.

The discovery by Edward Jenner in 1796 that humans could be protected against smallpox after immunization with cowpox was a historical milestone during the development of vaccine concept (Figure 1).[4] Since then, different types of vaccines, such as attenuated or inactivated vaccines,[5, 6] subunit vaccines[7–9] and DNA or RNA vaccines,[10–12] against various human pathogens have emerged. This review is focused on antibacterial vaccines, especially the subunit vaccines derived from bacterial polysaccharides and their repeating units.

The concept of carbohydrate-based antibacterial vaccines was first introduced by Avery and Heidelberger in the 1920s,[13] when they found that the capsular polysaccharides (CPSs) of the Gram-positive bacterium *Streptococcus pneumoniae* were immunogenic.[14] In addition, it was discovered that CPSs also played a critical role in the serotype specificity of different *S. pneumoniae* strains.[15,16] Francis and Tillett further observed that intravenous injections of the CPSs derived from various *pneumoniae* serotypes elicited respective CPS-specific antibodies in patients.[17] In 1935, after studying the antibody responses induced by type III and type VIII pneumococcal CPSs, Finland and Ruegsegger developed the first CPS-based pneumococcal vaccine.[18] A 6-valent CPS-based pneumococcal vaccine was developed by Heidelberger et al. in 1942-1945, which was used to immunize the US air force.[19] However, the development of antibacterial vaccines, including carbohydrate-based vaccines, stopped in the early 1950s due to the great success of antibiotics. It was soon discovered that antibiotics were not the panacea for all bacterial infections. Thus, the interest in antibacterial vaccines resurged. In particular, the ever-growing bacterial resistance to antibiotics in the past few decades has boosted the interest in developing antibacterial vaccines. In 1983, Merck introduced the first carbohydrate-based pneumococcal vaccine, PneumoVax, made up of purified CPSs from 14 pneumococcal serotypes.[20] Presently, the second-generation pneumococcal vaccine is composed of 90 pneumococcal CPSs from 23 serotypes.[21] Overall, carbohydrate-based antibacterial vaccines have contributed significantly to human health and welfare.

However, stand-alone polysaccharide vaccines are effective mainly in adult populations. They remain deficient in high-risk groups (e.g., children under five years of age) and

virtually ineffective in infants (below two years of age), mainly because polysaccharides alone cannot elicit strong T-cell dependent immune responses in these people.[22] To address this problem, carbohydrate-based conjugate (glycoconjugate) vaccines have emerged. Its basic concept is to covalently link a highly immunogenic carrier molecule (*e.g.*, a protein) to carbohydrate antigens to improve their immunogenicity and T-cell dependent immune responses.[23, 24] The first bacterial polysaccharide-protein conjugate vaccine against *Haemophilus influenzae* type b (Hib) was approved for clinical use in 1987.[25] Since then, a series of other bacterial polysaccharide-protein conjugate vaccines have been developed. These vaccines have been proved effective both in adults and in children and thus are widely used.

Despite the great advancement of conjugate vaccines made up of bacterial polysaccharides, these vaccines have their limitations mainly because the polysaccharides produced by bacteria are complex mixtures that are difficult to control in quality and in the conjugation with carrier proteins (CPs). Consequently, the resultant conjugates are complex and ill-defined cross-linked lattices.[26–28] To address this issue, currently, great effort has been devoted to the synthesis of oligosaccharides containing the repeating units of bacterial polysaccharides and applying them to the development of oligosaccharide-based antibacterial conjugate vaccines.[29] This type of conjugate vaccines that contain synthetic and structurally defined carbohydrate antigens can have several advantages, such as controlled conjugation chemistry and product quality, reduced contamination, and feasibility to perform structural and structure-activity relationship studies and vaccine optimization.[30,31] A successful example in this field is the vaccine made up of fully synthetic oligosaccharides of Hib polysaccharides approved by Cuba and other countries for clinical use in 1999.[25] Recently, an oligosaccharide-based vaccine for shigellosis has reached phase II clinical trials.[32,33] In the literature, there are several reviews about carbohydrate-based vaccines and therapeutics,[34,35,36,37,38] including a recent review by Adamo et al.[39] The present review is unique in that it focuses mainly on antibacterial conjugate vaccines made of synthetic oligosaccharides and their structure–immunogenicity relationships.

2. Development of Carbohydrate-Based Antibacterial Conjugate Vaccines

2.1 The general structure of carbohydrate-based conjugate (*i.e.*, glycoconjugate) vaccines:

A glycoconjugate vaccine is usually composed of three components, a carbohydrate antigen, a carrier molecule, and a linker. In the process of vaccine development, the first step is to identify the target antigen, which, theoretically, should be antigenic, conserved, and exposed on the cell surface to facilitate immune recognition. The next step is to select the proper carrier molecule, which should be an immunostimulator to help enhance the immunogenicity of the carbohydrate antigen. Finally, a linker is identified for carbohydrate antigen and carrier molecule coupling, which should be governed by the conjugation chemistry and related reaction conditions.

Based on the properties of the carbohydrate antigens, carrier molecules, and the preparation methods of glycoconjugate vaccines, they are divided into three different types (Figure 2). One type of glycoconjugate vaccines has polysaccharides derived from natural sources,

e.g., bacterial cultures, directly conjugated with CPs (Figure 2a).[40] Most FDA-approved carbohydrate-based antibacterial conjugate vaccines belong to this type. In this case, the polysaccharide antigens are complex mixtures and, more importantly, need to be activated by methods to enable their conjugation with CPs,[41] which further increases the degree of complexity of the polysaccharide mixtures. In addition, the conjugation sites for both the carbohydrate antigens and the CPs are uncontrollable. As a result, this type of glycoconjugate vaccines is complex and batch-to-batch different. Another type of glycoconjugate vaccines has synthetic oligosaccharide antigens linked to CPs, which are called semi-synthetic conjugate vaccines (Figure 2b).[42–44] In these vaccines, the structure of carbohydrate antigens and their linkage forms to CPs are well defined, although the protein conjugation sites are usually undefined. The third type of glycoconjugate vaccines has synthetic carbohydrate antigens conjugated with synthetic carrier molecules, such as lipids, glycolipids,[45] glycopeptides,[46] oligosaccharides or polysaccharides[23] and synthetic polymers,[47] as well as other synthetic materials,[48] which are called fully synthetic glycoconjugate vaccines (Figure 2c).[49] These vaccines have well-defined structures that can be fully characterized by modern analytical techniques, including NMR and MS. This review is mainly focused on semi- and fully synthetic antibacterial glycoconjugate vaccines.

2.2 Bacterial carbohydrate antigen:

Bacteria express diverse polysaccharides, such as CPSs, lipopolysaccharides (LPSs) and exopolysaccharides (EPSs). CPSs and LPSs are conserved and exposed on the bacterial cell surface, making them useful immunological targets for vaccine development. However, bacterial polysaccharides are not only species-specific but also strain-specific. For instance, the antigenic diversities of bacterial polysaccharides have resulted in 13 meningococci serotypes[50] and 90 pneumococcal serotypes.[51] Therefore, it should be noted that a vaccine derived from a specific bacterial polysaccharide is only functional for that specific bacterium and strain, and an effective vaccine against an infectious disease may need to be of multivalent property, *i.e.*, vaccines derived from the polysaccharide antigens of multiple strains of the disease-causing bacterium.

Although bacterial polysaccharides are useful for the development of vaccines, they are not necessarily the optimal immunogens, because large polysaccharides may affect the interactions of carrier molecules in the resultant glycoconjugates with immune cells. Furthermore, although bacterial polysaccharides are large and complex, they are usually composed of small repeating units of monosaccharides or oligosaccharides. Most likely, the immune system only recognizes some unique epitopes of a polysaccharide, often the non-reducing end epitope[52] or the epitope containing one to several repeating units,[53] instead of the full glycan. Thus, the glycan length is always one of the concerns in the design of carbohydrate-based vaccines. Deciphering the optimal number of repeating units of a polysaccharide or glycan length required for immune recognition and vaccine development is not easy, because this needs in-depth analysis of the structure-activity relationships of carbohydrate antigens, which is challenging. Nevertheless, there is sufficient information to suggest that it may not be necessarily “the longer glycans, the better” for vaccine development. Moreover, it seems that there is not a golden standard about the optimal glycan length as antigens, thus the best oligosaccharide substitute for each

specific polysaccharide antigen may need to be determined individually. For example, in semi-synthetic Hib vaccine Quimi-Hib, the optimal oligosaccharide epitope ranges from six to eight repeating units of the Hib polysaccharide,[54] but in other cases, the oligosaccharide length varies.[55,56,57] In addition, other factors, such as the structural uniqueness, frameshift[58] and functionalization of carbohydrate antigens[59,60,61,62] and the epitope stability that may affect the immunogenicity and antigenicity,[63] should also be taken into consideration during the design and development of carbohydrate-based antibacterial vaccines.

2.3 Vaccine carrier:

In glycoconjugate vaccines, carrier molecules also play a critical role in stimulating the immune system and enhancing immune responses, especially T-cell dependent immune responses, to the targeted carbohydrate antigens. Thus, the carrier molecule needs to contain T-cell helper epitopes and be immunologically active. Other properties that a vaccine carrier should possess are that it should be safe and easily produced in low cost and consistent quality. Among various vaccine carriers, proteins are the most commonly used for antibacterial vaccine development. In addition to the necessary properties for carrier molecules as mentioned above, proteins contain many and different functional groups, such as amino, thiol, carboxylic and hydroxyl groups, which enables various conjugation methods. Currently, there are several common CPs, such as mutant cross-reacting material (CRM) from diphtheria toxin, diphtheria toxoid (DT), tetanus toxoid (TT), meningococcal outer membrane protein complex (OMPC), and *H. influenzae* protein D (HiD), which are all used in licensed bacterial vaccines. Besides these established CPs, many other CPs are in preclinical studies or clinical trials.[48] For example, recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA) is utilized as a carrier for *Shigella O*-antigens.[64] Clinical results have demonstrated the effectiveness of glycoconjugate vaccines, as well as the long-term and protective immune responses that they induce. However, it should be noted that CPs can also induce specific antibody responses that may supress the immune response to the conjugated carbohydrate antigens.[65–67] Repeated immunizations using vaccines containing the same CP can reduce the effectiveness of vaccination as well. Thus, research to discover new CPs or non-protein carrier molecules is important.

2.4 Glycoconjugate vaccine linker:

It has been well established that carbohydrate antigens must be covalently linked to carrier molecules to exhibit enhanced immunogenicity. The choice of a proper linker between carbohydrate antigens and CPs depends on several factors. First, it should be non-immunogenic so that it will not cause unwanted antibodies or immune response. It was also shown that strong immune reactions to the linker can supress immune responses to the target carbohydrate antigen.[68–70] Second, it needs to be bi- or multi-functional, as it has to bridge two distinctive biomolecules, *i.e.*, the carbohydrate antigen and the CP. Furthermore, the conjugation reactions should be highly efficient and selective, which can help reduce the complexity and heterogeneity and achieve desired and consistent antigen loading of resultant glycoconjugates. It has been demonstrated that the antigen loading of glycoconjugate vaccines can have a big impact on their immunological properties.[71–73] Many linkers and the associated conjugation methods have been developed for the preparation of glycan-

protein conjugates.[74,75] The selection of a proper linker and conjugation method for a vaccine is largely decided by the structure of carbohydrates and sometimes by the CP as well. For polysaccharide antigens, their activation method and the resulting functional groups dominate the conjugation method since the functionalized sugar units in the polysaccharide antigen usually serve as the linker directly. For synthetic oligosaccharide antigens, it is flexible to install various functional groups in the synthetic process to facilitate specific linkage forms and conjugation methods. In the literature, there are several well-established methods developed for carbohydrate-protein conjugation, and each method has its advantages and limitations.[76] Generally, the amino and thiol groups in CPs are frequently used for the attachment of carbohydrate antigens. Activated acyl groups, aldehydes or alkenes are also introduced to the carbohydrate antigens to enable their coupling with protein via chemoselective *N*-acylation, reductive animation, Michael addition, etc. Other conjugation methods involving click reaction and other chemistry are also reported.[76]

In summary, in the development of antibacterial glycoconjugate vaccines, the selection of proper carbohydrate antigens is critical. With polysaccharide antigens, their activation method to facilitate the conjugation with CPs is also important, as it will not only decide the properties (*e.g.*, the structural integrity and molecular size) of the to-be-conjugated carbohydrate antigens but also the linker and linkage form, conjugation chemistry, antigen loading, chemical stability, product quality and biological activity consistence and, thereby, the immunological properties of the resultant glycoconjugate vaccines. Furthermore, the properties of linker and CP also have a significant impact on the immunological properties of glycoconjugate vaccines. Therefore, all these factors should be considered and optimized during the design and development of new glycoconjugate vaccines.

3. Immunology of Carbohydrate-Based Antibacterial Vaccines

Bacterial polysaccharides are promising targets for the development of glycoconjugate vaccines for infectious diseases. In as early as 1930’s, antibodies against pneumococcal polysaccharides were already observed,[75] and the antibody-mediated immunity was found to last at least several months. Ultimately, bacterial CPSs have been developed into antibacterial vaccines. However, a major problem for polysaccharide vaccines, or carbohydrate antigens in general, is that they are not highly immunogenic and induce only poor quality of antibody responses, especially in children, largely due to their inability to be properly presented to T cells[77] and provoke T-cell dependent immune responses and immune memory.[78, 79] As shown in Figure 3a, polysaccharide vaccines mainly interact with B-cells to elicit quick and arbitrary innate immune responses and generate T-cell independent non-specific immunoglobulin M (IgM) antibodies, which have a low affinity toward the carbohydrate antigens.[80] T-cell independent responses are less robust than adaptive immune responses and short-lived. Moreover, even repeated vaccination will not establish strong enough immune responses to fight pathogens.[81]

On the other hand, the covalent coupling of carbohydrates with immunogenic CPs, which contain T-cell epitopes, can convert them from T-cell independent to T-cell dependent antigens to induce adaptive immune responses involving both B- and T-cells (Figure 3b).[82] Adaptive immunities are antigen-specific, robust, and persistent but take longer

time to establish, when compared to innate immunities. In the development of adaptive immunity, antigen-presenting cells (APCs), such as dendritic cells (DCs)—the most important professional APCs, play a key role. APC presentation of the antigenic epitopes of glycoproteins to T-cell enables the cross-linkage of T-cell immunogens with B cell receptors (BCRs) to initiate the signalling processes of adaptive immune responses.[83] The co-stimulatory signals from DCs and macrophages help stimulate B-cell activation and maturation into plasma cells and release specific antibodies.[84,85] Other B-cells reproduce independently, and marginal zone B-cells and non-circulating mature B-cells are segregated in the spleen and other lymphoid tissues. Specifically, glycoconjugate vaccines are internalized by APCs and processed in endosomes, and the resulting carbohydrate epitopes are presented to T-cells along with peptides generated from CP digestion.[71,86] These epitopes bind to major histocompatibility complex class II (MHC-II) ligand present on the T-lymphocyte surface through the peptide portion with the carbohydrate epitopes recognized by T-cell receptors (TCRs). The recognition of the glycan epitope with MHC-II ligand by $CD4^+$ T-cell receptors and the secreted signaling cytokines help B-cell maturation and secretion of carbohydrate-specific high-affinity IgG antibodies and generation of memory B cells (MBCs). However, the role of B- and T-cell co-signaling is unclear. A study suggests that glycoproteins are processed in B-cells into small glycopeptides that bind MHC-II ligand with the hydrophilic glycan exposed to TCRs to interact specifically with carbohydrate-specific $CD4^+$ cells, elicit T-cell responses, and promote immunoglobulin switching from IgM to IgG.[87]

Of course, there are exceptions to the above scenarios. For example, the meningococcal A polysaccharide was observed to elicit immune responses in children and stimulate MBCs.[88] In addition, zwitterionic polysaccharides (ZPS) also induce T-cell dependent immune responses with the zwitterionic epitopes involved in the activation of T-cells. In the activation step, MHC-II ligand is needed to present the processed antigens. Subsequently, they are recognized by the APCs and B-cells, macrophages, and TCRs.[89,90]

4. Bacterial Polysaccharide-Protein Conjugate Vaccines

Due to the advantages of conjugate vaccines over polysaccharide vaccines, great efforts have been devoted to developing antibacterial glycoconjugate vaccines with natural polysaccharides as antigens. As a result, notable advancements have been achieved in this field. For example, many antibacterial glycoconjugate vaccines, *e.g.*, Hiberix® (Glaxo Smith Kline), Menjugate® (Chiron), PedvaxHIB® (Merck), Prevenar 13® (Wyeth Pharmaceuticals), etc., as listed in Table 1, have been approved for clinic use.[91] In the meantime, many new antibacterial glycoconjugate vaccines are presently at various stages of clinical studies.

5. Semi- and Fully Synthetic Carbohydrate-Based Antibacterial Conjugate Vaccines

Despite the great success of bacterial polysaccharide-protein conjugate vaccines, they still have limitations as noted above.[42, 92, 93] As a result, the interest in glycoconjugate vaccines containing structurally well-defined carbohydrate antigens, which possess several

potential advantages, is growing rapidly. For example, since the first oligosaccharide-protein conjugate vaccine against Hib was licensed in the late 1990's,[94] several oligosaccharide-based conjugate vaccines against different infectious diseases have been developed or are evaluated in clinical trials (Table 1).[68] Moreover, fully synthetic glycoconjugate vaccines, such as that against group C meningitis using monophosphoryl lipid A (MPLA) as the vaccine carrier, were demonstrated to exhibit promising immunological properties.[49] This section is devoted to the review of the progresses made in this specific area.

5.1 Hib vaccine.

Hib is a Gram-negative bacterium in both encapsulated and unencapsulated forms. It causes severe infections such as meningitis. Hib used to be a leading cause of bacterial meningitis in younger children (up to five years of age) in the USA before the introduction of Hib vaccines. Presently, several carbohydrate-based Hib vaccines are commercially available in the single form or in combination with other vaccines (Table 1). These vaccines are derived from the poly-ribosyl ribitol phosphate (PRP) epitope. Vaccines made up of natural PRPs are inconsistent in carbohydrate antigen size and linkage to CPs, and thus exhibit different immune responses in the population[40,95] Nonetheless, these glycoconjugate vaccines have been adopted by many countries and various immunization programs to successfully and drastically reduce the number of Hib infections. For example, in the USA, by 1997 the number of reported Hib cases in children under five years of age had decreased by 99%.[96] Similarly, in the UK, the frequency of Hib infections in children under five years of age reduced from over 20/100,000 to below 1.0/100,000 within three years after vaccines were introduced.[97] The first synthetic oligosaccharide antigen-based conjugate Hib vaccine, Quimi-Hib, developed in Cuba was able to help reduce the cost of Hib vaccine significantly.[94] Its efficiency is proved to be about 99.7% in children, and it shows an excellent safety profile.

Quimi-Hib (**1**, Figure 4a) comprises of synthetic oligosaccharide antigens with an average of seven repeating units of PRP conjugated to TT through a maleimido linker.[94] To further investigate the appropriate length of PRP oligosaccharides as antigens for the development of Hib conjugate vaccines, the Seeberger group synthesized various sizes of PRP oligosaccharides by a [2 + 2], [4 + 2], [6 + 2], and [8 + 2] elongation strategy and conjugated them with CRM_{197}. Immunological evolutions of the resultant glycoconjugates **2**-**5** (Figure 1b) with the Zika rabbit model showed that **2** containing a tetramer of the PRP repeating unit was an excellent antigen for developing semisynthetic glycoconjugate Hib vaccines.[98] On the other hand, the Pozsgay group reported the first total synthesis of PRP oligosaccharides up to twelve repeating units,[99] among which the octamer and dodecamer were linked to bovine serum albumin (BSA) but no immunological results of these conjugates were reported.

5.2 Meningitis vaccine.

Meningococci are commensal bacteria in humans and observed in diversity. Some of the bacteria cause invasive diseases. For example, *Neisseria meningitidis* is a Gram-negative bacterium and the cause of various infections, predominantly meningococcal meningitis, in young children and older adults worldwide.[100] One of the significant difficulties in

diagnosing meningococcal diseases is that its clinical manifestations are hard to differentiate from common and less severe illnesses. To date, 13 serogroups of *N. meningitidis* have been identified based on the chemical composition of their CPSs.[101] Among them, six serogroups (A, B, C, W135, X, Y) are mainly responsible for invasive meningococcal infections.[102] The global picture of these serogroups is: serogroup W135 (MenW) is mainly found in African and South American regions; serogroup X (MenX) is found in some areas of Africa; serogroup A (MenA) is found in Asia and Africa, while serogroup B (MenB), C (MenC), and Y (MenY) are usually observed in Europe and North America.[103]

Currently, several vaccines are available for the disease-causing serogroups A, B, C, W135, Y of *N. meningitidis*, but there is no vaccine for serogroup X yet. These vaccines have helped dramatically decrease invasive meningococcal infections and associated diseases. They are all glycoconjugate vaccines made up of purified polysaccharides derived from pathogens.[104] There are three licensed quadrivalent meningococcal glycoconjugate vaccines for serotypes A, C, Y, and W135 marketed by different manufacturers, *i.e.*, Menveo® (MenA/C/W135/Y-CRM197) by GSK, Nimenirix® (MenA/C/W135/Y-TT) by Pfizer, and Menactra® (MenA/C/W135/Y-DT) by Sanofi Pasteur. One monovalent glycoconjugate vaccine (MenAfriVac*)* is available for serotype A and three for serotype C. Among the monovalent vaccines for serotype C, NeisVac-C® (Pfizer) employs TT as the CP, whereas the other two, Menjugate® (GlaxoSmithKline) and Meningtec® (Pfizer), use CRM_{197}.[105] Because natural polysaccharides are used to produce these vaccines, they are heterogenic in respect to antigen, linkage, and conjugation site. However, they are uniform in immunogenicity against bacteria of specific serotypes and eligible for all age groups from 2 months to 55 years. In addition, other licensed monovalent conjugate vaccines against serogroups A and C are also available.

As shown in Figure 5, the MenA CPS is composed of repeating →6)-2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate(→ with about 70–80% *O*-acetylation of the 3-OH group.[106] The Pozsgay and Oscarson groups independently reported the synthesis of oligosaccharides of MenA CPS.[106,107] It was demonstrated that oligosaccharides higher than trisaccharide of MenA CPS repeat were unstable due to the hydrolysis of anomeric phosphates, which is enhanced by the adjacent NHAc group.[108] This explains the poor stability of innate MenA CPS in aqueous medium. To alleviate this problem and develop stable glycoconjugate vaccines, several groups explored the mimics of MenA CPS oligosaccharides. Replacing the ring oxygen atom of the pyranose and/or the anomeric oxygen atom with methylene groups led to the stable carbocyclic and 1-*C*-phosphono analogs, which were conjugated with CPs to obtain respective conjugates (Figure 5).[109,110] *In vivo* studies of CRM_{197} conjugates **6**-**8** containing the carbocyclic analogs of MenA CPS oligosaccharides revealed that only antibodies induced by the trimer conjugate **8** could recognize MenA polysaccharide and exhibit *in vitro* antibacterial activity, although all other conjugates elicited specific antibodies against the synthetic antigens.[111] *In vitro* biological studies of the 1-*C*-phosphono analogs **9**-**11**, which can mimic MenA CPS oligosaccharides more closely, showed that these unnatural antigens were recognized by human polyclonal anti-MenA CPS antibodies and inhibited the binding of antibodies to MenA polysaccharide. This activity was independent of the anomeric configuration of the *N*-acetyl mannosamine

in **9**-**11** but affected by the carbohydrate chain length.[112] Similarly, it was shown that the human serum albumin (HSA) conjugates **12**-**14** of oligosaccharides **9**-**11** induced T-cell proliferation *in vitro* by 40% at a concentration of 10 μM, in contrast to 28% induced by phosphonodisaccharide conjugate at the same concentration, and stimulated specific IgG production *in vivo*.[113] These results suggested that the above synthetic analogues of MenA CPS oligosaccharides might be useful for developing anti-MenA vaccines.

The MenC CPS is a homopolymer of α-(2→9)-linked sialic acids with irregular 7/8-*O*-acetylations (Figure 6). Deacetylated MenC CPS is more immunogenic than the natural glycan, but both can elicit protective antibodies with bactericidal activities.[114] The Wu and Wong group developed a convergent and effective route to synthesize a series of α-(2→9)-linked sialic acid oligomers **15**-**20** (Figure 6).[115] The Guo group also prepared several α-(2→9)-linked oligosialic acids and conjugated them with keyhole limpet hemocyanin (KLH).[116] *In vivo* studies showed that the resultant glycoconjugates **21**-**24** elicited strong T cell-mediated immune responses that recognized α-2,9-polysialic acid. Furthermore, the synthetic oligosialic acids were found to be more immunogenic than natural polysialic acid.

The synthetic α-(2→9)-oligosialic acids were also coupled with synthetic monophosphoryl lipid A (MPLA) to generate glycoconjugates **25**-**28**, which represent the first fully synthetic antibacterial vaccines.[49] Immunological studies in mice showed that conjugates **25**-**28** alone provoked robust T cell-dependent immune responses comparable to the corresponding KLH conjugates. Out of these conjugates, tri- and tetrasaccharide conjugates **26** and **27** induced a higher titer of antibodies. These results suggested that MPLA was not only an excellent carrier molecule for antibacterial glycoconjugate vaccines but also a potent adjuvant to construct self-adjuvanting vaccines. The elicited antibodies were able to bind to *N. meningitidis* cells that expressed α-(2→9)-polysialic acid, suggesting the potentials of this type of fully synthetic glycoconjugate vaccines.

The MenW CPS is composed of a disaccharide repeating unit, →6)-α-D-Gal*p*-(1→4)-α-D-Neup5Ac(7/9*O*Ac)-(2→ (Figure 7). To facilitate the development of oligosaccharide-based conjugate vaccines against MenW, the Wu group first developed a [2 + n] synthetic strategy to obtain oligomers (*e.g.*, monomer to pentamer **29**-**33a**) of the disaccharide repeating unit of MenW CPS via stereoselective glycosylation using a disaccharide donor.[117] The synthesized oligosaccharides was conjugated with CRM_{197}, and the immunological properties of resultant glycoconjugates **29**-**33b** were evaluated in mice. It was demonstrated that conjugates **30**-**33b** induced robust immune responses and the induced antibodies could recognize both the di- and the tetrasaccharide epitopes. However, antibodies induced by conjugate **29b** recognized only the disaccharide epitope but not the tetrasaccharide and other longer oligosaccharides. Among these glycoconjugates, antibodies elicited by **32b** exhibited more potent antibacterial activity. These results imply that the minimum length of an oligosaccharide required to represent the characteristic antigenic epitopes of MenW CPS is a tetrasaccharide and that the oligosaccharide containing four disaccharide repeating units of MenW CPS is a promising candidate antigen for the development of anti-MenW vaccines.

MenX infections spread worldwide, but a significant prevalence is observed in the region of sub-Saharan Africa known as the “meningitis belt”.[118] For example, the outbreaks of MenX infections occur frequently in African counties such as Uganda,[119] Niger,[120] Kenya,[119] Togo,[50] etc. In the past few decades, its occurrence in America, Europe,[121] and China[122] is also raising. However, currently, there is no vaccine to prevent MenX. Therefore, many studies are focused on developing anti-MenX vaccines.

The MenX CPS is a homopolymer of the →4)-2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate-(→ repeating unit (Figure 8). MenX CPSs of varied chain length were coupled with CRM_{197} to form glycoconjugate vaccines (Figure 8). The conjugates induced high titers of IgG antibodies in mice, and the elicited antibodies exhibited significant bactericidal activities.[123] Chhikara et al. developed a synthetic route for MenX CPS oligosaccharides for the preparation of semi-synthetic glycoconjugate vaccines.[124] Both the MenX tetrasaccharide-TT conjugate **34** and trisaccharide-CRM_{197} conjugate **35** exhibited lower immunogenicity than the conjugates of natural MenX CPSs.[125] However, when the length of the oligosaccharide increased further, the resultant conjugates were able to induce immune responses comparable to that induced by the conjugates of natural CPSs. Recently, Adamo et al. developed a one-pot chemoenzymatic strategy for the synthesis of MenX oligosaccharides.[126] This strategy allows for rapid access to complex bacterial oligosaccharides or CPS fragments of various lengths under pathogen-free conditions. The oligosaccharides were conjugated to CRM_{197}, and the resultant conjugates were evaluated in mice. Glycoconjugate **36** induced antibodies comparable to those induced by the conjugates of natural CPSs.

5.3 Vaccine against *Acinetobacter baumannii*.

A. baumannii is a pathogenic aerobic Gramnegative bacterium discovered in 2003 during the Iraq War.[127] It belongs to the *Neisseriaceae* family. Its outbreak occurred in Germany in 2013, and it is multi-drug resistant (MDR), thus it is becoming a global threat. In literature, twenty structures[128] and >90 serotypes[129, 130] of *A. baumannii* are reported. The major component of the EPS of *A. baumannii* strain 54149 has →3)-[Pse5*N*Ac7*N*Ac-α-(2→6)-Glc*p*-β-(1→6)]-Gal*p*-β-(1→3)-Gal*N*Ac*p*-β-(1→ (Figure 9, **37a**) as its repeating unit.[131] *A. baumannii* strain ATCC17978 expresses mainly **37c**, consisting of a trisaccharide core →3)-α-D-Gal(1→6)-β-D-Glc(1→3)-β-D-GalNAc-(1→ and a triacetylated 2,3-diamino-D-glucuronate and a GlcNAc residues as branches β-linked to the Gal 4-O- and 6-O-positions of the core, respectively. The core trisaccharide is also observed in the EPSs of other *A. baumannii* strains, such as ATCC NIPH146,[132] 17961,[133] SMAL,[134] LUH5537,[128] KL22 and PSgc9.[135] Several groups are engaged in synthesizing and using the oligosaccharides of *A. baumannii* EPSs as antigens for the design of new glycoconjugate vaccines. For example, Li et al. prepared the conjugates (Figure 9) of pseudaminose (Pse) that mimics the side chain non-reducing end monosaccharide epitope of the polysaccharide.[136] These conjugates had different sugar/protein ratios (4.76, 8.27, and 14.34 for conjugates **38**-**40**, respectively), aiming to disclose the impact of antigen loading on the immunological properties of glycoconjugate vaccines. Pse-BSA conjugate **41** was used as a coating antigen to detect anti-Pse-antibodies during immunological studies. All the three conjugates elicited glycan-specific IgG antibodies in mice and protected mice from *A. baumannii* infection.

These results suggested that a partial structure of a bacterial polysaccharide antigen, so long as its epitope is sufficiently unique, may be sufficient to induce antibacterial immune responses and useful for vaccine development.

Recently, the Seeberger group designed and synthesized a series of well-defined mono- and oligosaccharide units of *A. baumannii* ATCC17978 EPS, including four pentasaccharides, three tetrasaccharides, four trisaccharides, four disaccharides and one monosaccharides, with an aminopentyl linker at the reducing end for microarray printing and protein conjugation.[137] The study aimed to define the key epitopes and better understand the role of the acetyl groups in the tri-acetylated glucuronic fragment of **37c** in antibody binding. The synthetic glycans were printed onto microarray slides and screened with diseased patient sera and a monoclonal antibody (mAb) C8. Among the sixteen glycans, acetylated oligosaccharides were specifically recognized by antibodies and constituted the promising vaccine candidates. Furthermore, they have also proposed the study of conjugates of these synthetic glycans *in vivo* for development of vaccines against *A. baumannii* infection.

5.4 Pneumonia vaccine.

S. pneumoniae infections remain a major challenge globally. These Gram-positive bacteria mainly cause invasive diseases like pneumonia, septicemia, meningitis, and otitis media in newborns or infants. *S. pneumoniae* bacteria are classified into 97 serotypes based upon their CPSs. Among them, the most virulent 20 serotypes cause more than 90% of pneumococcal infections.[138] In 2016, a worldwide survey showed that *S. pneumoniae* was the leading cause of lower respiratory infection morbidity and mortality, contributing to more than one million deaths (1,189,937 deaths) in all age groups.[139] About 36% of the global burden of pneumonia and 27% of the global burden of otitis media are due to *S. pneumonia*.

To reduce the disease burden globally, pneumococcal vaccination is crucial, especially in underdeveloped countries. Currently, two types of vaccines are available on the market, *i.e.*, the polyvalent polysaccharide vaccine PPV23 and the glycoconjugate vaccines Prevnar13® and Synflorix®. The most used PPV23 vaccine (Pneumovax®23) is composed of 23 native CPSs, and it is used to immunize people above 50 years of age. Pneumococcal glycoconjugate vaccine (PCV) Prevnar13® contains 13 glycoconjugate vaccines with CRM_{197} as the CP. It is approved for all age groups (infant, children, and adults over 65 years of age), while Synflorix® (PCV10) contains 10 glycoconjugate vaccines with three different CPs, and it is permitted for children under five years old.[140] Recently, Merk has developed two new 15-valent glycoconjugate PCVs (PCV15-A and PCV15-B), which have been verified in phase I and II clinical trials and should be available for vaccination soon.[141] PVC15 uses CRM_{197} as the CP, and is formulated with aluminum phosphate adjuvant.

A primary concern about these vaccines is that they do not provide protections against all serogroups of *S. pneumoniae*.[142] For example, PCV7 is a 7-valent vaccine that only protects serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, while PCV13 protects serotypes 1, 3, 5, 6A, 7F, and 19A in addition.[143] Another concern about the multivalent vaccines is that some conjugates have low immunogenicity in the formulation, *e.g.*, the vaccine against serotype ST3, or cannot protect some strains with structurally similar polysaccharides, *e.g.*, ST19A and 19F.

To develop more effective PVCs, many research groups have been engaged in establishing effective synthetic methods for oligosaccharide segments of *S. pneumoniae* polysaccharides and using them as antigens for the design of novel glycoconjugate vaccines (Figure 10). In this respect, serotype 1 (ST1) ZPS is an attractive target due to its unique immunological properties. For instance, it is the first known carbohydrate antigen that induces T-cell dependent immune responses without conjugation with a CP.[144] Several research groups have developed synthetic methods for ST1 zwitterionic oligosaccharides for vaccine design.[66, 145,146,147] To gain a better understanding of the immunomodulatory activity of ZPSs, the Seeberger group prepared an ST1 zwitterionic trisaccharide with a thioether spacer and conjugated it with CRM_{197} to obtain glycoconjugate **42**.[148] It was revealed that **42** induced a robust antibody response in rabbits comparable to the control vaccine PCV13. In addition, the synthetic trisaccharide in **42** was recognized by an antiserum against the native polysaccharide.

The Seeberger group also synthesized and explored oligosaccharide-protein conjugates as vaccines for *S. pneumoniae* serotypes ST2 (**43**), ST3 (**44**), ST5 (**45**), ST8 (**47**), and ST14 (**48**) (Figure 10).[149] All these glycoconjugates induced high levels of protective antibodies against specific *S. pneumoniae* serotypes. Furthermore, combining these glycoconjugate vaccines with commercial PCV13 and PCV10 resulted in robust protective immunities in mice. It provides an example of a fully synthetic carbohydrate-based multivalent vaccine in combination with conjugates made up of native polysaccharides.

The Kamerling group synthesized a series of protein conjugates, such as **46** (Figure 10), of *S. pneumoniae* serotype ST6B CPS oligosaccharides.[150] Immunological evaluations of these conjugates in rabbits revealed that they induced high levels of pneumococcal ST6B antibodies that could accelerate type-specific phagocytosis. Moreover, the rabbit antisera could passively protect mice against pneumococcal ST6B. In addition, tetrasaccharide conjugate **46** provoked phagocytic and protective anti-ST6 B antibodies in mice as well.

S. pneumoniae serotypes 19A (ST19A) and 19F (ST19F) can cause pneumococcal diseases even after immunization with PCV13. To address this problem, the Morelli[151] and Seeberger[152] groups designed chimeric oligosaccharide-containing protein conjugates as candidate vaccines against ST19A and ST19F. Glycoconjugate **49** synthesized by the Seeberger group comprised of the repeating units of ST19A and ST19F CPSs (Figure 10).[152] Immunological studies of **49** showed that it induced high titers of antibodies in rabbits that were bactericidal to both ST19A and ST19F strains in vitro and protected animals from infection in vivo.

In addition, other vaccine carriers or antigen delivery methods are explored for developing new anti-*S.pneumoniae* conjugate vaccines as well. For example, gold nanoparticles have been used to construct multivalent vaccines that carried the CPS motifs of different serotypes (e.g., ST14 and ST19F).[153] Similarly, virus-like particles (VLP) have been utilized as carriers along with the carbohydrate antigens of ST3 and ST14 to construct vaccines that initiate both B- and T-cell dependent immune responses.[154] These new vaccine carriers or antigen delivery methods gave positive results and may be further exploited in vaccine design.

5.5 Vaccines against Shigella group bacteria.

In underdeveloped countries, Shigellosis is a common and devastating diarrheal disease in the paediatric population—children under the age of five years old. A survey of the mortality and morbidity rate caused by Shigella showed that this pathogen was responsible for 212,438 deaths and the second leading cause of diarrhea in all age groups (2.69 million people) worldwide in 2016.[155] Shigella bacteria are Gram-negative and have 50 serotypes, falling into four distinct groups, namely, *S. dysenteriae* (15 serotypes), *S. flexneri* (15 serotypes), *S. boydii* (19 serotypes), and *S. sonnei* (1 serotype). The strains *S. dysenteriae* and *S. flexineri* are more infectious and invasive, while *S. sonnei* is less virulent than others.[156] *S. flexneri* 2a (SF2a) is the most virulent and a primary cause of Shigellosis.[157]

Shigella infections result in the expression of Shiga toxins that can cause severe intestinal symptomatology with many complications in humans. However, currently, there is no vaccine against Shigella. Most vaccine candidates are still in developmental or clinical trial stages.[157] SF2a, the most virulent strain, expresses a LPS with *O*-polysaccharide consisting of a branched pentasaccharide repeating unit: →2)-α-D-Glc-(1→4)-[α-L-Rha-(1→2)-α-L-Rha-(1→3)]-α-L-Rha-(1→3)-β-D-GlcpNAc-(1→.[158] To develop anti-Shigella glycoconjugate vaccines, Mulard et al. synthesized a series of protein conjugates **51**-**53** having one, two, and three repeating units of the SF2a *O*-antigen linked to TT (Figure 11). Immunological studies of these conjugates in mice showed that they elicited robust IgG responses and the immune responses increased with the size of oligosaccharide antigens from **51** to **53**. Glycoconjugate **53** containing a trimer of the pentasaccharide repeating unit was the best vaccine candidate, since it induced specific and long-lasting anti-SF2a *O*-polysaccharide antibodies and protected mice from Shigella infection. Currently, **53** is in Phase II clinical trials (NCT04078022).[159]

The Mulard group has recently reported a scalable process for the preparation of another synthetic glycan-based anti-Shigella conjugate vaccine **54** with TT as its CP (Figure 11).[33] The oligosaccharide antigen in **54** is a trimer of the pentasaccharide repeating unit of SF2a *O*-antigen featuring with nonstoichiometric *O*-acetylation, which is observed in various strains of SF2a.[160,161] This synthetic procedure has enabled the scale-up GMP production of the conjugate vaccine with uniform glycan/protein ratio. Conjugate **58** has been shown to induce bactericidal antibody responses in mice and cleared all toxicity-related criteria. Furthermore, the synthetic glycoconjugate vaccine was proved to be stable for at least 66 months.

S. dysenteriae type 1 (or *Shiga Bacillus*) is mainly responsible for epidemic dysentery and diarrhea diseases in developing countries.[162] The LPS expressed by *S. dysenteriae* type 1 is a virulence factor and protective antigen, and its *O*-specific polysaccharide antigen consists of a tetrasaccharide repeating unit: →2)-α-L-Rha-(1→2)-α-D-Gal-(1→3)-α-D-GlcNAc-(1→3)-α-L-Rha-(1→. Pozsgay and co-workers reported the first experimental glycoconjugate vaccines **55**-**58** (Figure 11) for *S. dysenteriae* type 1, which were made of synthetic oligosaccharide antigens carrying a monomer, dimer, trimer, and tetramer, respectively, of the tetrasaccharide repeating unit of the *S. dysenteriae* type 1 (*O*-antigen, Immunological studies of these glycoconjugates in mice showed that *55* induced a

weaker immune response than other conjugates. Conjugate **58** containing the tetrameric oligosaccharide antigen was the most immunogenic and provoked high levels of antigen-specific IgG antibodies that were cross-reactive with *S. dysenteriae* type 1 LPS. To study the impact of the glycan non-reducing end sequence on the immunological properties of oligosaccharide antigens and glycoconjugate vaccines, they also synthesized a series of oligosaccharides (hexa- to tridecasaccharides) with different non-reducing ends and their protein conjugates.[163] It was shown that the protein conjugates of glycans with Glc*N*Ac or Gal at the non-reducing end elicited the highest levels of anti-LPS antibodies, but still, their antigenicity was lower than that of native (*O*-antigen-protein conjugates.[164] Very recently, Yin and co-workers accomplished the first total synthesis of the tetrasaccharide of *S. dysenteriae* serotype 10 (*O*-antigen in a stereoselective manner.[165] Additionally, corresponding (*R*)-4,6-*O*-pyruvylated and non-pyruvylated tetrasaccharides, as well as (*S*)-4,6-*O*-pyruvylated mannose, were also synthesized and analyzed for their antigenicity through glycan microarray screening. The results indicated that the tetrasachharide repeating unit is a crucial antigenic epitope of the *S. dysenteriae* serotype 10 O-antigen. Importantly, the direct comparison of native (*S*)-4,6-*O*-pyruvylated mannose with its analog (*R*)-4,6-*O*-pyruvylated mannose revealed that this motif was responsible for the antibody-binding of the *O*-antigen. This finding is helpful to understand the biological significance of this rare pyruvyl ketal functional group in the existing pathogenic bacterial surface glycans.

5.6 Anthrax vaccine.

Anthrax is caused by a highly lethal Gram-positive bacterium *Bacillus anthracis* that has three well-known strains, Ames, Sterne, and Vollum, among which Amens is the most virulent.[166, 167] The main virulent factors of *B. anthracis* are its CPSs and anthrax toxins.[168] *B. anthracis* spores are refractive to extreme conditions, such as stresses, desiccation solvents, high temperature, pressure and pH, UV light and even ionizing radiation.[169] These properties of *B. anthracis* ensure its perseverance in the environments. For example, it can survive thousands of years in the wet state, and when favourable conditions return, the spores become active and undergo germination and growth processes. As a result, *B. anthracis* is a choice for biological weapons. To address the threat of *B. anthracis*, significant efforts have been made to diagnose the disease early and develop anti-anthrax vaccines.

Cell-free vaccines containing the anthrax toxin protective antigen (PA) components have been proven safe and effective. On the other hand, glycans in the exosporium nap layer of *B. anthracis* are also extensively explored to identify new antigenic targets. It was found that the non-reducing end of its poly-β-L-rhamnose (Rha) constituent is capped with a rare sugar, 2-*O*-methyl-4-(3-hydroxy-3-methylbutana-mido)-4,6-dideoxy-D-glucopyranose, known as anthrose (Figure 12). The unique structure of anthrose makes it a useful target for anti-anthrax vaccine development. The Seeberger group synthesized an anthrose-containing tetrasaccharide and conjugated it with KLH to get conjugate **59** (Figure 12). Its immune-easy adjuvant (QIAGEN) formulation elicited tetrasaccharide-binding IgG antibodies that recognize B. *anthracis*.[170] This work has proven the feasibility of anthrose-containing oligosaccharides as antigens for anti-*B. anthracis* glycoconjugate vaccine development. In the meantime, the Boons group prepared several KLH conjugates **60a-d** (Figure 12) of the anthrax trisaccharide carrying differently acylated anthrose and analysed their

structure-activity relationships. This study had disclosed that even the simple *N*-acetylated trisaccharide in conjugate **60** was sufficient to elicit a desired immune response in rabbits. More importantly, structure-activity studies demonstrated the significance of the *N*-(3-methylbutyryl) moiety as an antigenic epitope in immune recognition of the bacterial spores.[171] Djedaïni-Pilard et al. synthesized a simple anthrose-KLH conjugate **61** (Figure 12) and found that it induced specific immune responses against anthrax spores in rabbits.[172] Other studies gave additional information about the immunological functions of the 2-*O*-Me, 4-*N*-(3-methylbutyryl), and 6-Me groups in anthrose and the poly-Rha moiety.[173,174] It was shown that all the substituents on anthrose, except for 2-*O*-Me, were necessary for the oligosaccharide antigenicity and anti-spore antibody recognition. Moreover, without anthrose, the poly-Rha moiety also induced immune responses against the spores of *B. anthracis*.

5.7 Vaccine against *Clostridium difficile*.

C. difficile is an anaerobic Gram-positive pathogen. Its spores are essential to cause infection and gastrointestinal diseases,[175] such as hypervirulent antibiotic-associated diarrhea, pseudomembranous colitis, and toxic megacolon in humans.[176] Like *B. anthracis*, the spores of *C. difficile* are robust and dormant, and can survive indefinitely outside their hosts. The asymptomatic persistence and resistance of *C. difficile* to disinfectants have made *C. difficile* infections (CDIs) a serious public health problem.[177] *C. difficile* secretes two potent toxins, TcdA and TcdB, which can enormously damage intestinal epithelium to lead to diarrhea and colitis.[178] In addition, both toxins induce the release of various chemokines and cytokines, which causes acute inflammation of the large intestine.[179] The exact strains of *C. difficile* are difficult to diagnose; more importantly, they are resistant to antibiotics. Therefore, many efforts are directed toward the development of effective vaccines against CDI.

In this regard, toxoid-based anti-CDI vaccines are extensively explored, and several are in various clinical stages. These vaccines are mainly made of formalin detoxified TcdA and TcdB derived from the bacterial culture. Safety concerns and the low yields in large-scale production of toxins, stability issues, and other limitations related to storage and so on compel people to look for alternatives. Thus, their unique glycans have become targets for developing anti-CDI glycoconjugate vaccines. Structural studies on the glycocalyx of *C. difficile* cell revealed three types of important glycans, PS-I (**62**), PS-II (**63**), and PS-III (**64**) (Figure 13a).[180] Particularly, PS-II is a highly conserved polysaccharide on the cell surface of different strains of *C. difficile* and hence represents a useful target for vaccine development.[181] Several PS-II oligosaccharides with or without a phosphate group at their non-reducing end have been synthesized and coupled with CRM_{197} via different linkers.[182, 183] *In vivo* studies on the resultant glycoconjugates **65-67** (Figure 13b) in mice revealed that they were highly immunogenic. Especially, the Seeberger group found that **66** induced high levels of specific IgG antibodies.[183] A comparison between conjugates **65**, **67**, and **66** containing oligosaccharides without and with the phosphate group, respectively, showed that the negatively charged phosphate was vital for the elicitation of IgG antibodies and the recognition of the whole polysaccharide of PS-II. A comparison between conjugates **66** and **68** containing only one and multiple repeating units of PS-II, respectively, revealed

that they induced comparable antibody responses. Therefore, it was concluded that the negatively charged hexasaccharide epitope in glycoconjugate **66** should be sufficient for the design and development of conjugate vaccines against *C. difficile*.

In addition, Monteiro et al. discovered that horse sera that contained natural anti-PS-I IgG antibodies could detect both the synthetic non-phosphorylated repeating unit of PS-I and the native PS-I with a slightly higher affinity for the latter.[184] These results further demonstrated the potential of phosphorylated glycans in the development of anti-CDI vaccines.

5.8 Brucella vaccine.

The Brucella genus contains ten Gram-negative proteobacterial species, *Brucella abortus, melitensis, suis, ovis, canis, neotome, microti, inopinata, pinnipedialis*, and *ceti*. Five of them cause infections in both animals and human.[185] They are facultative intra-cellular pathogens that can cause severe zoonotic bacterial diseases like brucellosis.[186] Brucella species are genetically similar to each other, which makes the diagnosis of human brucellosis challenging in the laboratory and clinic.[187] Many efforts have been devoted to developing new tools and methods to identify brucella infections. Furthermore, treating human brucellosis by antibiotics is a lengthy and costly process. Thus, vaccination is considered the best alternative to fight this disease, although currently no anti-brucellosis vaccine is available.

To identify proper antigens for the design of vaccines against brucella, its DNA sequences and surface protein and carbohydrate epitopes have been extensively analyzed. It was found that the brucella cell wall contains a unique LPS with the *O*-antigen composed of a rare sugar, 4,6-dideoxy-4-formamido-α-D-mannopyranose (Rha4NFo), in two distinctive linking patterns, known as antigen A and antigen M (Figure 14).[188] Antigen A is composed of only α-1,2-linked Rha4NFo, which forms the long inner sequence of *O*-antigens, and the shorter antigen M has an α-1,3-linked D-Rha4NFo after every four α-1,2-linked D-Rha4NFo units, which forms the cap of *O*-antigens.[189] It was found that both antigen A and antigen M were virulent.[190]

The *O*-antigens of Brucella are structurally unique, and this results in its complex serology. Since the antibodies to the Brucella *O*-antigens are useful for the diagnosis of infections, the ideal vaccine candidates should be built upon a protective epitope that does not interfere with the diagnosis. Early structural studies established that two epitopes, A and M, exist in the *O*-polysaccharide. The A epitope is a 1,2-linked homopolymer of 4,6-dideoxy-4-foramido-α-D-mannopyranose,[191] and the M epitope is 1,3-linked.[191, 192] It was later realized that, in fact, the *O*-polysaccharide consists of α–1,2-linked polysaccharide capped by a 1,2-/1.3-/1,2-linked tetrasaccharide sequence.[189] Prior to these definitive structural studies, a series of well-defined oligosaccharides **69**-**75a** (Figure 14) were designed and synthesized to represent the structural epitopes of antigens A and M in different settings.[193] For example, **69a** was considered as a mimic of mainly antigen M, while **70a** contained the epitopes of both antigens A and M.

Oligosaccharides **69**-**75a** (Figure 14) and other structures were used to probe mAbs raised against the whole bacteria.[194] With BSA conjugates **69b** and **70b** as the probes, two

mAbs, YsT9-1 and Bm 10, were discovered in animals. It was demonstrated that the hexasaccharide antigen **69a** preferentially bound the M antibody while the nonasaccharide antigen **70a** bound A- and M-antibodies in equal affinity. Further studies using conjugates **71**-**74b** as the probes showed that the M-type disaccharide **71Da** and tetrasaccharide **73a** could detect antibodies in the sera of human and animals infected with *B. suis* and *B. abortus*. Both conjugates **71Db** and **73b** had strong binding to M-specific mAbs and weak to A-specific mAbs. Glycoconjugate **75** with an α-1,2-linked hexasaccharide was prepared and shown to elicit A-specific antibodies that also recognized M-type oligosaccharides **71a** and **73a**.[193] This result suggested that α-1,2-Rha4NFo may represent the epitopes of both A- and M-antigenic serotypes and, thus, be useful for the development of broadly applicable anti-Brucella glycoconjugate vaccines.

However, these reports preceded the correct structural elucidation of the Brucella A and M epitopes by Vinogradov and co-worker[189] and illustrated the hazard of conducting synthesis without full knowledge of the complete structure.[189,193] Subsequent publications employed the structure identified by Kubler-Kielb and Vinogradov and established the structural basis for understanding the fine specificity of mAbs and polyclonal antibodies (pAbs) that bind the M antigen. This resulted in the discovery of a disaccharide that shows considerable potential as a diagnostic antigen for the detection of brucellosis in human and animals.[195] The ability to create discrete *O*-polysaccharide antigens identified an *O*-polysaccharide epitope equally common to all *Brucella abortus* and *Brucella melitensis* strains but unique to *Brucella*. The previously untapped diagnostic potential within this key diagnostic structure also holds significance for the design of brucellosis vaccines and diagnostics that enable the differentiation of infections from vaccinated animals.[196]

Currently available whole cell vaccines induce anti-A and M antibodies and thus infected animals cannot be differentiated from vaccinated animals as both produce A- and M-specific antibodies. It was hypothesized that chemical synthesis of a pure A vaccine would offer unique identification of infected animals by a synthetic M diagnostic antigen that would not react with antibodies elicited by the vaccine. TT conjugates of two forms of the A antigen, hexasaccharide **74a** and heptasaccharide **75b** linked to TT via the reducing and non-reducing terminal sugars, respectively, were synthesized and explored as vaccine candidates. Mouse antibody profiles to these immunogens showed that, to avoid reaction with diagnostic M antigen, it was essential to maximize the induction of anti-A antibodies that bind internal oligosaccharide sequences and minimize production of antibodies directed toward the non-reducing monosaccharide. This objective was achieved by conjugation of *Brucella O*-polysaccharide to tetanus toxoid via its periodate-oxidized non-reducing monosaccharide moiety, thereby destroying terminal epitopes to focus the antibody response to internal A epitopes. This established a method to resolve the decade-long challenge of how to create effective brucellosis vaccines without compromising diagnosis of infected animals.[188, 197]

5.9 Vaccines against *Burkholderia pseudomallei and B. mallei*.

B. pseudomallei (*Bp*) and *B. mallei* (*Bm*) are two highly virulent Gram-negative bacteria, which are serious threat to human and animal lives.[198] *Bp* and *Bm* are sporadically found in tropical and subtropical regions of the world, and if infected patients are left

untreated, the fatal rate is up to 50%.[199] *Bp* causes serious melioidosis, and *Bm* causes glanders in solipeds, both leading to death. These diseases are difficult to diagnose as their clinic symptoms are multifaceted, from skin abscess to acute pulmonary infection and fulminating septicemia. Moreover, both bacteria are CDC "Tier 1" select agents because of their high infectivity via inhalation, low infectious doses, and potential for misuse as biothreat agents. Antibiotics can be used to control these diseases, but not entirely due to drug resistance. However, currently, there is no approved prophylactic vaccine for these infections. Therefore, the development of effective countermeasures to combat these diseases, including vaccines, is of utmost importance.

The attractive outer surface LPS *O*-antigens of *Bp* and *Bm* (**76a** and **76b**, respectively) as targets for the development of vaccines are depicted in Figure 15. Both *O*-antigens are linear polysaccharides with the backbone composed of a disaccharide repeating unit →3)-6-deoxy-*α*-L-Tap-(1→3)-*β*-D-Glc-(1→, with 2-*O*-acetylation of the talose (Tal) residue. However, their non-reducing end capping disaccharide is distinctive since it contains a unique methylation and acetylation pattern with the terminal Tal residue and this pattern varies from species to species. It has been shown that, in *Bm*, the terminal Tal residue is 3-*O*-methylated and 2-*O*-acetylated, whereas it is also *O*-4-acetylated in *Bp*.[200]

To study the significance of *O*-acetyl and *O*-methyl modifications for the immunology of *Bp* and *Bm* LPSs and for the recognition of *Bp* and *Bm* by mAbs obtained with vaccinations, Gauthier et al. synthesized a library of di- and trisaccharides containing minimal structures of all reported acetylation and methylation patterns associated with *Bp* and *Bm* LPS *O*-antigens and coupled them with CRM_{197} to afford glycoconjugates **77a** and **77b** (Figure 15).[201] They used the synthetic conjugates to characterize the minimal epitopes required for binding with a series of *Bp* and *Bm* LPS-specific mAbs, which were passively protective in mice models of melioidosis and glanders. It was demonstrated that *Bp* and *Bm* LPS-specific mAbs could bind to the terminal sugar residues of the *O*-antigens. Furthermore, immunizing mice with conjugate **77a** containing a *Bm*-like disaccharide elicited a high level of antibody response that protected mice from the infection. In contrast, conjugate **77b** with a *Bp*-like disaccharide could not induce a strong antibody response in BALB/c and C57BL/6 mice. Recently, the same group synthesized two larger oligosaccharides, **78a** and **78b** (Figure 15), comprising both the internal and terminal epitopes of *Bp* and *Bm O*-antigens.[202] These *Bp* and *Bm*-related tetrasaccharides exhibited high reactivity towards the serum of Thai melioidosis patients and the similar antigenicity as native *Bp O*-antigen. These findings suggested that tetrasaccharides **78a** and **78b** could mimic the epitopes of *Bp* and *Bm O*-antigens and be useful antigens for the development of functional oligosaccharide-based conjugate vaccines against melioidosis and glanders.

On the other hand, Scott et al. synthesized a *Bm* CPS-associated linear pentasaccharide of β-(1→3)-linked 2-*O*-acetyl-6-deoxy-D-manno-heptopyranose.[203] Its conjugate with the nontoxic Hc-domain of tetanus toxin (HcTT), namely **79** (Figure 15), was shown to elicit IgM and IgG antibodies, which recognized the native CPS and effectively protect against *B. pseudomallei* (strain K96243) in a mouse model. In addition, recently, Gu et al. developed an efficient strategy to access various oligosaccharide analogs of *Bp* and *Bm* CPS in a large scale. Using this strategy, they prepared a series of mono-, di-, tri-, and tetrasaccharides of

Bp and *Bm O*-antigens with and without 2-*O*-acetylation and coupled these oligosaccharides with CPs to afford glycoconjugates **80a-h** and **81a-h**.[204] The CRM_{197} conjugates **80a-f** were found to elicit robust antigen-specific T-cell dependent IgG antibody responses, demonstrating its desirability as a new vaccine candidate. Comparing the immunological properties of **80a-d** and **80e-f** containing 2-*O*-acetylated and deacetylated oligosaccharides, respectively, led to a conclusion that the acetyl groups at the oligosaccharide 2-*O*-position had a rather small impact on their immunogenicity. The study further reveals that gly coconjugates **80c** and **80g** are the most immunogenic among all synthetic conjugates and are promising vaccine candidates for **BP** and **BM**.

5.10 Cholera vaccine.

Cholera is an acute watery diarrheal disease caused by the pathogenic Gram-negative bacterium *Vibrio cholerae*.[205] Cholera causes widely publicized epidemics and have an especially large burden in sub-Saharan Africa and South Asia countries. Thus, it is a challenge to the public health in many parts of the world.[206, 207] According to the World Health Organization (WHO), in 2018, 34 countries reported a total of 1,227,391 cholera cases and 5654 deaths, with case-fatality rate (CFR) of 0.6%.[208] Current cholera vaccines are oral, either attenuated or killed whole cell vaccines with or without the cholera toxins B subunit (CtxB).[209] Although the existing cholera vaccines are able to control the pandemic of infection but they have limitations. For example, they cannot effectively protect younger populations, the major health tension in many cholera-endemic countries.[210] Therefore, it is necessary to develop new and effective vaccines that can provide high-level and long-term immunity.

Over 200 serogroups of *V. cholerae* have been identified, and toxigenic strains of O1 and O139 are the major causes of epidemic diseases. *V. cholerae* O1 strain has two serotypes, *i.e.*, Ogawa and Inaba, which are classified based on the structures of their LPS O-antigens, which are linear homopolymers of α-1,2-linked 3-deoxy-L-glycero-tetronamido-D-perosamine with or without a 2-*O*-methyl group on the non-reducing end perosamine moiety (Figure 16). The Ogawa serotype LPS O-antigen has been exploited for the design and development of cholera O1 vaccines. For example, the Kovac group has been engaged in developing synthetic methods to access the O-antigen oligosaccharides of *V. cholera* equipped with a squaramide linker.[211,212] The BSA conjugates (Figure 16) of these synthetic antigens were immunologically studied to reveal that only the hexasaccharide conjugate **83c** showed the vibriocidal humoral response, suggesting that shorter oligosaccharides lacked the required epitope. It has also been found that conjugate **83c** with the lowest carbohydrate loading provided a higher protective capability in mice.[213] Furthermore, they compared immunologically the *P. aeruginosa* rEPA conjugates of Ogawa and Inaba serotype O-antigen oligosaccharides and found that the conjugates of Ogawa oligosaccharides boosted Inaba-primed mice, whereas the conjugates of Inaba oligosaccharides could not boost Ogawa-primed mice. This result implies that Ogawa and Inaba LPSs contain different immunodominant epitopes.[214]

The first emergence of a new *V. cholerae* serotype O139 was noticed in the southern part of India in 1992 and becomes the non-O1 *V. cholera* serotype to cause epidemic cholera.[215]

V. cholerae O139 possess a high molecular weight CPS, in distinctive composition from the LPS antigen.[216] This O139 CPS consists of uniquely branched hexasaccharide **88** (Figure 16), which features two units of a rare deoxysugar, 3,6-dideoxy-l-xylohexose (colitose), and a 4,6-cyclic phosphate group on the Gal unit. The Kovác group completed the first chemical synthesis of the fully protected oligosaccharide that is equipped with linker, making it ready for conjugation to CPs.[217] Later on, an alternative synthesis of the hexasaccharide of O139 antigen and its ester form was reported, which were then coupled with BSA via squaric acid chemistry to generate glycoconjugates **89a-c** that were immunologically investigated.[218]

5.11 Tuberculosis vaccine.

Tuberculosis (TB) is a contagious, fatal, and devastating disease caused by bacillus *Mycobacterium tuberculosis* (Mtb). It is one of the leading causes of human death. The Global Tuberculosis Report 2021 states that a total of 1.5 million people died from TB in 2020 worldwide.[219] For TB treatment, antibiotic drugs, such as rifampin, pyrazinamide etc., have been proven to be effective, but prolonged regimens of multiple antibiotic treatments result in multiple-drug resistance.[220] Moreover, TB and HIV co-infection is a huge problem.[221] Therefore, TB is an important burden of the health care system worldwide.

To control TB, a safe and effective vaccine is highly desired. Currently, the most broadly used vaccine for the protection against TB, especially in children, is Bacilli Calmette-Guerin (BCG). However, the efficacy of this vaccine is wildly variable (from 0 to 80%).[222] Therefore, other approaches have also been explored for vaccine development. For example, attenuated auxotrophic strains of *M. tuberculosis*,[223] DNA vaccines,[224, 225] and sub-cellular (protein and peptide antigens) vaccines,[226] have been investigated. Recent advancement in glycomics and accumulated information on the carbohydrate antigens of *M. tuberculosis* bacilli allow a new view on the development of carbohydrate-based anti-TB vaccines.

The cell envelope of *M. tuberculosis* consists of mainly three distinguished components, the plasma membrane, the cell wall core, and the outer layer, while its outer surface is covered by a thick layer of complex carbohydrates.[227,228] Within the bacterial wall envelope, mycolic-arabinogalactan (mAG) complexes and lipoarabinomannan (LAM)-related glycolipids are the two major constituents,[229] which protect the bacteria and assist their survival.[230] Furthermore, LAMs are also implicated in the immunogenicity of *M. tuberculosis* and have been shown to inhibit T-cell activation[231] and various microbicidal activity.[232] Therefore, LAMs are excellent targets for vaccine development.

As shown in Figure 17, LAMs contain a phosphatidyl *myo*-inositol (PI) anchor that is non-covalently associated with the plasma membrane of *M. tuberculosis*.[229] The PI unit is extended at the inositol 2-*O*-position with an α-mannose (Man) residue to generate phosphatidylinositol mannose known as PIM_1. Adding another Man unit to the inositol 6-*O*-position of PIM_1 leads to PIM_2, and adding more α1,6-linked Man residues to the 6-O-Man of PIM_2 gives PIM_3 and PIM_4. Further extension of the sugar chain from PIM_4 is diverse. For examples, *a*-1,2-linked Man residues are added to PIM_4 to form PIM_5 and PIM_6. PIM_6 and more complex PIMs serve as key intermediates for the biosynthesis of polysaccharides, such as LAMs and ManLAMs, through a series of enzymatic mannosylation and arabinosylation.[233, 234] The exact structures of the mannan

and arabinan polymer of the LAM backbone still remain unclear but the glycan structures of PIMs are well characterized.[235, 236] Earlier studies have confirmed the crucial role of α-1,3, α-1,5, and β-1,2-linked oligoarabinofuranosyl motifs of LAMs in the immunological events related to mycobacterial infections.[237–239] Owing to the interesting biological properties of LAMs, significant efforts have been devoted to the synthesis of related structures, such as mycobacterial PIMs, lipomannan (LMs), and LAM.[240,241]

Here, we will only cover the synthetic studies on LAM-related oligosaccharides and their conjugates that have been subjected to immunological studies as TB vaccines. The Seeberger group has chemically synthesized several PIM derivatives and investigated their activities as potential adjuvants or antigens for vaccine development.[242] All of the synthetic PIMs contained a thiol group that enabled their immobilization onto microarray chips and/or conjugation with carrier molecules. Conjugation of the synthesized oligosaccharides with KLH through the thio-linker gave conjugates **90b**-**g** (Figure 17). Biological studies showed that PIM_5 and PIM_6 has the highest binding affinity to dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) receptor. Moreover, the PIM-KLH conjugates were proved as strong immunostimulators during the immunization of mice. PIM_6 conjugate **90g** induced a marked increase in anti-KLH antibodies compared to KLH alone. The adjuvant property of PIM_6 was also confirmed by measurement of the cytokine production.

To facilitate development of LAM-based TB vaccines, Guo and Gu established an efficient method to synthesize LAM arabinofuranosyl oligosaccharides, and then conjugated them with BSA and KLH via a dicarboxyl linker (Figure 18).[243] The resultant glycoconjugates **91**-**93a** were immunologically evaluated with conjugates **91**-**93b** as coating antigens. It was found that all the synthetic glycoconjugates could elicit carbohydrate antigen-specific immune responses. Remarkably, **92a** induced higher antibody titers than **91a** and **93a**. Overall, these studies have validated that LAM oligosaccharides are useful epitopes for TB vaccine development.

The Guo and Gu group also conjugated a LAM tetrasaccharide antigen with MPLA at its 6'-*O*- and 1-*O*-positions to get conjugates **94** and **95**, respectively (Figure 18), as fully synthetic glycoconjugate vaccines. They anticipated these conjugates to have self-adjuvanting activity, and immunological comparison of the two conjugates was expected to also reveal the impact of conjugation sites to MPLA on the immunological properties resultant conjugate vaccines.[244] It was found that both conjugates induced strong antibody responses against LAM compared to the mixture of tetrasaccharide and MPLA. Furthermore, 6'-*N*-linked MPLA-conjugate **94** induced significantly higher IgG titer than corresponding 1-*O*-linked MPLA-glycoconjugate **95**. The outcome of this study proved the vital role of MPLA as a vaccine carrier molecule and as a self-adjuvant and the influence of linkage site between carbohydrate antigen and MPLA on their immunological properties.

In addition to vaccine development, early and easy diagnosis of TB is also a curial step in the control of TB spread. This relies on the specificity and sensitivity of tests. Previous studies on TB diagnosis have shown that anti-LAM IgG antibodies are present in most patients and LAM-related arabinofuranosyl oligosaccharides can selectively bind

to anti-LAM antibodies. Therefore, anti-LAM antibodies are considered as useful tools for TB diagnosis.[245] As a result, several groups have synthesized protein conjugates of LAM oligosaccharide to investigate their application to TB diagnosis.[246]

For example, the Kononov group synthesized two LAM arabinofuranosyl hexasaccharide conjugates **96a** and **96b** (Figure 19) employing MPB-64 and Rv0934 recombinant protein as carriers, respectively, and examined them as antigens for serological assays of TB.[246] These conjugates bound to the targeted antibodies. A series of α-linked linear oligoarabinofuranosides with a 4-(2-chloroethoxy)phenyl aglycon were also synthesized and coupled with BSA to get glycoconjugates **97a-c**, which are useful for biological study and application in TB diagnosis and vaccine development.[247] The Bundle group has developed the biocompatible Copovidone polymer as a non-protein carrier for conjugation with carbohydrate epitopes and investigation of antibody binding.[248] It was discovered that, the polymer-based glycoconjugate **96d** showed similar binding properties as BSA conjugate **96c**. Some important features of the copovindone conjugates are that the glycan is stable to acid and the assay plate can be reused up to 3 times without any degradation in quality. In addition, Derda et al. linked this LAM hexasaccharide to several peptide carriers derived from phage display library.[249] The resultant glycoconjugates **96e-h** were utilized as ligands for selective detection of anti-LAM antibodies generated from mycobacterial infection. This study has demonstrated the potential of structurally defined LAM oligosaccharide-peptide conjugates as diagnostic reagents for active and latent tuberculosis.

5.12 Group A *streptococcus* vaccine.

Group A streptococcus (GAS) is a Gram-positive bacterium that can cause a wide spectrum of clinical syndromes.[250] The severe infections range from pharyngitis, cellulitis, and pyoderma to necrotizing fasciitis, sepsis, pneumonia, and streptococcal toxic shock syndrome.[251] Annually, over half a million people die from GAS infections.[252] The economic burden from these infections in both developed and developing countries is significant. GAS infections are usually treated with antibiotics, but drug resistance has become a major issue. To develop GAS vaccines, many virulent components of GAS have been identified as potential targets. Among them, the cell wall M protein is one of the leading candidates and several M-protein vaccine candidates have entered clinical trials.[253] However, a major concern about these vaccines is immunological safety due to the cross-immune reaction with human tissues.[254, 255] Therefore, other GAS cell surface components, such as other surface proteins like C5a peptidase,[256, 257] toxins and polysaccharides,[56, 258] have been considered as attractive alternatives for GAS vaccine development.

One of the major polysaccharides on the cell surface of GAS is **98** (Figure 20), composed of a branched trisaccharide repeating unit. The Pinto group has explored the potential of this polysaccharide for vaccine development. Accordingly, they prepared several oligosaccharides and linked them to CPs to get glycoconjugates that were evaluated as GAS vaccines.[259] Their results indicated that the branch and size of the oligosaccharide antigens are central elements of the epitope recognized by both rabbit polyclonal and mouse monoclonal antibodies that bind to native polysaccharide antigens.[259–262] Moreover, synthetic hexasaccharide-TT conjugate **99** containing in average 30 hexasaccharide haptens

per TT molecule elicited specific antibodies in mice and response surge with booster immunization, suggesting immunological memory.[263] This study validated glycoconjugate **99** as a promising GAS vaccine candidate.

Costantino and co-workers[264] synthesized a series of oligosaccharides of this cell wall polysaccharide of different chain lengths (hexa- vs nonasaccharides) and non-reducing end terminal sequences, and coupled them with CRM197 to get conjugates **100a,b** and **101a,b** (Figure 20). The immunoprotective efficacies of these semi-synthetic conjugate vaccines were evaluated by *in vitro* opspnophagocytosis assays. It was disclosed that conjugates **100** and **101** displayed equal protections in mice. Their studies further indicated the potential of oligosaccharide-based anti-GAS conjugate vaccines.

The Guo and Gu group[69, 265] designed and synthesized a series of GAS oligosaccharide-protein conjugates **102**-**104a**-**c** (Figure 20) to systematically examine the structure-activity relationships of GAS oligosaccharide-based conjugate vaccines. The streptococcal C5a peptidase (ScpA)-193 conjugates **102a**-**c** of GAS oligosaccharides were specially designed as bivalent vaccines to boost immune responses against both the cell wall polysaccharide and ScpA. Immunological studies in mice revealed that Scp193 conjugates **102a**-**c** elicited robust antibody responses specific to not only the carbohydrate but also ScpA, suggesting ScpA as a promising target antigen for the development of anti-GAS vaccines. Their results indicate that nanasaccharide-ScpA193 conjugate **102c** is a hopeful GAS vaccine candidate. In addition, the CRM_{197} conjugates **103a**-**c** and the TT conjugates **104a**-**c** induced even higher titers of antibodies, suggesting the influence of CPs on the immunological properties of glycoconjugate vaccines.

5.13 Vaccine against Group B *streptococcus:*

Since the early 1970's, group B *Streptococcus* (GBS), a Gram-positive bacterium, has been one of the major concerns of bacterial infections in neonates and pregnant women. It causes many severe diseases, such as sepsis, meningitides, pneumonia, abortion, etc.,[266–268] and *S. agalactiae* is one of the strains mainly responsible for morbidity.[269, 270] Globally, about 18% of pregnant women are colonized by GBS,[271] which has remarkably increased the infection risk of pregnant women and babies. In 2015, GBS-caused diseases affected 319,000 newborns and resulted in 90,000 deaths, as well as up to 3.5 million preterm births and around 57,000 fetality.[272] The preventive measures aimed to reduce the risk of invasive GBS diseases in infants have been focused on intrapartum antibiotic prophylaxis (IAP).[273] However, IAP is effective for early-onset GBS diseases but cannot prevent late-onset GBS diseases. Consequently, effective vaccines against GBS are highly desirable.

GBS isolates from humans express unique CPSs as their major virulence factors to support their evasion of the host defense mechanisms.[274] Based on the structures of their specific CPSs, GBS is classified into ten serotypes, including Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX (Figure 21).[275,276] Meta-analysis data revealed that five serotypes, including Ia, Ib, II, III and V, account for the vast majority of GBS infections.[277] Most GBS CPSs are composed of Glc, Gal, GlcNAc, and Neu5Ac, which are differently arranged with diverse branches that are typically terminated with a Neu5Ac residue. These structurally unique CPSs can be useful immunotargets for the development of GBS vaccines. However, the oligosaccharide

repeating units of GBS CPSs, especially their densely branched oligomers, are challenging synthetic targets. Therefore, many research groups have been focused on the synthesis of GBS oligosaccharides,[39] as well as their application to vaccine developmemt.[268, 273, 277, 278] This is a huge subject, hence we have to limit our discussion on only a few examples to demonstrate the recent development in the synthesis and immunological evaluations of oligosaccharide-based conjugate vaccines against GBS.

As depicted in Figure 22, Adamo et al. synthesized glycoconjugates **116**-**119** containing oligosaccharides of different lengths and frameshifts of the repeating units of GBS serotype III CPS, with CRM_{197} as the CP.[279] Both glycoconjugates **117** and **119**, but not **116**, could bind to polyclonal antibodies derived from anti-GBS serotype III murine sera. This result suggests that the branching motif β-linked to the GlcNAc 6-*O*-position is important for antibody recognition. STD-NMR and X-ray studies further implicate that a hexasaccharide (shown in **119**) spanning two repeating units of the native CPS is responsible for interactions with a protective mAb.[280] Studies on CRM_{197}-conjugates carrying 2 to 6 repeating units of GBS CPS III, obtained from degradation of natural polysaccharides, suggest that an oligosaccharide containing 2 repeating units is enough to represent the antigenic epitope of GBS CPS III.[281] Moreover, hexasaccharide conjugate **119** was able to elicit functional immune responses comparable to that induced by the native CPS conjugates. These results have confirmed that the hexasaccharide is the minimal GBS III CPS epitope as a functional antigen for synthetic conjugate vaccine development.[282] In addition, they found that the oligosaccharide loading degree of glycoconjugate vaccines also has a significant impact on their immunological properties, and a high loading is required to elicit robust immune responses probably due to multivalent effects.[281]

Recently, the Guo group reported a chemical synthesis of the pentasaccharide repeating unit of GBS serotype Ia CPS,[283] as well as a decasaccharide representing a dimer of the repeating units of GBS Ia CPS. Subsequently, the synthetic oligosaccharides, both monomer and dimer, were conjugated with CRM_{197} and HSA to generate glycoconjugates **120a**-**d** (Figure 22). The CRM_{197} conjugates **120** and **120c** were immunologically investigated in mice, both induced robust IgG and IgM antibody responses. In addition, the antibodies elicited by these conjugates were cross-reactive, suggesting the potential of the synthetic oligosaccharides as antigens for vaccine development.

The cell glycocalyx of GBS is complex. Another unique and group-specific polysaccharide on GBS cell surface shared by all serotypes is comprised of a rhamnose-rich oligosaccharide interspaced with a glucitol phosphate.[284, 285] Each repeating unit is made up of five L-rhamnose, one D-*N*-acetylglucosamine, one D-galactose, and one D-glucitol residues (Figure 23). The repeating units are linked through a phosphate diester group between the glucitol residue and the 6-O-position of the galactose residue. This unique polysaccharide plays a key role in GBS cell growth and immunological property. However, this polysaccharide is difficult to isolate from natural sources in homogeneous form and sufficient quantity, which hinders its potential application.[286, 287] Recently, the Codee group reported the synthesis of several oligosaccharides of this polysaccharide and coupled them with CPs to generate conjugates **121**-**123a,b** (Figure 23).[288] CRM_{197} conjugates **121**-**123a** were immunologically evaluated, and the results showed that each conjugate

could elicit a specific immune response and the induced antibodies were cross-reactive with the other oligosaccharides. Immunization of mice with conjugate **123a** that contained a large oligosaccharide elicited antibodies that recognized and bound the bacterium. It was also demonstrated that the glycoconjugates elicited functional immune responses against different GBS serotypes. Therefore, these oligosaccharides are identified as promising antigens for the development of multivalent vaccines against all clinically relevant GBS serotypes such as Ia, Ib, II, III, IV, and V.

6. Concluding Remarks

Prophylactic vaccines are useful tools to control infectious diseases and prepare people against epidemic situations.[289] In history, vaccines have helped successively restrain and exterminate many fatal infectious diseases, such as smallpox, polio, rubella, etc.[290] The power of vaccine has been demonstrated again during the recent pandemic of COVID-19. Therefore, vaccination is considered the most effective and cost-efficient platform against infectious diseases. Among various technologies for vaccine development, glycoconjugate vaccines are attractive because carbohydrates as antigens are abundant and exposed on the cell surface for the recognition by the host immune system, highly conserved on bacterial cells, and essential virulence factors for bacterial survival. In this regard, the unique polysaccharides on the bacterial cell surface are particularly useful. As a result, many bacterial polysaccharides and their protein conjugates have been successfully developed into vaccines and used in clinic to control various bacterial infections. However, as discussed earlier, these vaccines still have their limitations, and hence conjugate vaccines based on oligosaccharide analogues of bacterial polysaccharides have been actively pursued in the past decades. Some of the oligosaccharide-based vaccines have been approved for clinical use and more are in clinical trial stages.

Despite these great advancements, there are still a number of hurdles remaining in the field. Particularly, to design and develop effective glycoconjugate vaccines, it is necessary to know the structural epitopes of bacterial polysaccharides required for inducing protective immune responses to targeted pathogens. This is not easily achievable as it needs extensive structural studies and structure-activity relationship analysis of the polysaccharide antigens. In addition, the immunology and the exact functional mechanisms of carbohydrate-based vaccines are still poorly understood. Another problem is related to the synthesis of complex oligosaccharides, especially oligomers of the repeating units of bacterial polysaccharides, which remains challenging and therefore has hindered the access to these molecules in large quantity and to their derivatives. Furthermore, the carrier molecules for conjugate vaccines also need new alternatives during the development of glycoconjugate vaccines because repeated uses of the same CPs in various glycoconjugates can cause concerns, such as immunotolerance and decreased efficiency of vaccines.

On the other hand, we believe that, currently, the rapid and continuous progress and expansion of new methods for carbohydrate synthesis, technologies for structural study of carbohydrates, and in-depth understanding of the interactions between carbohydrate antigens and antibodies or the host immune system and of the functional mechanisms of carbohydrate-based vaccines, will provide more effective platforms for the design of

new vaccines, *e.g.*, through rational design, and speed up the research and development process and result in more and efficacious vaccines. In the meantime, new biotechnologies are emerging as attractive alternatives to the currently chemical strategies for more flexible and cost-effective vaccine production.[291] For example, new enzymatic strategies for protein glycosylation have been employed to access glycoconjugates. One of the strategies exploited oligosaccharyltransferase-dependent *N*-linked glycosylation process to produce glycoconjugate vaccines against bacterial pathogens and humanized glycoproteins. A recent application of this highly innovative glycoengineering method is to produce a *Shigella dysenteriae* O1 conjugate vaccine from *E. coli*,[292] and the vaccine has cleared Phase 1 clinical trials. Vaccines against other pathogens, e.g., *Francisella tularensis, Staphylococcus aureus*, etc. are currently under development.[293,294] Although the bacterial glycosylation systems are attractive and have advantage over chemical methods, there are still some technical limitations associated with it, e.g., (i) the assignment of a glycosylation sequon to a particular site within the target protein is sometimes difficult, (ii) complications were observed while working with mammalian cell lines; (iii) the products generated by this approach has the concern of spreading viral diseases. Therefore, more glycosylation systems remain to be explored. Nonetheless, the new technologies will promote vaccine development and help fight various infections that are threatening human life and further reduce the social and economic burdens caused by infectious diseases, thereby strengthening the health and welfare of humankind.

Finally, we want to point out that the topic that this article tries to review is very broad, and the research area has been developing rapidly in the past few decades and is still growing. For example, in the process of preparing this article, Pereira and co-workers reported the synthesis and evaluation of some new glycoconjugate vaccine candidates for *Escherichia coli* O25B.[295] Therefore, it is challenging to cover the conjugate vaccines against all bacterial pathogens, and we choose to review only some common bacteria and their vaccine development. Furthermore, even among the few bacterial pathogens selected, it is impossible to include all the researches related to glycoconjugate vaccine development. Here, we try to include the reports that contain biological evaluation of the conjugates as many as we can but still, some of the excellent works may be uncited. Additionally, in the literature, there are many excellent synthetic works about bacterial oligosaccharides and their conjugates that are included herein due to the limited scope of this article. We sincerely apologize for all those authors.

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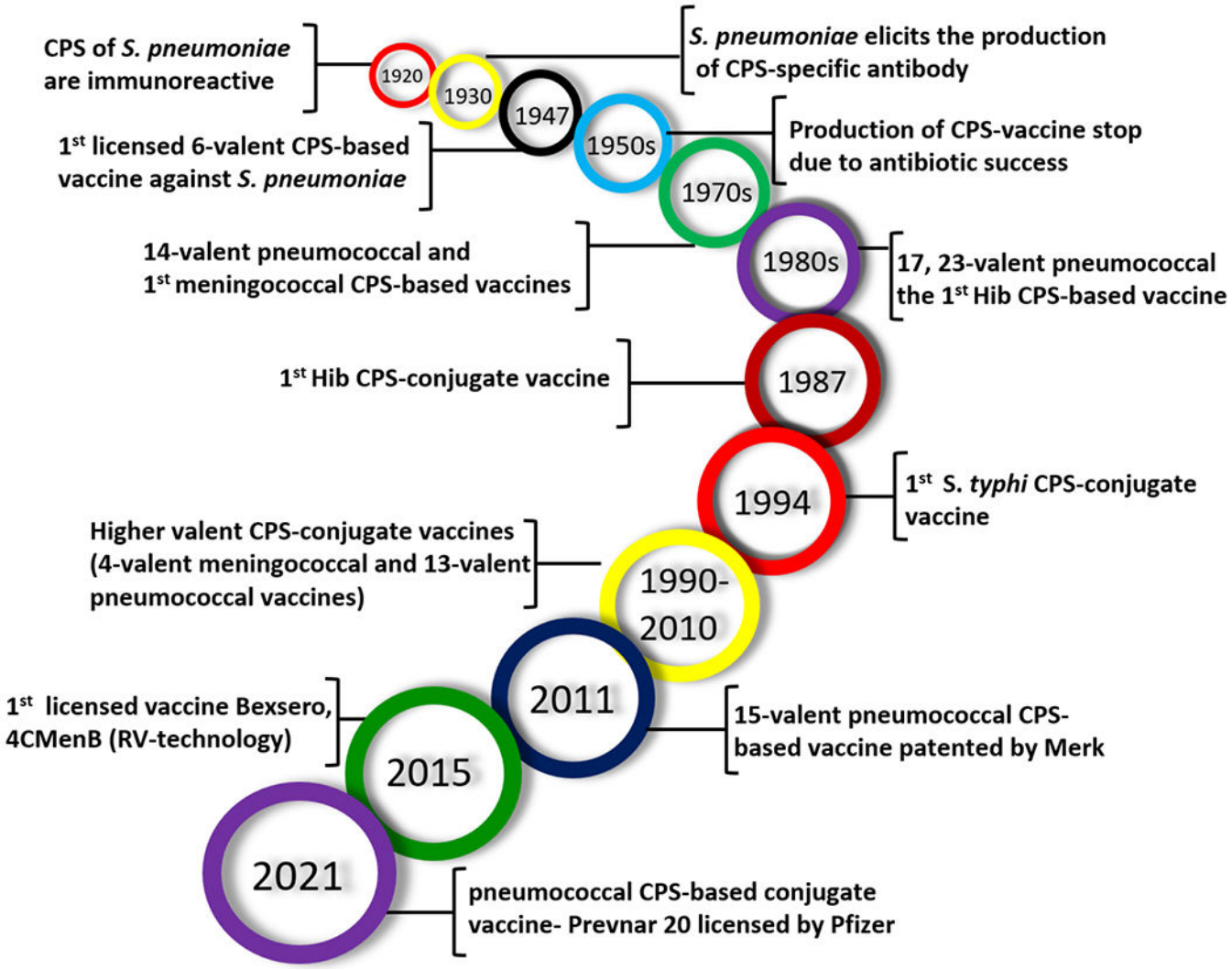

Figure 1:
Major milestones in the history of carbohydrate-based antibacterial vaccine development.

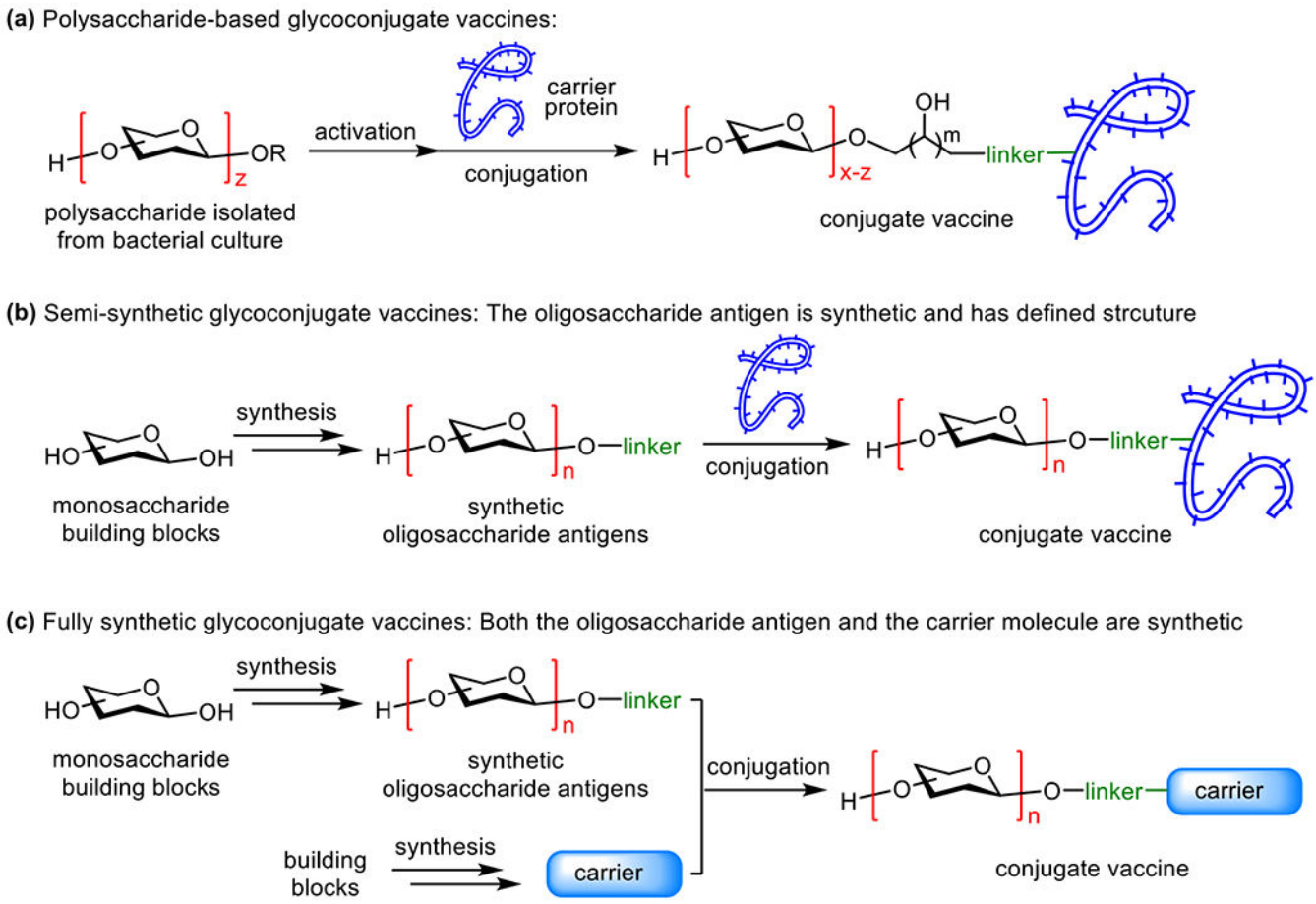

Figure 2.

Three major classes of glycconjugate vaccines. (**a**) The traditional polysaccharide-based glycoconjugate vaccines; (**b**) semi-synthetic glycoconjugate vaccines consisting of synthetic, strcuturally well-defined oligosaccharide antigens; (**c**) structurally defined fully synthetic glycoconjugate vaccines consisting of synthetic oligosaccharide antigens and synthetic carrier molecules.

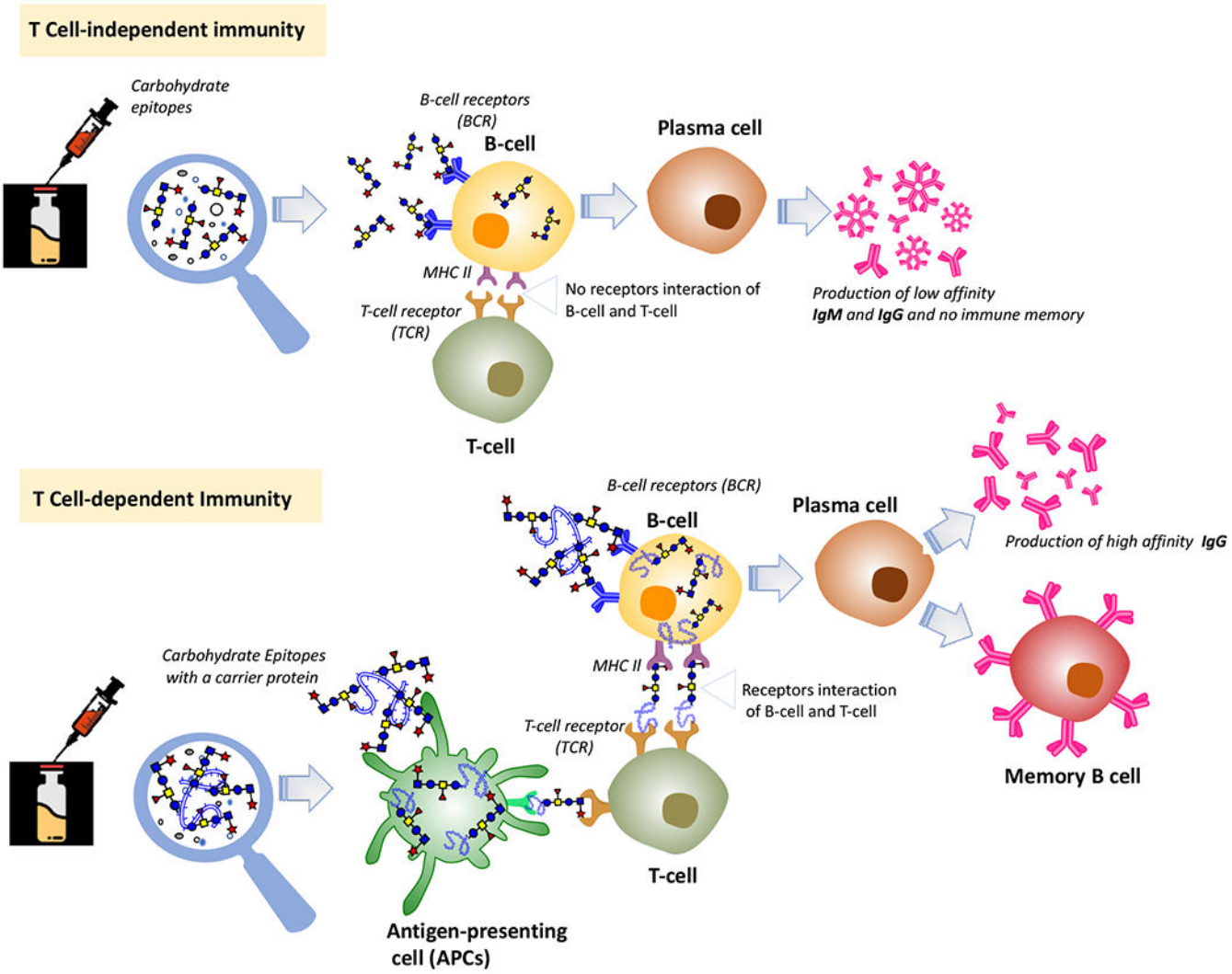

Figure 3.
The general immunology of carbohydrate-based antibacterial vaccines in human.

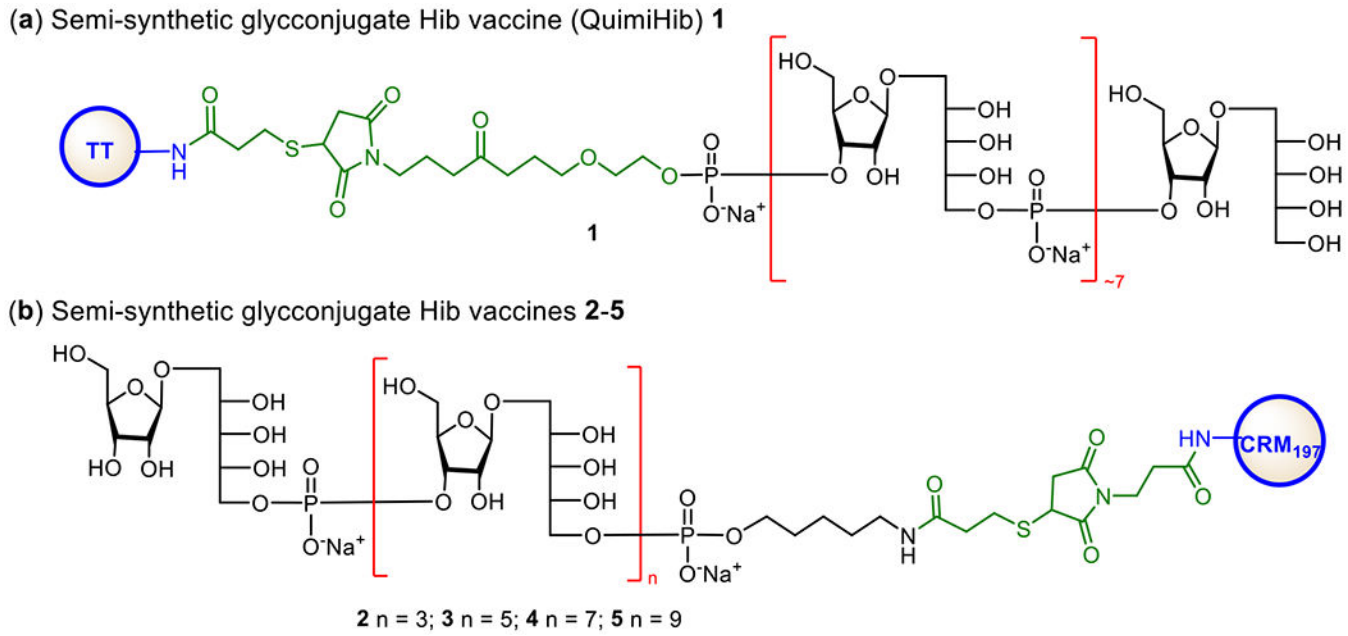

Figure 4.

Structures of commercialized semi-synthetic glycoconjugate Hib vaccine Quimi-Hib **1** (**a**) and semi-synthetic glycoconjugates **2**–**5** explored as Hib vaccines by the Seeberger group (**b**).

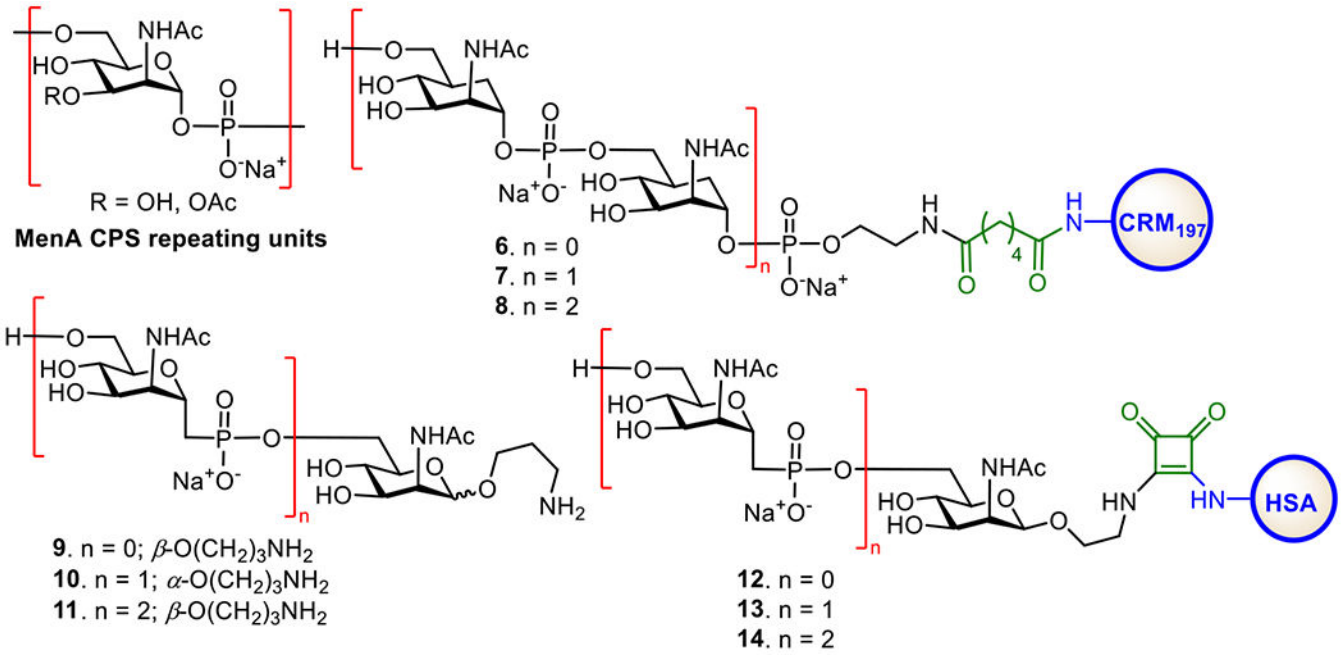

Figure 5.

Structures of MenA CPS and its synthetic 1-*C*-phosphono and carbocyclic analogues, as well as their protein conjugates explored as MenA vaccines.

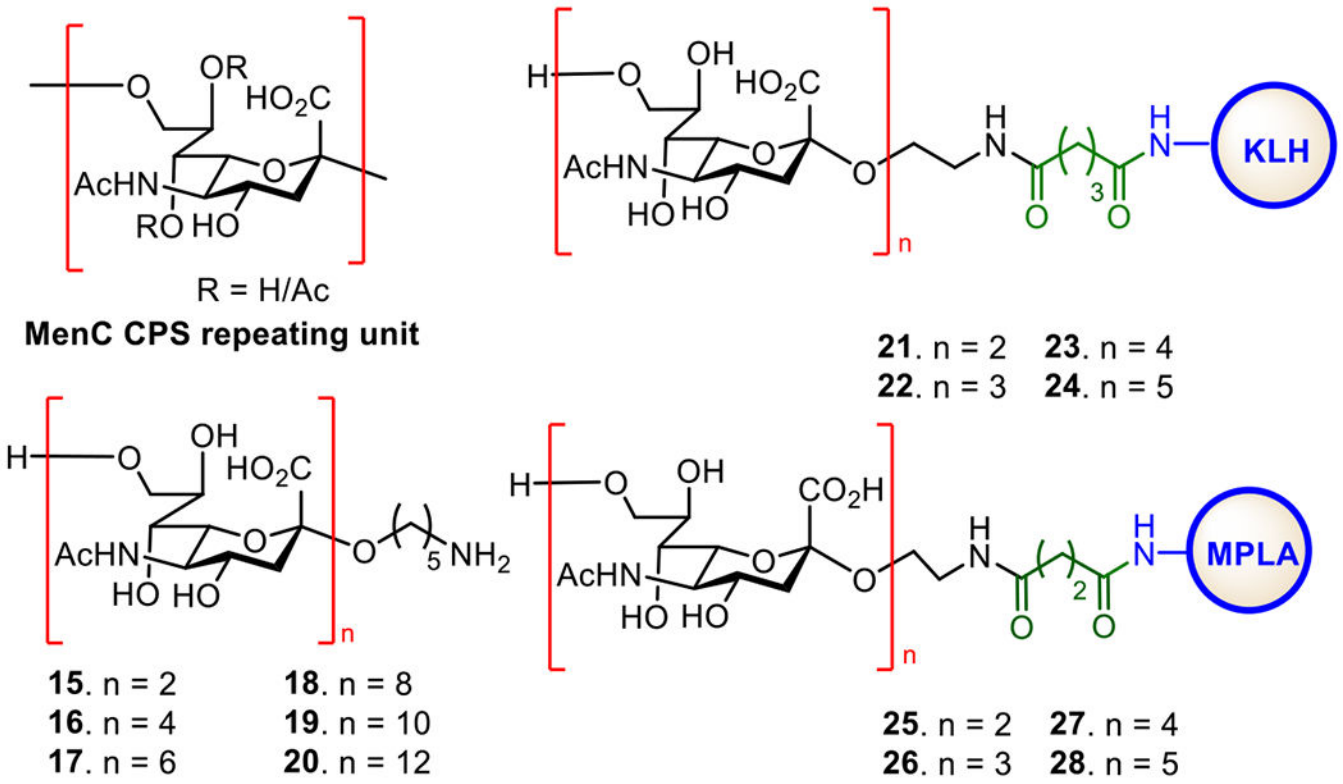

Figure 6.

Structures of MenC CPS, its synthetic oligosaccharides **15-20**, and the protein conjugates **21-28** of oligosaccharides **15-20**.

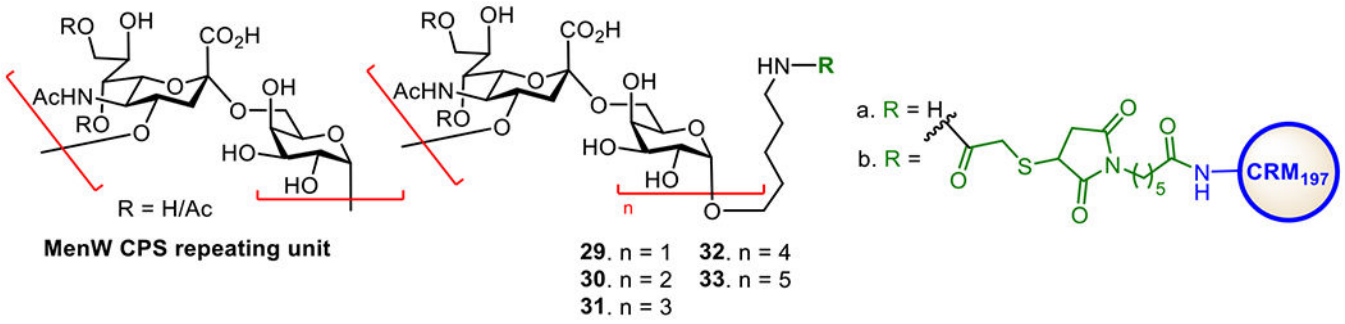

Figure 7.

Structures of MenW CPS, its synthetic oligosaccharides **29-33a**, and the CRM_{197} conjugates **29-33b** of oligosaccharides **29-33a**.

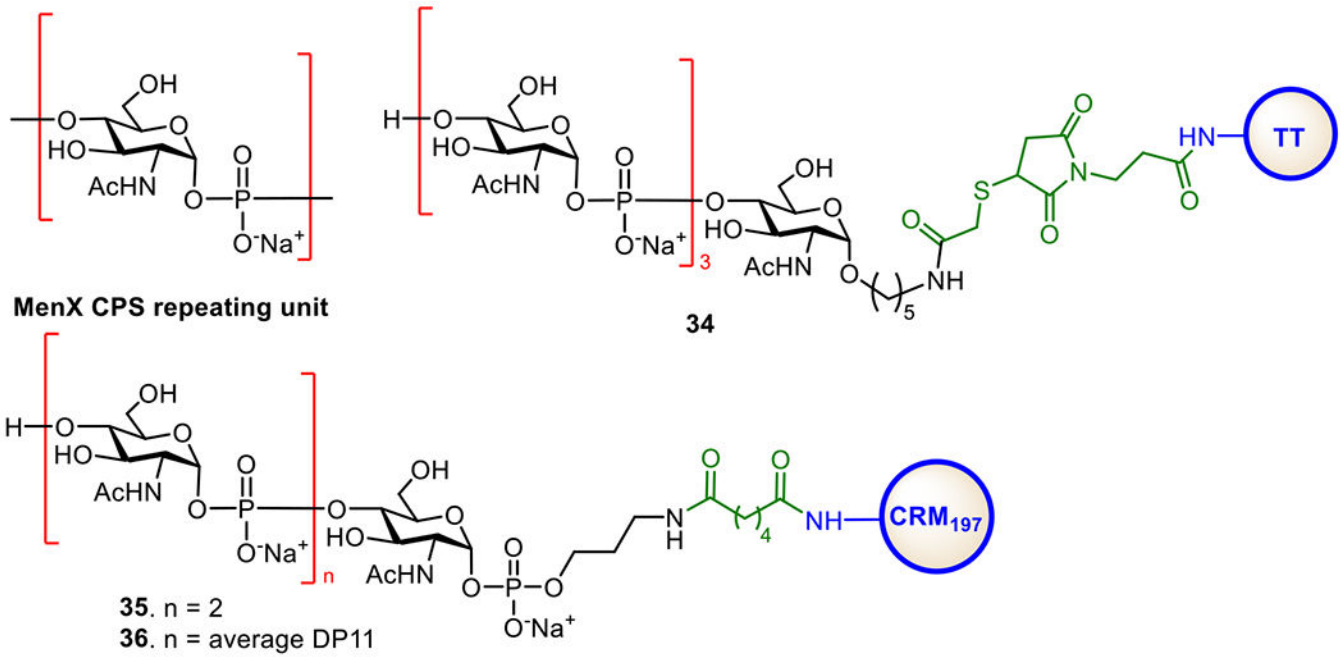

Figure 8.
Structures of the MenX CPS and protein conjugates **34–36** of MenX CPS oligosaccharides.

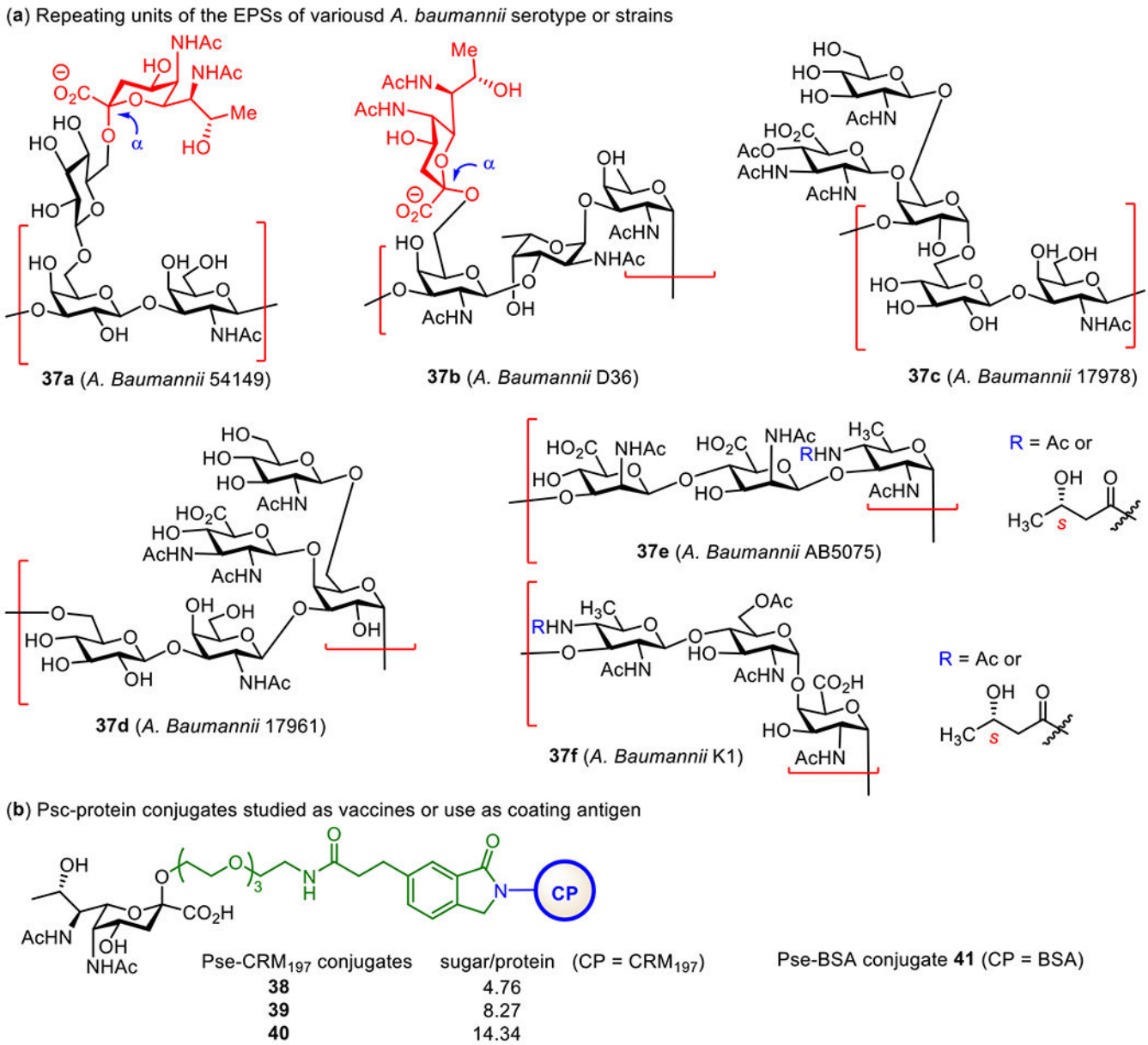

Figure 9.

Structures of the repeating units of *A. baumannii* EPSs and their protein glycoconjugates.

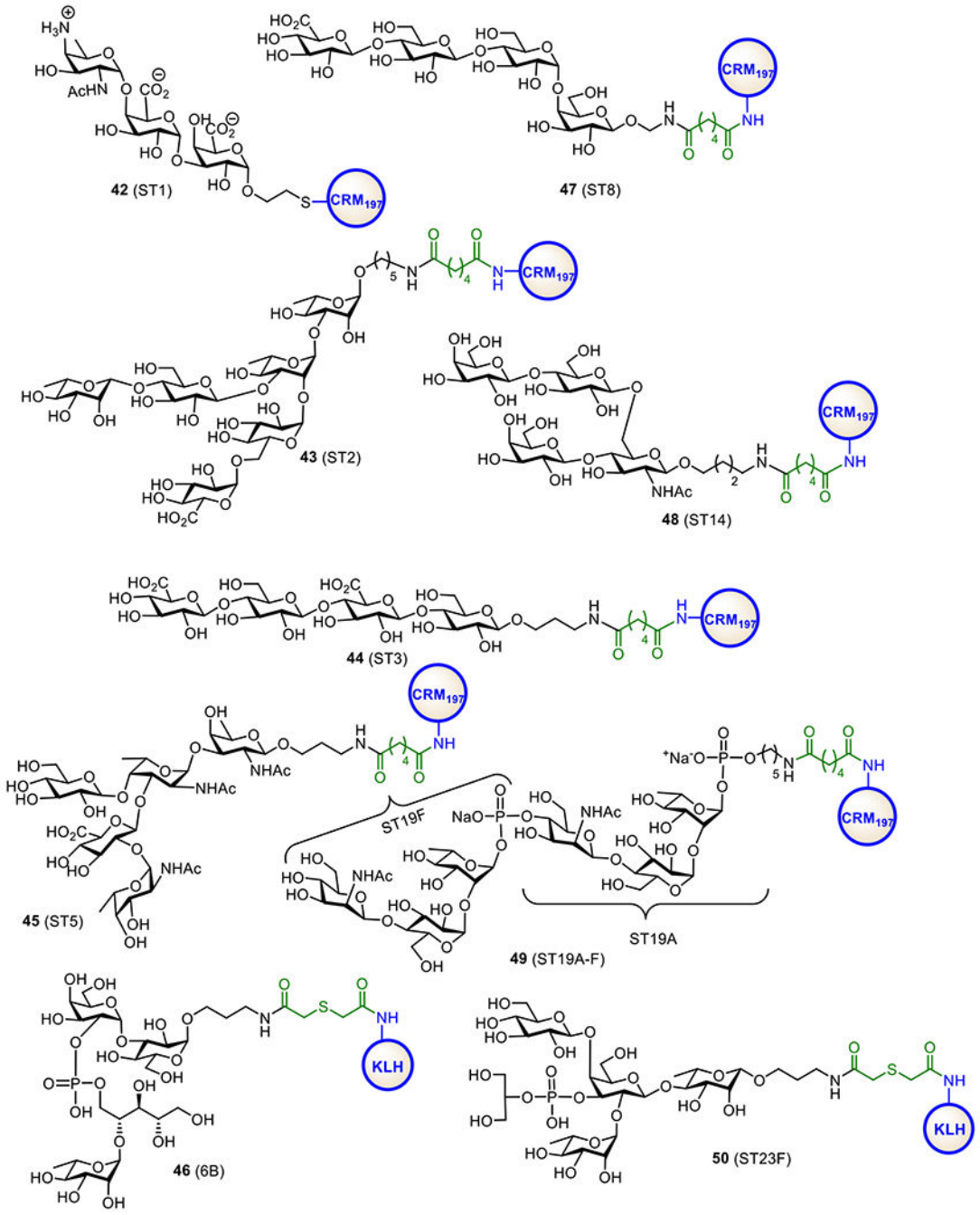

Figure 10.

Structures of the synthetic oligosaccharide-protein conjugates **42-50** as vaccine candidates for *S. pneumoniae* serotypes ST1, ST2, ST3, ST5, ST8, ST6B, ST14, ST19A-F, and ST23F.

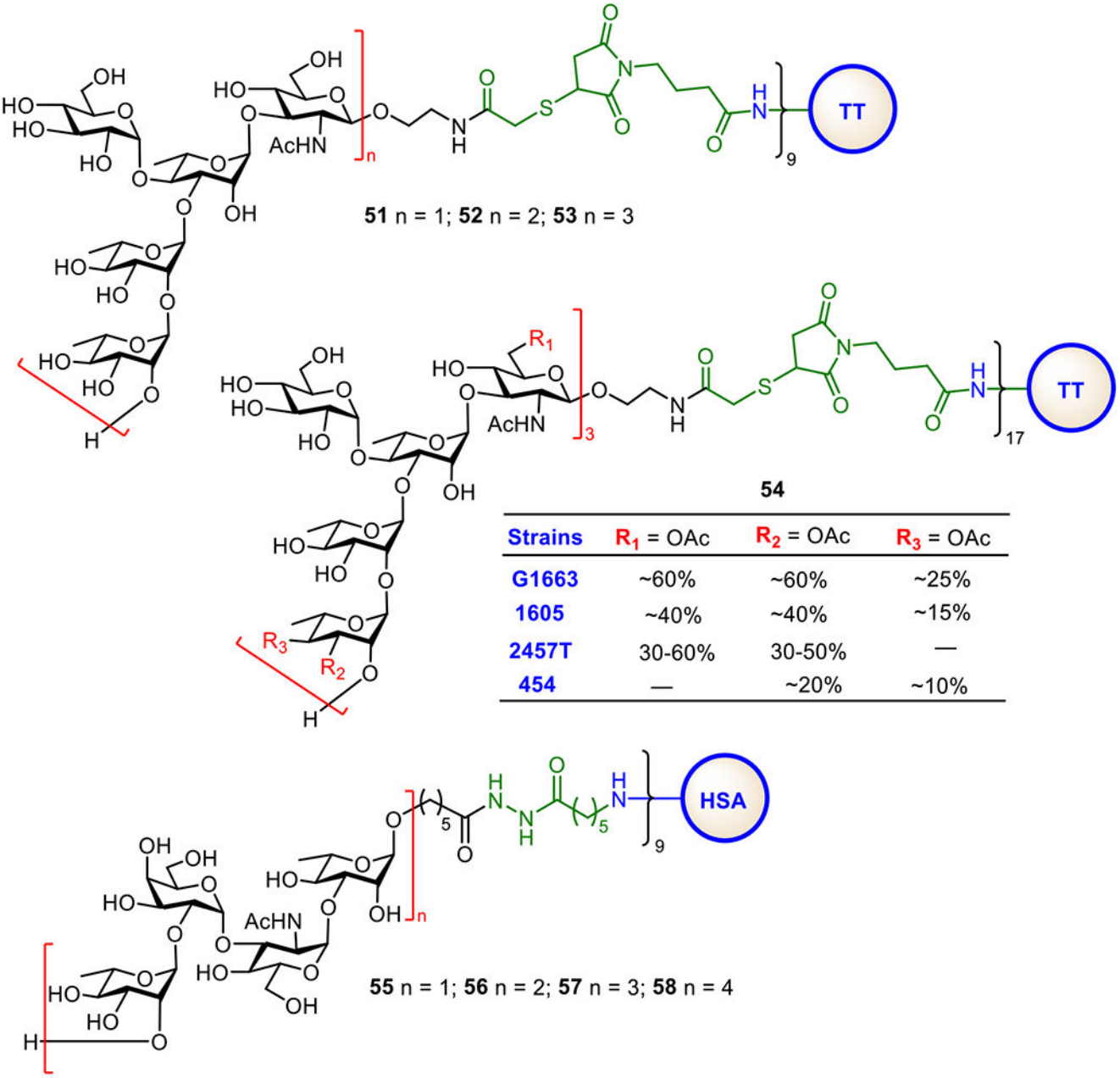

Figure 11.

Structures of synthetic conjugate vaccines **51**–**53** for *S. flexneri* 2a made of oligosaccharides of its *O*-antigen and **54** made of *S. flexneri* 2a *O*-antigen oligosaccharides with nonstoichiometric *O*-acetylation, as well as glycoconjugate vaccines **55**–**58** for *S. dysenteriae* type-1.

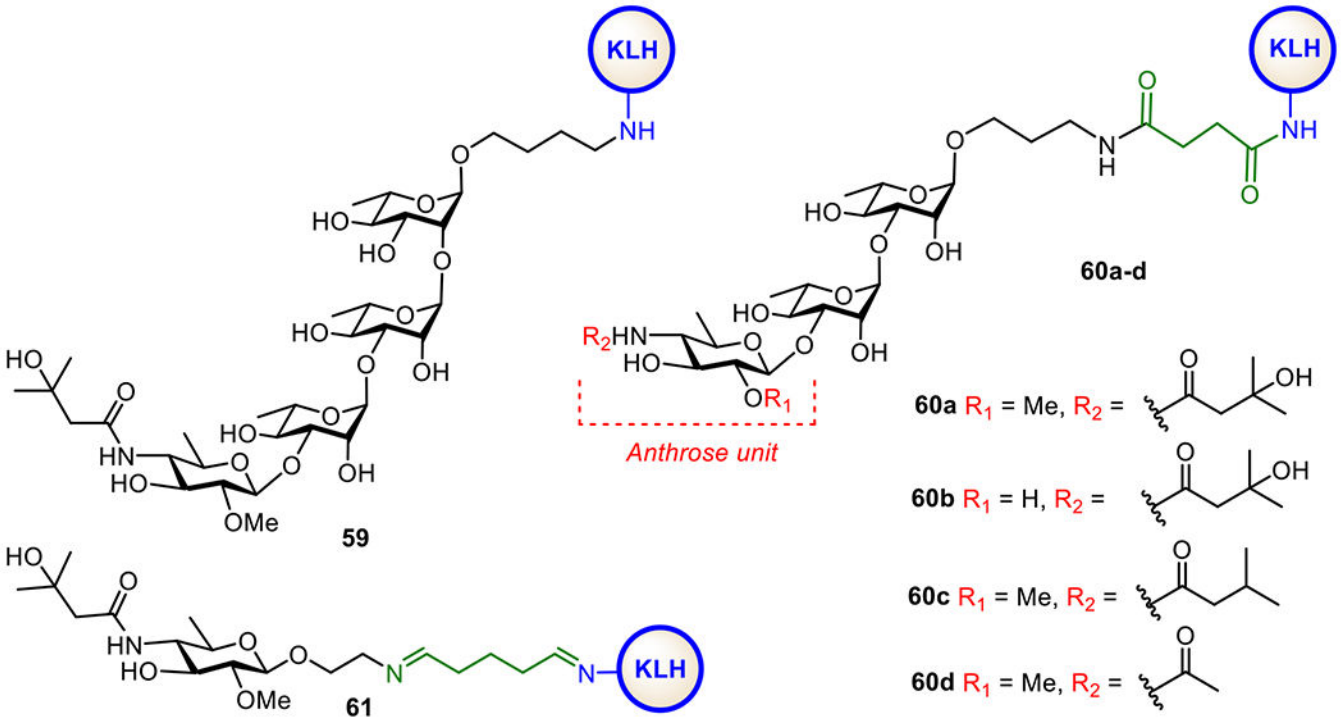

Figure 12.
Structures of synthetic glycoconjugates **59**, **60a-d,** and **61** explored as anti-anthrax vaccines

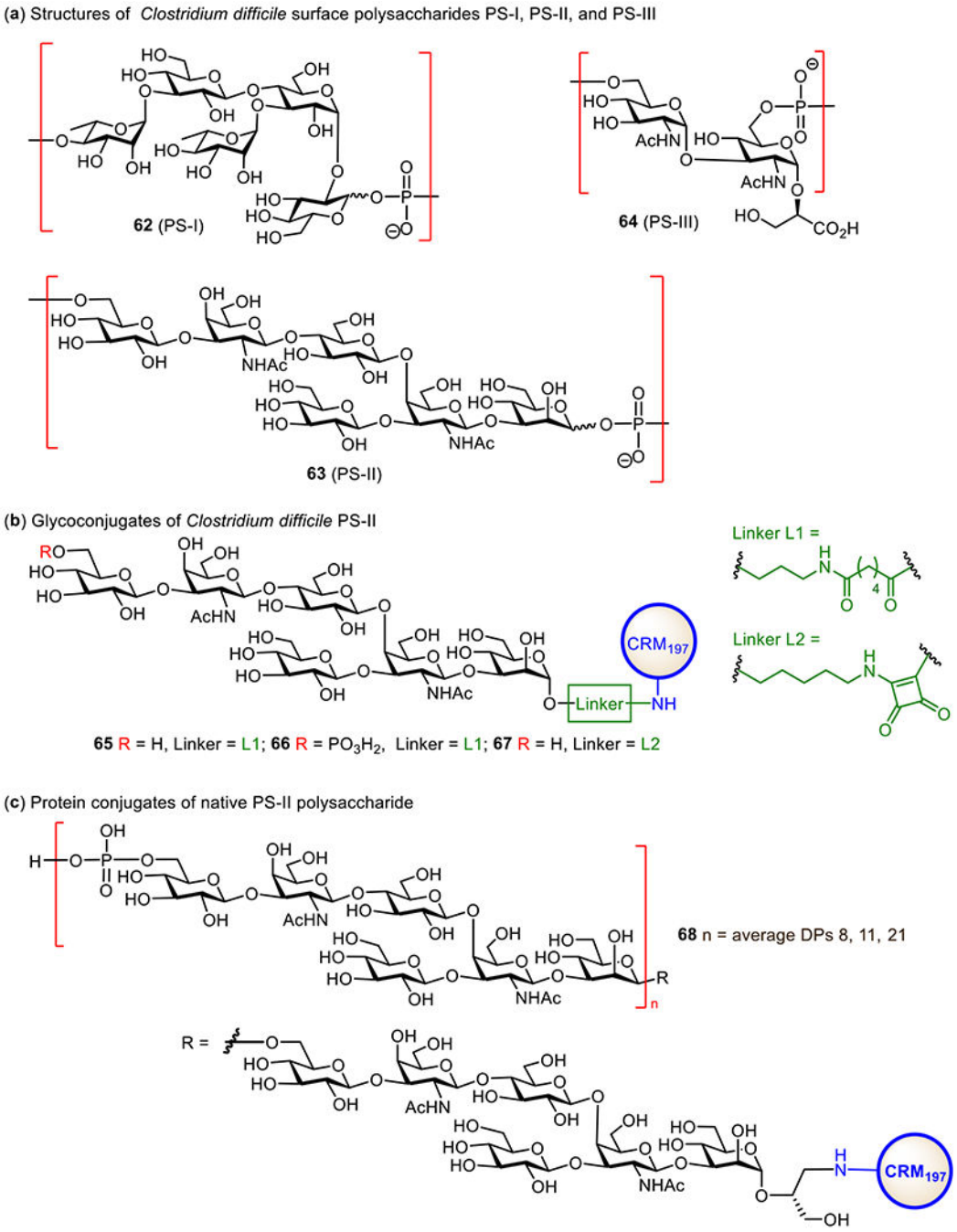

Figure. 13.

Structures of the surface polysaccharides of *C. difficile* PS-I, PS-II, and PS-III **62**–**64** (**a**), synthetic glycoconjugates **65**–**67** consisting of PS-II oligosaccharides (**b**), and native polysaccharide PS-II-CRM_{197} glycoconjugate **68** (**c**) investigated as vaccines for *C. difficile*.

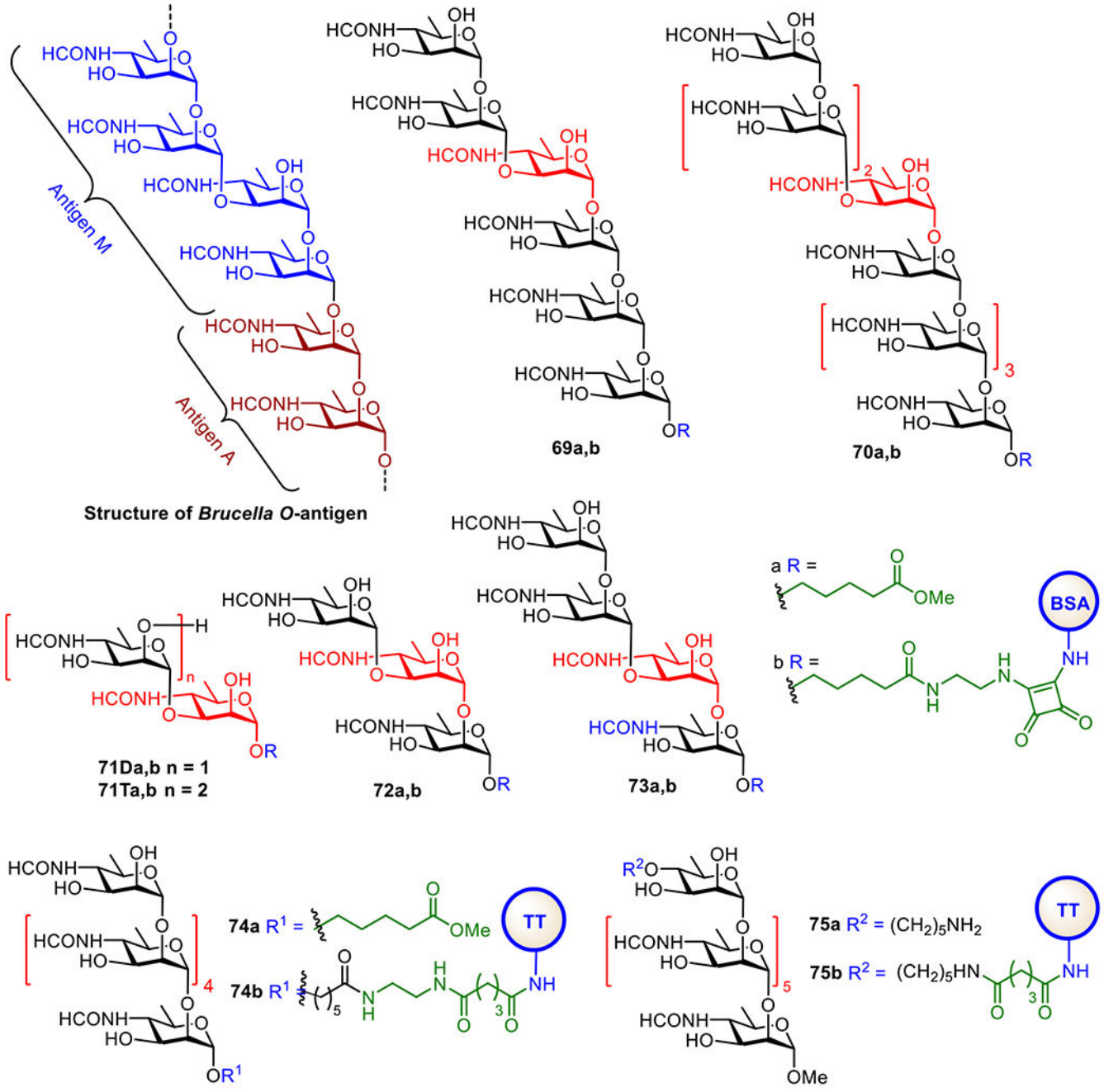

Figure 14.

Structures of *Brucella O*-antigen and its synthetic oligosaccharide analogs **69-75a**, as well as their protein conjugates **69-75b**.

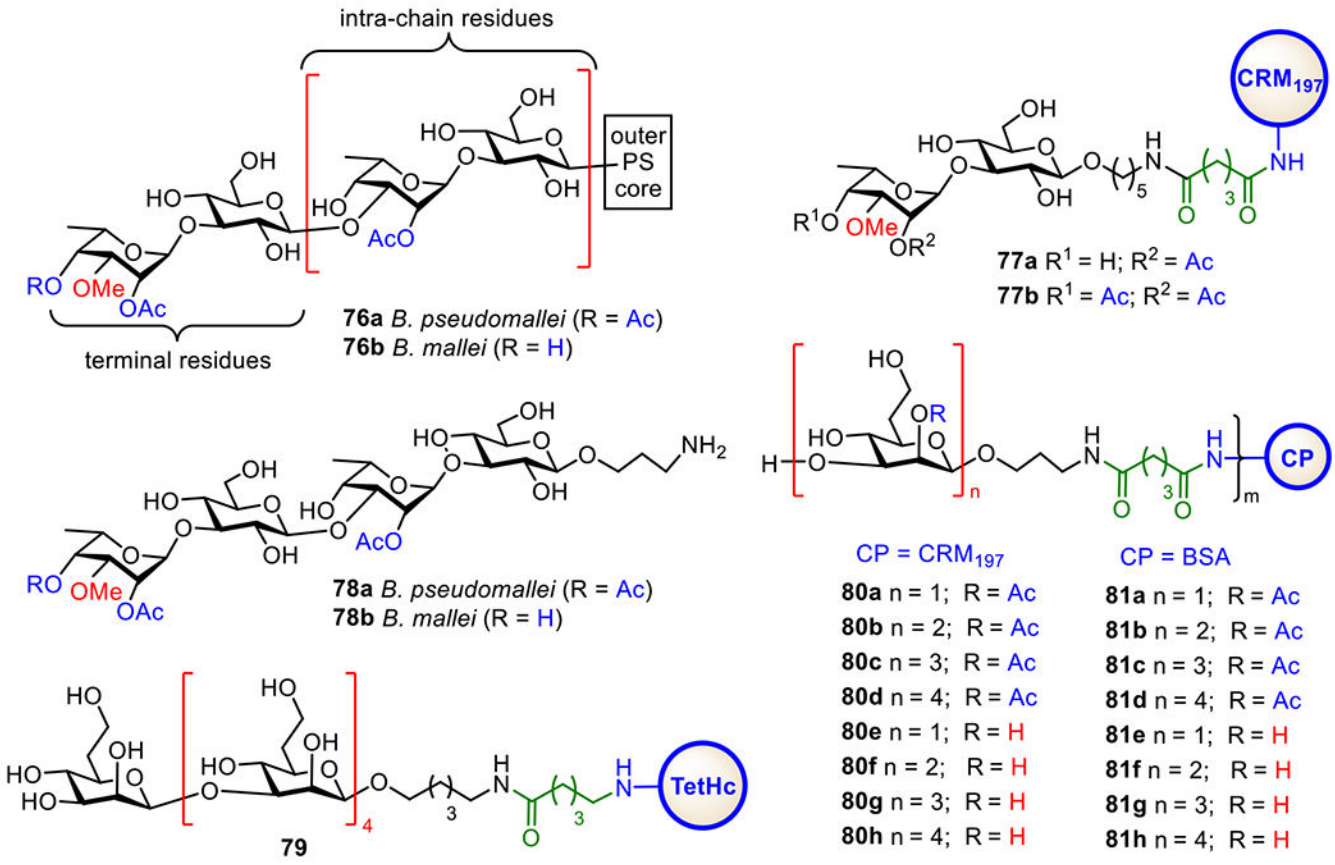

Figure 15.

Structures of the type I O-PS **76a** and **76b** of *B. pseudomallei* and *B. mallei* and the protein conjugates **77a-b, 78a-b, 79, 80a-f,** and **81a-f** of *B. pseudomallei* and *B. mallei* CPS *oligosaccharides*.

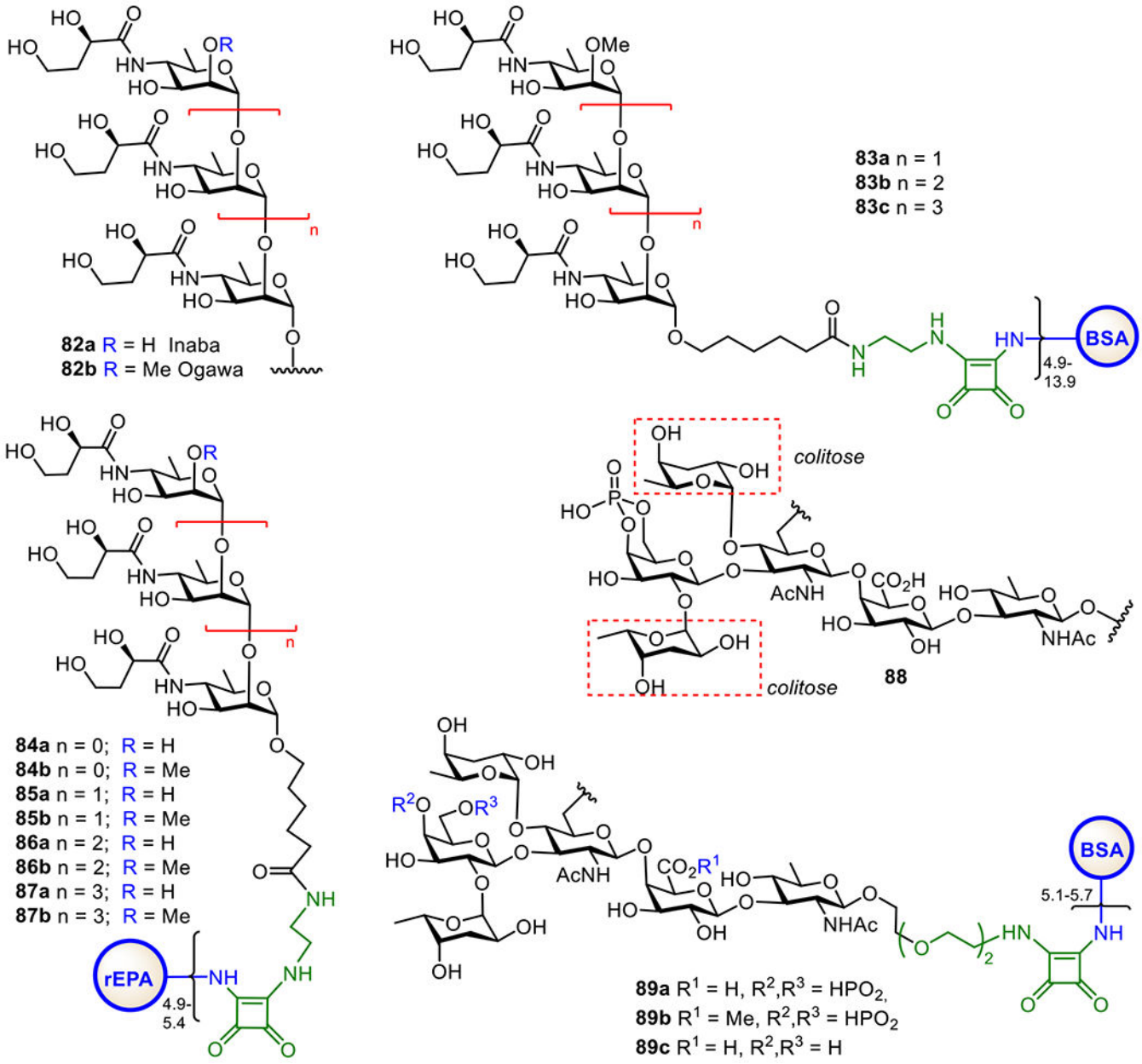

Figure 16.

Structures of the *O*-antigens **82a** and **82b** of *Vibrio cholerae* O1 Inaba and Ogawa serotypes, respectively, the BSA-Ogawa serotype oligosaccharide conjugates **83a-c**, and rEPA-Inaba and Ogawa serotype oligosaccharide **84-87a,b**, as well as structures of the *V. cholerae* O139 *O*-antigen **88** and its protein conjugates **99a-c**.

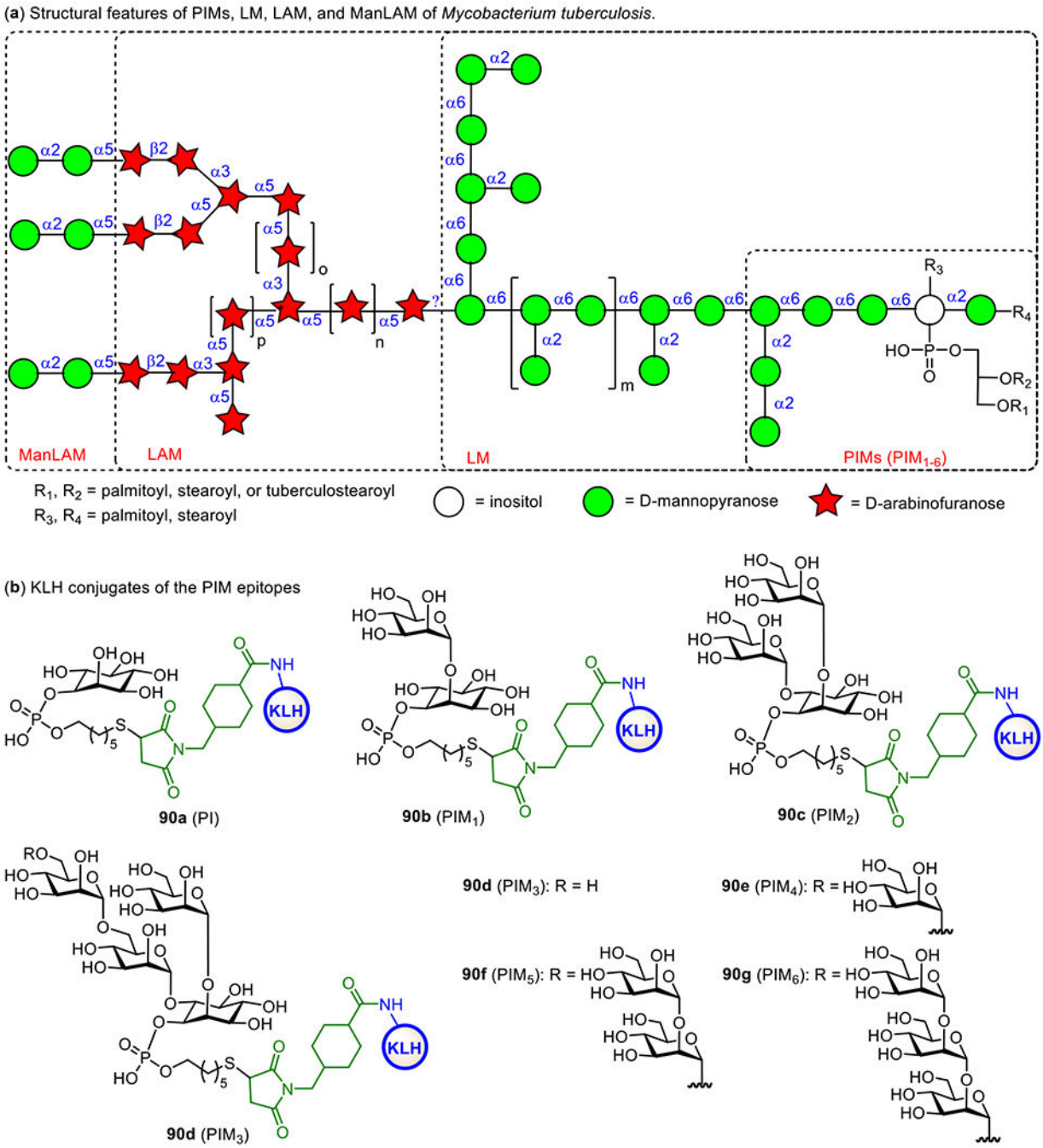

Figure 17.

Structures of mycobacterial LAMs and related molecules (**a**) and the KLH conjugates **90a-f** of synthetic PIM derivatives (**b**).

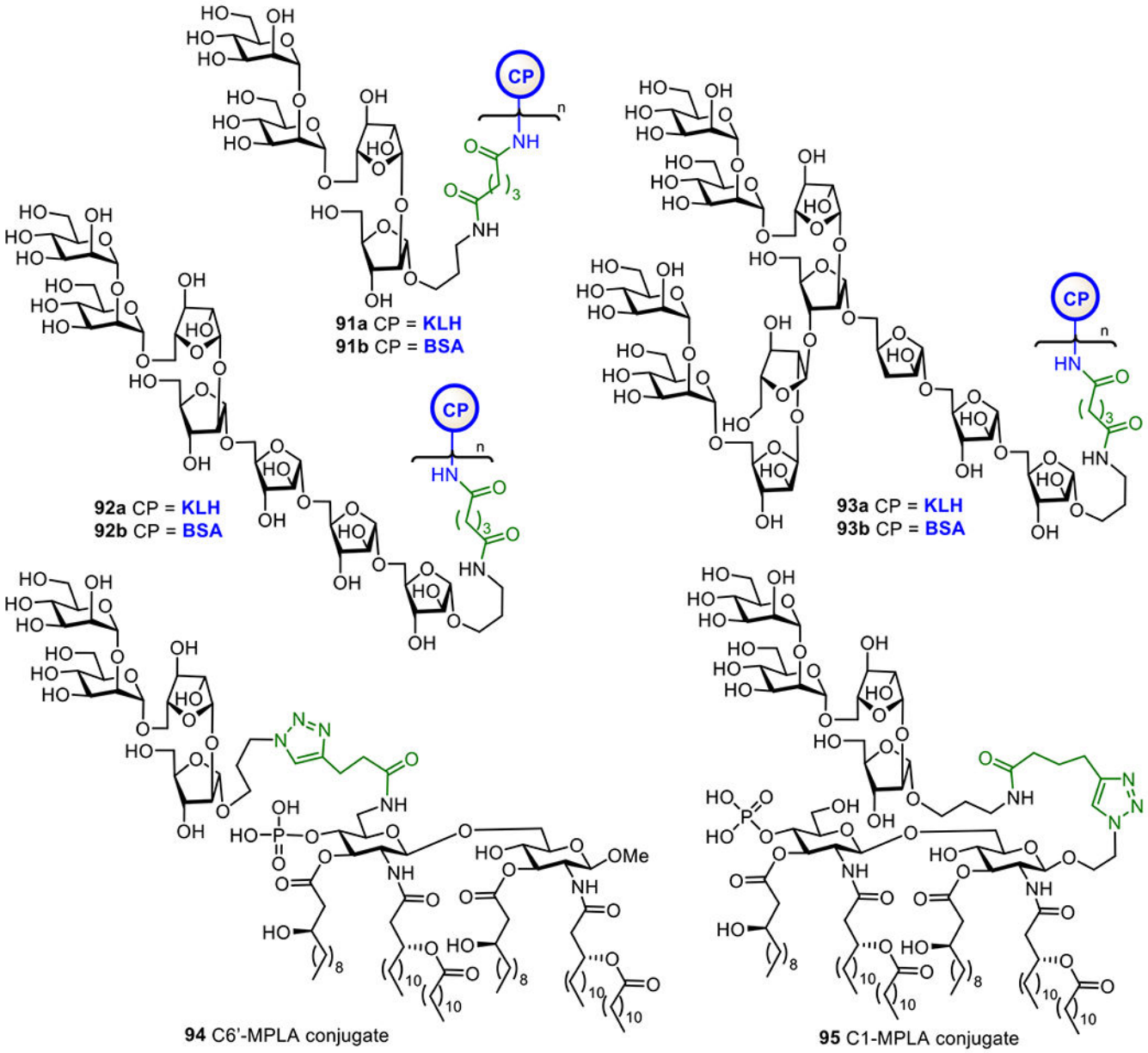

Figure 18.

Structures of KLH and BSA glycoconjugates **91-93a** and **91-93b** of the synthetic LAM oligosaccharides and MPLA conjugates **94** and **95** of LAM tetrasaccharide

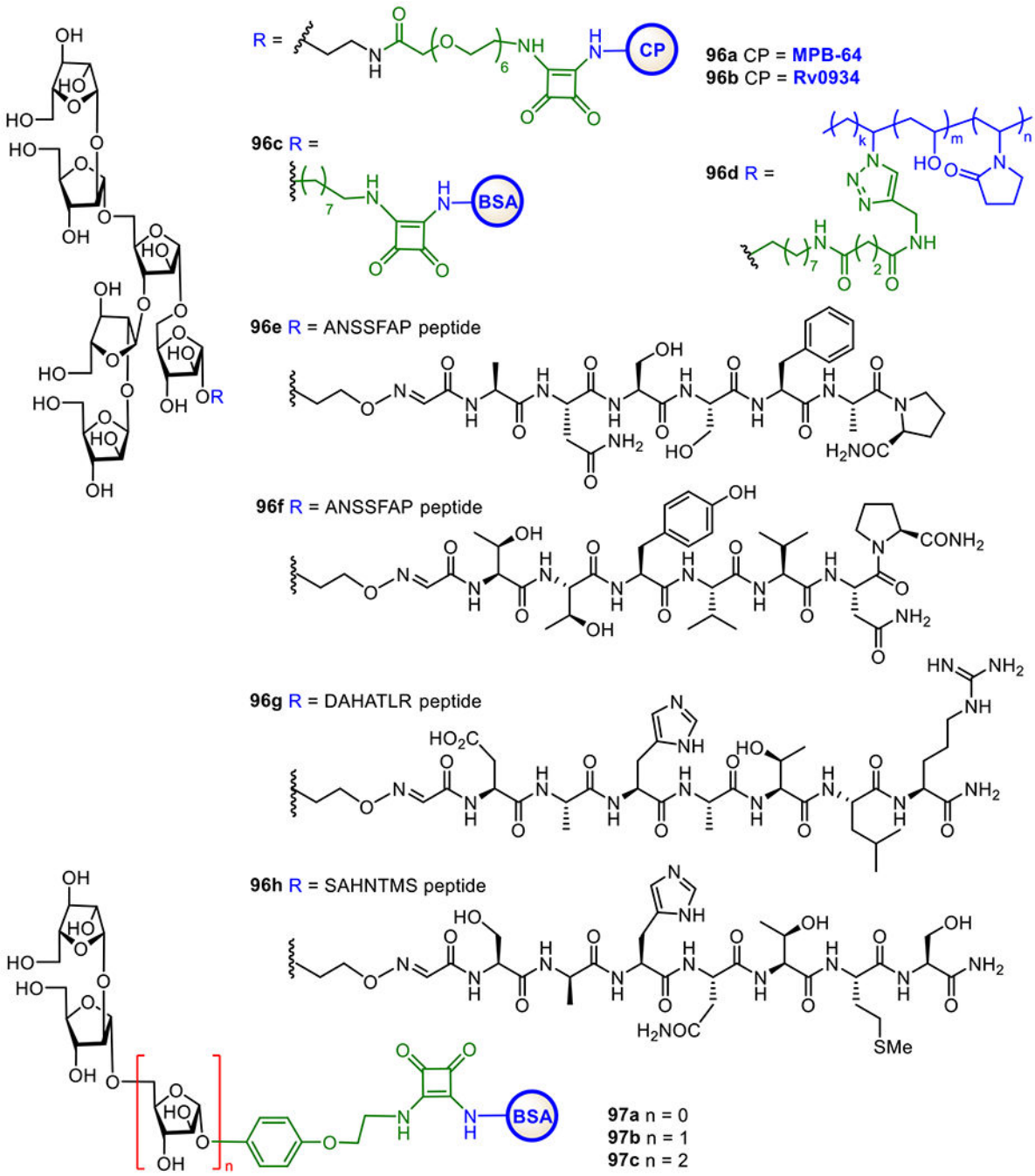

Figure 19:

LAM-related arabinofuranosyl oligosaccharides conjugates **96a-h** and **97a-c** studied as TB diagnostic agents.

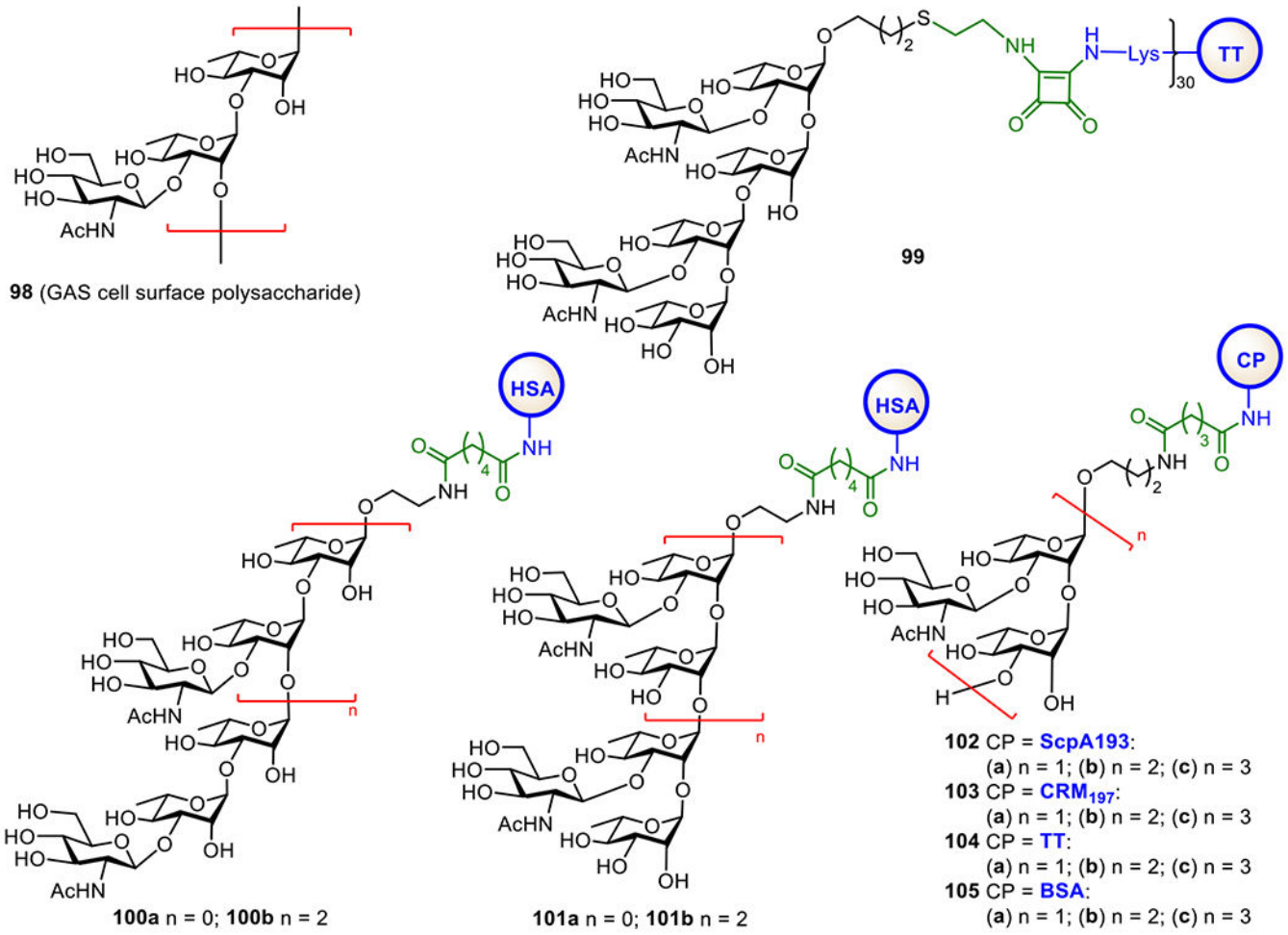

Figure 20.

Structures of the conserved GAS cell wall polysaccharide **101** and GAS oligosaccharide–protein conjugates **102-108** investigated as anti-GAS vaccines.

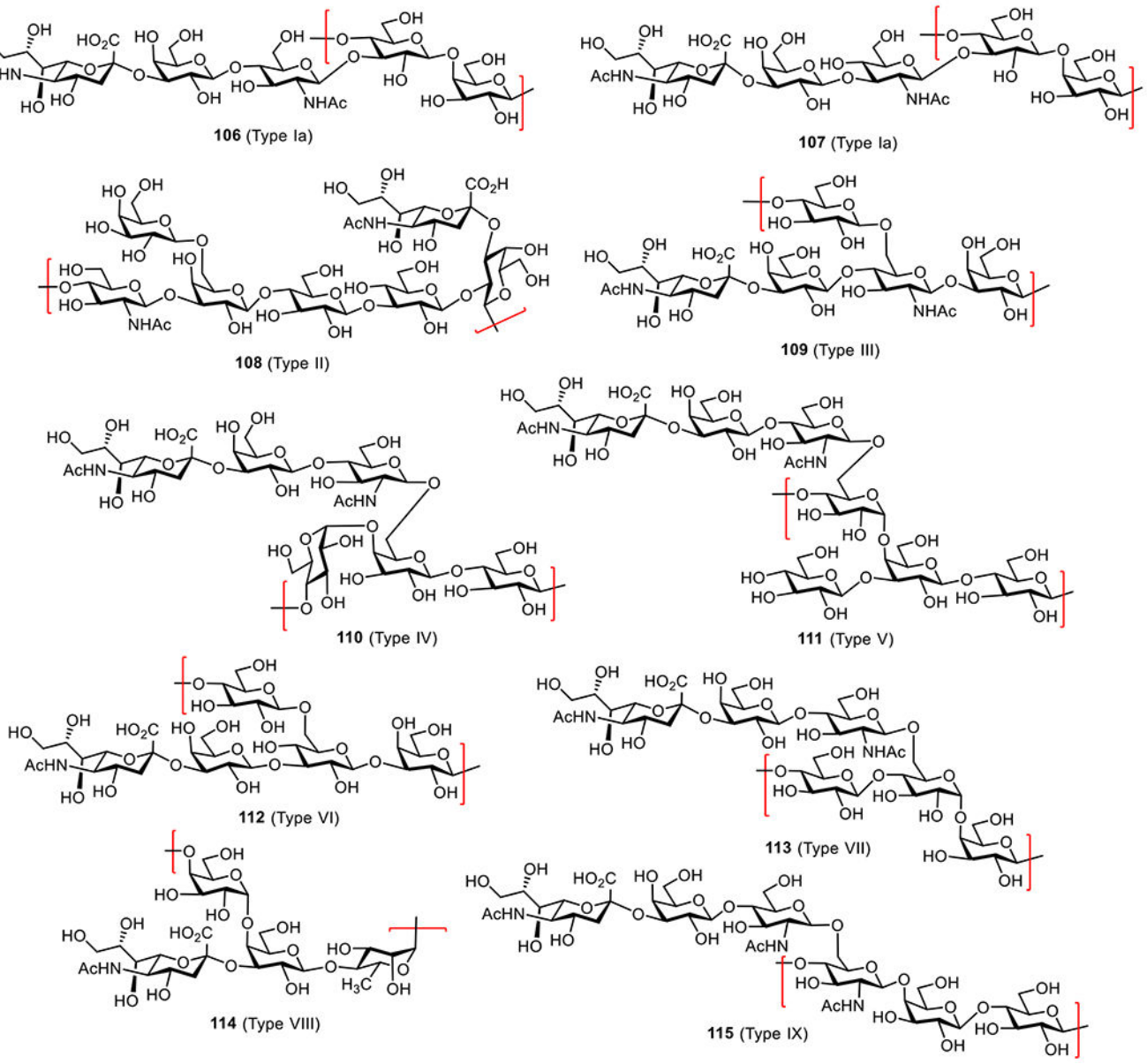

Figure 21.

Structures of the conserved CPSs on the cell surface of GBS serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX.

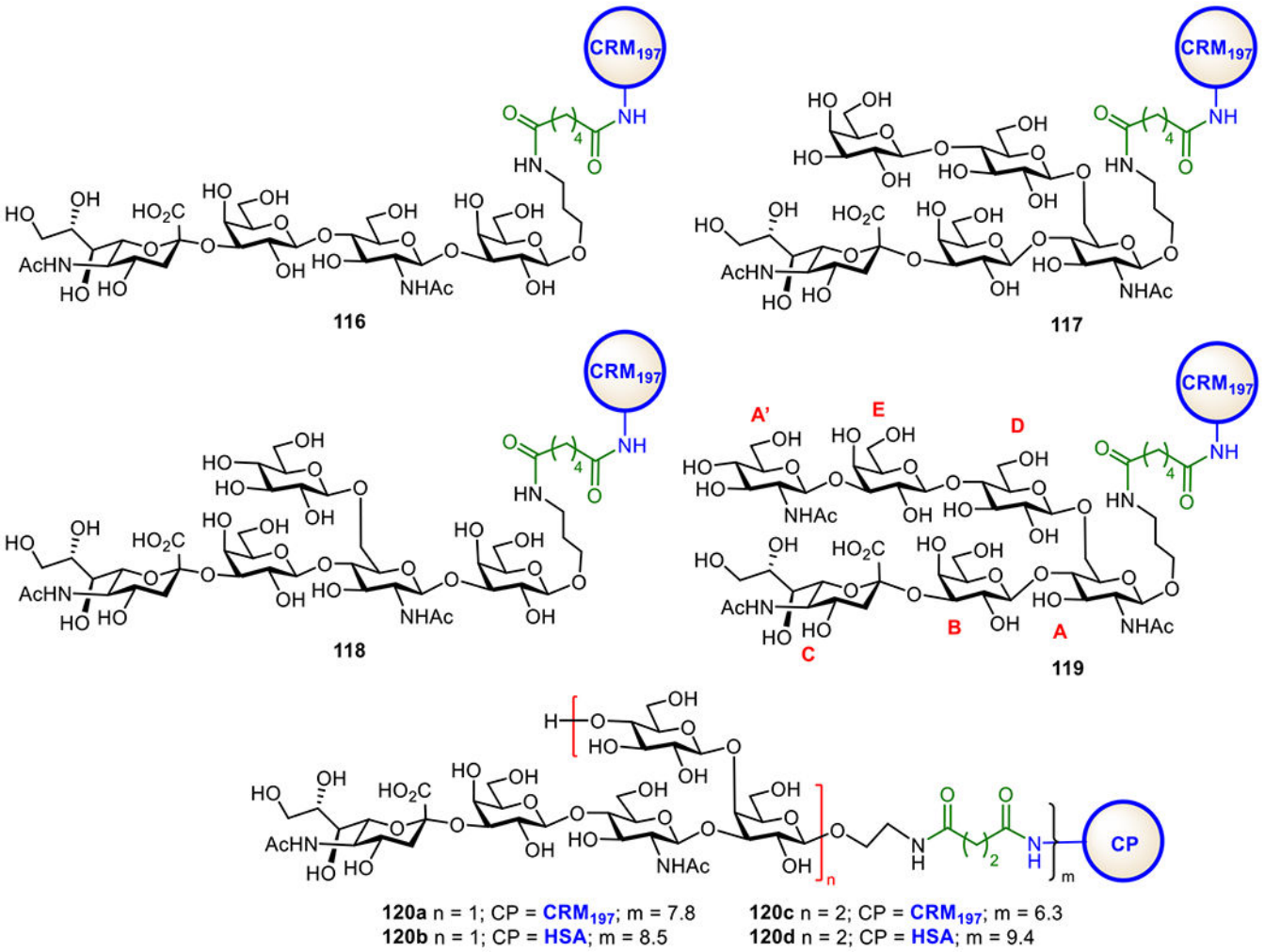

Figure 22.

Structure of serotype III GBS oligosaccharide-CRM_{197} conjugates **116-119** and serotype Ia GBS oligosaccharide-protein conjugates **120a-d**.

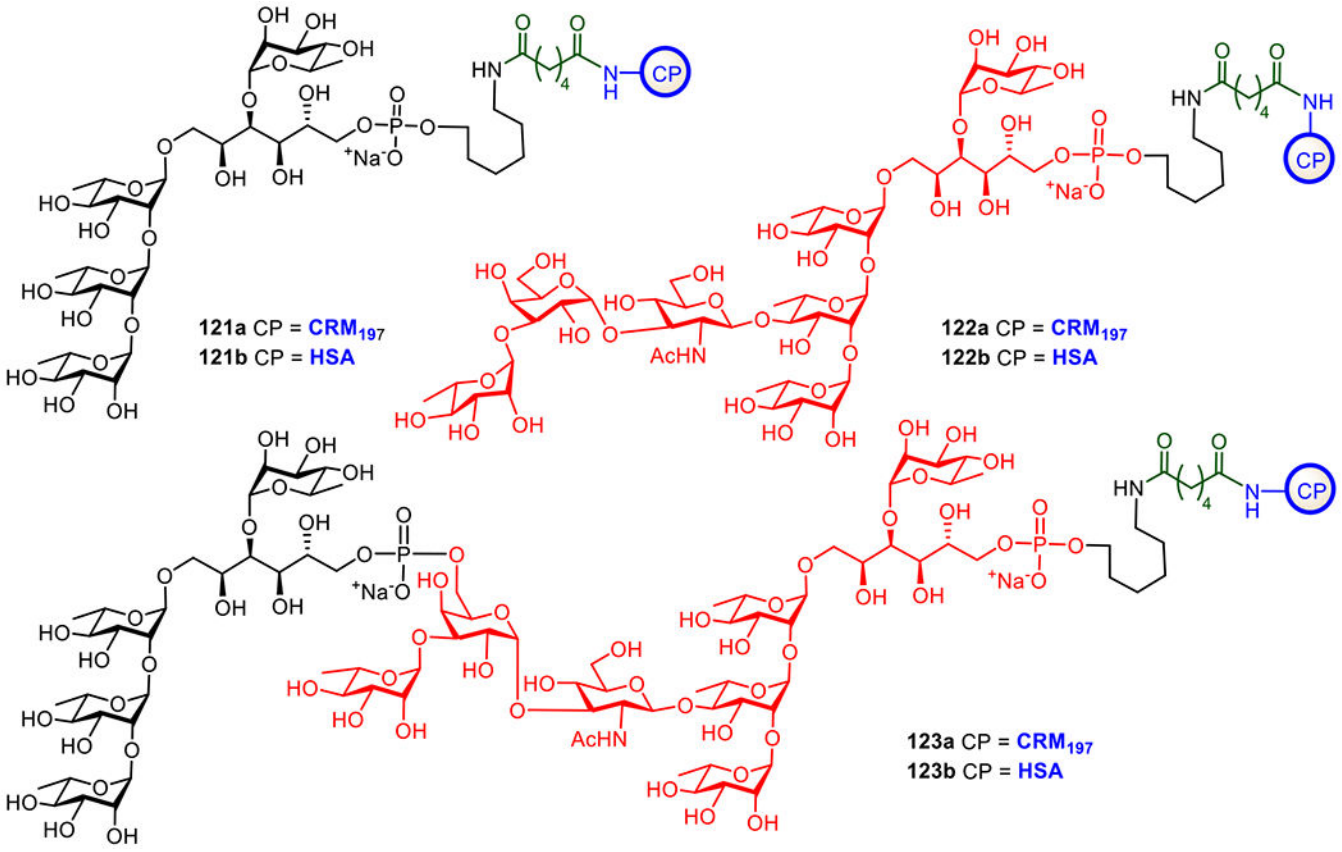

Figure 23.

Structures of the protein conjugates of oligosaccharide epitopes of a group-specific cell surface polysaccharide shared by different GBS serotypes.

Table 1.

Licensed or in-clinical-trial carbohydrate-based antibacterial conjugate vaccines

Bacteria/Pathogens	Carbohydrate antigens	Carrier proteins	Clinical phases	Trade names, Manufactures	Targeted age groups
Haemophilus influenzae type B	Natural polysaccharides	TT	Licensed	ActHIb®, Sanofi Pasteur	Children (2 months to 5 years)
	Natural polysaccharides	TT	Licensed	Hiberix®, GSK	Children (15 months to 4 years)
	Medium-length capsular polysaccharides	OMP	Licensed	PedaxHib®, Merck	Children (2 to 71 months)
	Capsular oligosaccharides	CRM_{197}	Licensed	HibTiter®, Pfizer	Children (2 to 71 months)
	Capsular oligosaccharides	CRM_{197}	Licensed	VaxemHib®, Novartis	Children (2 months to 4 years)
	Synthetic oligosaccharides of capsular polysaccharides (eight repeating units in average)	TT	Licensed	Quimi-Hib®, CIGB, Cuba	Children
Neisseria meningitidis	Capsular oligosaccharides of MenA/C/W/Y	CRM_{197}	Licensed	Menveo®, GSK	Children (from 2 months), adults (up to 55 years)
	Capsular polysaccharides of MenA/C/W/Y	DT	Licensed	Menactra®, Sanofi Pasteur	Children (from 9 months), adults (up to 55 years)
	Medium-length capsular polysaccharides of MenA/C/W/Y	TT	Licensed	Nimenrix®, Pfizer	Children (from 12 months), adults (up to 55 years)
	Capsular oligosaccharides of MenC strain C11	CRM_{197}	Licensed	Menjugate®,	Children (from 2 months), adults, seniors (up to 64 years)
	Capsular oligosaccharides of MenC	CRM_{197}	Licensed	Meningite®, Pfizer	Children (from 6 weeks), adults, seniors (up to 64 years)
	De-*O*-acetylated polysaccharides of MenC strain C11	TT	Licensed	NeisVac-C®, Pfizer	Children (from 2 months), adults, seniors (up to 64 years)
	Medium-length (100–200 kDa) capsular polysaccharides of MenA	TT	Licensed	MenAfriVac®, Ser. Ins. India	Children (from 1 year), adults (up to 29 years)
Streptococcus pneumoniae	Native capsular polysaccharides of *pneumococci* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9 V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F	CRM_{197}	Licensed	Prevnar 20®, Pfizer	Adults (from 18 year or older)
	Native capsular polysaccharides of *pneumococci* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19A, 19F and 23F	CRM_{197}	Licensed	Prevnar 13®, Wyeth/Pfizer	Children (6 weeks to 17 years), adults (50 years or older)
	Native capsular polysaccharides of *pneumococci* serotypes 4, 6B, 9 V, 14, 18C, 19F, and 23F	CRM_{197}	Licensed	Prevnar®, Wyeth/Pfizer	Children (up to 9 years)
	Native capsular polysaccharides of pneumococci serotypes 1, 4, 5, 6B, 7F, 9 V, 14, 18C, 19F and 23F	Protein D, TT, DT	Licensed	Synflorix®, GSK	Children (up to 5 years)
	Natural polysaccharides of serogroups 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9 V, 10A, 11A, 12F, 14, 15B, 177, 18C, 19A, 19F, 20B, 22F, 23F, 33F	Rhizavidin	Phase II	Affinia	Children

Bacteria/Pathogens	Carbohydrate antigens	Carrier proteins	Clinical phases	Trade names, Manufactures	Targeted age groups
	Natural polysaccharides of serogroups 1, 5, 14, 6B, 18C, 19F, 23F	TT	Phase III	QuimiVio/CIGB	Children
Group B *Streptococcus*	Natural polysaccharides of serotypes Ia, Ib, II, III, IV, V	CRM_{197}	Phase II	Pfizer	Children
Klebsiella 4V	Oligosaccharides of *O*-antigen	EPA	Phase I	LMTB-GSK	Children
Shigella 2a and 4V	Oligosaccharides	EPA	Phase I	LMTB-GSK	Children
Shigella 2a	Synthetic oligosaccharides	TT	Phase I	Pasteur Institute	Children
Extraintestinal pathogenic *Escherichia Coli*	Oligosaccharides of serogroups O1A, O6A, O18A, O25B, O2, O4, O8, O15, O16, and O75	EPA	Phase III	Johnson & Johnson	Children
PNAG (*Staphylococcus aureus, E. coli, Klebsiella pneumoniae, N. gonorrhea, N. meningitidis, S. pneumoniae*)	Synthetic oligosaccharides	TT	Phase II	Alopex	Children

TT: tetanus toxoid; DT: diphtheria toxoid; CRM_{197}: tetanus toxin mutant; OMP: outer membrane protein derived from *Neisseria meningitides* serotype B1; Protein D: conserved surface protein from non-typed *Haemophilus influenzae* (NTHi); MenA/C/W/Y: group A, C, W and Y *meningococci*; rEPA: *Pseudomonas aeruginosa* exotoxin A

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