# OLIGOSACCHARIDE–PROTEIN CONJUGATES AS VACCINE CANDIDATES AGAINST BACTERIA

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## I. OVERVIEW OF POLYSACCHARIDE-BASED ANTIBACTERIAL VACCINES*

Complex polymers of mono- and oligo-saccharides are ubiquitous components of the bacterial cell wall.[1–3] The three major classes are the capsular polysaccharides,

* Abbreviations in the text.—Abe, abequose; BSA, bovine serum albumin; CRM, cross-reacting mutant; Ds, degree of substitution; DTx, diphtheria toxin; *E., Escherichia;* Gal, galactose; Glc, glucose; *H., Haemophilus;* Hib, *Haemophilus influenzae* type b; HSA, human serum albumin; IgG, immunoglobulin G; IgM, immunoglobulin M; Kdo, 3-deoxy-D-*manno*-octulosonic acid; KLH, keyhole limpet hemocyanin; $LD_{50}$, 50% lethal dose; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; Man, mannose; MW, molecular weight; *N., Neisseria;* O-SP, O-specific polysaccharide; *S., Salmonella; Sh., Shigella; Str., Streptococcus;* TT, tetanus toxoid.

Abbreviations in the formulas and schemes.—Ac, acetyl; All, allyl; BDPS, $^t$butyldiphenylsilyl; Bn, benzyl; BOM, benzyloxymethyl; Bz, benzoyl; CA, chloroacetyl; CPG, controlled-pore glass; DMT, 4,4′-dimethoxytrityl; Et, ethyl; Gal, D-galactose; Glc, D-glucose; GlcA, D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose; Hep, heptose; Kdo, 3-deoxy-D-*manno*-octulosonic acid; Man, D-mannose; MBn, 4-methoxybenzyl; Me, methyl; MMT, monomethoxytrityl; PEA, phosphoethanolamine; Ph, phenyl; Phth, phthaloyl; *p*-$NO_2$Bz, 2-nitrobenzoyl; Pr, propyl; Rha, L-rhamnose; Tol, 2-methylbenzoyl; Ts, *p*-tolylsulfonyl; Z, benzyloxycarbonyl.

Single-letter notations for amino acids.—A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

the teichoic acids, and the lipooligo- and poly-saccharides. Bacterial cell-surface polysaccharides provide mechanical protection to the cell against the intracellular osmotic pressure, impair phagocytosis, and activate the alternate pathway of complement activation.[4] That pneumococcal capsular polysaccharides are carriers of type-specificity was recognized by the identification of the "soluble specific substances" isolated from pneumococci as polysaccharides devoid of proteinaceous material.[5] This observation was extended to both gram-positive and gram-negative bacteria expressing capsular polysaccharides or teichoic acid-type polymers. Lipopolysaccharides of gram-negative bacteria exhibit the same principle: their O-specific polysaccharide domains alone define serotype specificity. Only few instances are known when different bacteria carry identical capsular polysaccharides (e.g., *E. coli* K12 and K82, and *E. coli* K1; group B *Neisseria meningitidis, Moraxella nonliquefaciens,* and *Pasteurella haemolytica* A2;[6,7] *Enterococcus faecalis,* and *Enterococcus faecium*[8]), or O-specific polysaccharides (e.g., *Shigella sonnei, Plesiomonas aeruginosa*[9]). Fully expressed capsular polysaccharides or teichoic acids of gram-positive bacteria and the O-specific polysaccharides of gram-negative enteric bacteria are essential for virulence of "primary" pathogens.[10] Bacterial cell-surface polysaccharides that have close structural similarity to saccharides found in human tissues may protect the bacteria from response by the host's immune response, and this molecular mimicry may be related to bacterial virulence. Because carbohydrates are secondary gene products and require several enzyme systems even for the construction of a monosaccharide, cell-surface carbohydrate epitopes are highly conserved and, in contrast to proteins, no mutation of bacterial polysaccharides has so far been reported. This permanency of the structures of surface polysaccharides has made them a target for immunoprophylaxis since the discovery by Francis and Tillet[11] that purified capsular polysaccharides are able to elicit anti-polysaccharide antibodies in healthy humans at a single dose of 10 μg. Subsequent work by Heidelberger et al.[12] firmly established that antibodies induced by pneumococcal capsular polysaccharides provide type-specific immunity that may last for up to 8 years. Antibodies to other cellular components of encapsulated bacteria are "of considerably less importance" for protection.[13,14] Recognition of increasing occurrence of bacterial resistance to antibiotics[15] emphasized the need for disease prevention by vaccination. Developments since the early explorations on bacterial polysaccharides as vaccine components led to the licensure of several vaccines as commercial pharmaceuticals consisting of purified cell-surface polysaccharides or teichoic acid-like polymers. These include 23-valent vaccines against pneumococci containing 25 μg of the capsular polysaccharide of each of the 23 most invasive types [16][Pneumovax (Merck) and Pnu-immune (Wyeth Lederle)], a bivalent vaccine against groups A and C *N. meningitidis* [AC Vax (SmithKline Beecham) and Mangivax (Pasteur Merieux)], a tetravalent vaccine against groups A, C, W-135, and Y *N. meningitidis*[16] [Menomune (Pasteur Merieux)], and a vaccine containing

the Vi polysaccharide[17] against typhoid fever [Typhim Vi (Pasteur Merieux Connaught)]. Despite their success, capsular polysaccharide vaccines have limitations because of their T-cell dependence (no booster response) and age-related immunogenicity: in contrast to proteins, immune responsiveness to polysaccharides does not usually develop in infants younger than 18 months of age.[17] Although the protective efficacy of the pneumococcal vaccine in infants is far from ideal, it has been suggested that their use in infants should be considered until a better vaccine becomes available.[18] The age-related immune response to plain polysaccharides may also be structure-dependent: in contrast to other polysaccharides, the capsular polysaccharides of group A *N. meningitidis*[19] and type 3 and 18C pneumococci[20] are good immunogens in infants starting at the age of 3 to 6 months and produce protective levels of IgG antibodies. In addition, the molecular size has a crucial role in that larger polysaccharides are generally more immunogenic than smaller ones, although no molecular weight range has been defined for polysaccharide immunogenicity.[21] The utility of capsular polysaccharides as vaccines is further limited by the fact that elderly persons and the those who are immunocompromised react poorly to plain polysaccharides,[22] and some polysaccharides, most notably the α-(2→8)-linked polymer of *N*-acetylneuraminic acid of group B *N. meningitidis,* are poor immunogens in humans of all ages.[23]

Based on Landsteiner and Lampl's[24] recognition that nonimmunogenic small molecules (haptens) may be rendered immunogenic by their covalent coupling to proteins, Avery and Goebel[25,26] showed in the 1930s that a conjugate of type-3 pneumococcal capsular polysaccharide with horse-serum globulin elicited anti-polysaccharide-specific antibodies in rabbits that conferred both active and passive immunity against the homologous organism. Neither the purified polysaccharide alone nor the intact bacterial cell from which it was obtained elicited polysaccharide-specific antibodies in rabbits. As a pioneering development in antibacterial immunoprophylaxis, a tetanus toxoid conjugate of the capsular material of the gram-negative bacterium *Haemophilus influenzae* type b (Hib) was prepared.[27] This conjugate offeres protection against infection by this pathogen, which is a major causative organism of childhood meningitis in young children, with high morbidity and mortality even with antibiotic therapy. Successors of this vaccine may be used in infants at the age of 2 months, who are at greatest risk to infection, to induce protective levels of humoral IgG antibodies against Hib. In countries where these vaccines are routinely used, cases of infant meningitis caused by Hib are virtually eliminated.[28] The spectacular success of the Hib vaccines in controlling a major childhood disease stimulated development of conjugate vaccines consisting of the capsular polysaccharides of numerous other bacterial pathogens. These include *Str. pneumoniae,*[29] *N. meningitidis,*[30,31] group B *Streptococcus,*[32,33] *Salmonella typhi,*[34] and *Staphylococcus aureus*.[35]

The O-specific polysaccharide components of the lipopolysaccharides of gram-negative bacteria fulfil an immunological role similar to that of the capsular

polysaccharides in that they are essential for bacterial virulence and express serotype specificity.[36–39] The purified O-specific polysaccharides, when devoid of Lipid A, are nontoxic, have no known pharmacological effects, and because of their low molecular weight (usually less than 30 kDa), they are not immunogenic. Similarly to other haptenic materials, they can be rendered immunogenic by covalent attachment to proteins. It has been proposed that serum antibodies against the O-specific polysaccharides of gram-negative enteric bacteria (e.g., *Salmonella*[40–43] and E. coli[44]) confer serotype-specific immunity, and it has been documented[10,44–49] that protection against infection by enteric bacteria correlates with the level of anti-O-specific polysaccharide-specific antibodies. Based on the hypothesis that a critical level of anti-O-specific polysaccharide-specific serum IgG antibody may protect against infection by the homologous organism by neutralizing the inoculum, a new generation of conjugate vaccines is being developed that contain either detoxified lipopolysaccharides or purified O-specific polysaccharides covalently linked to a bacterial toxoid.[10,48] These experimental vaccines were safe and immunogenic when tested in animals or humans, elicited anti-O-specific polysaccharide-specific IgG antibodies, and provided statistically significant protection against the homologous organism in human trials.[10]

Because of the poor immunogenicity of plain polysaccharides in the most vulnerable populations, carbohydrate vaccine technology in the future will focus on the design and synthesis of conjugate vaccines in which bacterial cell-surface carbohydrates or analogs are covalently linked to immunogenic proteins or their derivatives. In clinical trials, a vaccine is considered successful if it elicits high levels of anti-polysaccharide antibodies with an increased IgG/IgM ratio that can be boosted by a subsequent injection even in young infants, the immunocompromised, and the elderly.[50] Improvement of the current vaccines requires a better understanding of the role of the submolecular properties of glycoconjugates that influence the immune response. These include the length of the saccharide chain, the importance of the non-reducing terminal saccharide unit, the distance between the saccharide and the protein, the coupling chemistry, and the number of saccharide chains per protein molecule. Several studies indicate that, in glycoconjugate vaccines, the length of the saccharide chain may be less important than in plain polysaccharides. For example, albumin conjugates of dextran fragments obtained by depolymerization of the native dextran are immunogenic in animal models.[51] Conjugates of small dextran molecules (MW $\leq$4 kDa) were better immunogens in mice than those of large ones ($\leq$40 kDa).[50,52] Interestingly, chicken serum albumin conjugates of dextran-related oligosaccharides containing only 3–5 glucose units were more immunogenic than conjugates of dextran having MW ~40 kDa.[52] It should be noted that comparisons of these results should take into account the methods of conjugation: while the small oligosaccharides were coupled through their reducing end to chicken serum albumin, therefore exposing the non-reducing terminus and leaving the entire saccharide chain available for interaction with the B-cell

receptors, the dextran conjugates were prepared by multiple-point attachment, probably providing a network of undefined structure in which the available unchanged saccharide length is not known. In a study aimed at defining the saccharide length necessary for polysaccharide-specific immune response, tetanus toxoid conjugates of fragments of the capsular polysaccharide of type III group B *Streptococcus* were prepared. It was found that the conjugate of a fragment representing only $\frac{1}{10}$ of the native polysaccharide elicited anti-polysaccharide antibodies in rabbits, while the unconjugated native polysaccharide was nonimmunogenic.[53] In the Hib polysaccharide system, the extent of saccharide loading (saccharide molecules/protein) was more critical than other structural variables, including the chain length.[54] A similar trend was reported for capsular polysaccharide fragments of several *Str. pneumoniae* serotypes.[55]

The literature on bacterial polysaccharides as vaccine components has been reviewed in this series by Jennings.[56] Since then, the subject has been reviewed by Ala'Aldeen and Cartwright,[57] Dick and Beurret,[58] Dintzis,[59] Egan,[60] Goldblatt,[61] Jennings,[62–65] Lee,[66] Fattom,[35,67] Paradiso and Lindberg,[68] Peeters,[69] and Robbins.[10,45–47]

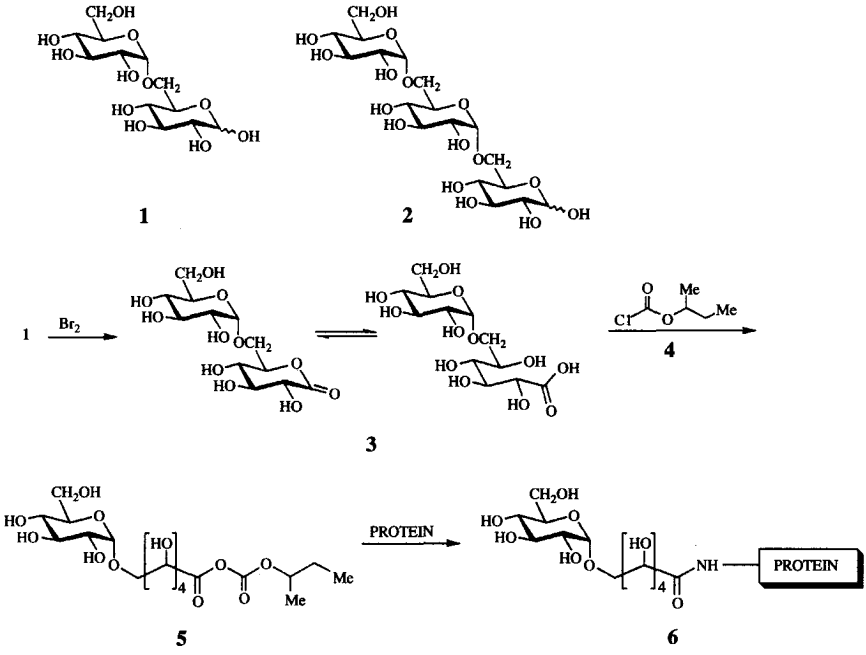
This chapter covers reports on the evaluation of the immunogenicity of protein conjugates of oligosaccharide components of bacterial cell-surface polysaccharides. Syntheses of representative oligosaccharides and their conjugation are included to illustrate the various chemical approaches used for the preparation of the synthetic immunogens. "Oligosaccharide" mixtures obtained by degradation of native polysaccharides and not purified to homogeneity are not covered. Oligosaccharides corresponding to mycobacterial polysaccharide antigens have recently been reviewed by Aspinall[70] and by Lipták.[71] Synthetic lipid A and its analogs have been reviewed by Rietschel.[38,39,72] Detailed description of the clinical relevance of individual pathogens can be found in the reviews just referred to.

## II. Synthesis and Immunological Evaluation of Protein Conjugates of Oligosaccharide Components of Bacterial Polysaccharides

### 1. Studies on Dextran-Related Oligosaccharide–Protein Conjugates

Although dextrans are not candidates for bacterial immunoprophylaxis, they represent simple, easily available, and useful models to study aspects of carbohydrate immunogenicity that may be relevant to the design of oligosaccharide-based conjugate vaccines. Structurally, native dextrans are homopolymers of α-(1→6)-linked D-glucopyranose residues that have α-(1→2), α-(1→3), and α-(1→4)-linked branches of varying length.[21,73,74] Kabat[75–77] and Richter and Eby[78] studied the minimal molecular requirements for antidextran antibody induction using protein and lipid conjugates of various epitope patterns of linear and branched dextrans. In Kabat's[75] approach, isomaltose (**1**) and isomaltoriose (**2**), prepared by

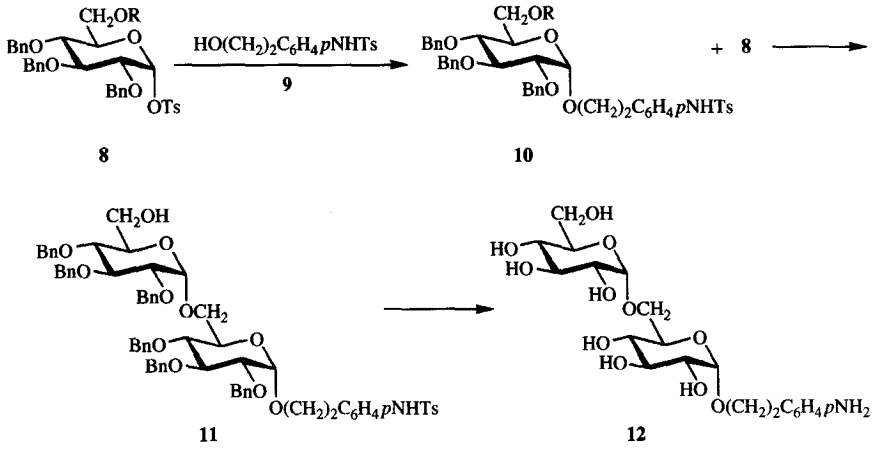
controlled acid hydrolysis of native dextrans, were covalently linked to BSA by the aldonic acid route using the mixed anhydride method. Thus, the disaccharide **1** was oxidized to an equilibrium mixture of the free and lactonized aldonic acid derivative **3** that was activated with isobutyl chloroformate **4** (→**5**) followed by condensation with the protein to afford the conjugate **6.** The BSA conjugates so prepared contained 8 and 14 moles of α-linked glucose and isomaltose, respectively, attached to the ε-amino group of the protein's lysine residues through the polyhydroxy spacer derived from the reducing-end glucose residue of the original di- and trisaccharides.[75] Although the antibodies raised in rabbits against the former conjugate having only one intact D-glucose residue in the hapten part did not precipitate dextran, the antibodies formed toward the disaccharide hapten exhibited extensive precipitation with native dextran. Dextran-precipitating antibodies were likewise elicited in mice and rabbit by protein conjugates of linear dextran fragments up to the hexasaccharide **7.**[76,79,80] An interesting finding in these early studies was that the linear dextran-derived di- to hexa-saccharides were able to inhibit precipitation of the antidextran antibodies by the homologous dextran as a function of their size: in the range studied, the linear hexasaccharide **7** was the best inhibitor.[81] Although dextrans offer a large reservoir of the various




α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→6)-]$_4$α-D-Glc

**7**

oligosaccharide epitopes, difficulties in specific degradation and isolation of the targeted oligosaccharides make chemical synthesis a better approach to sufficient amounts of the required fragments. Eby and Schuerch[82–84] synthesized protein conjugates of a range of dextran fragments that represent various epitope patterns of native dextrans. A typical synthetic sequence[82] started from the glucosyl donor[85] **8** that was condensed with the spacer **9** followed by deprotection. Subsequent glycosylation of compound **10** with the donor **8** afforded **11,** from which removal of the protecting groups provided isomaltose **12** having a terminal free amino group in its aglycon.




Dextran fragments prepared by chemical synthesis and their protein conjugates obtained either by the diazo coupling[25] or by the isothiocyanate protocol[86] (**13–23**) are listed in Table I. In the diazo-coupling method, a *p*-aminophenyl glycoside **24** is converted into the diazo intermediate **25,** which reacts with the protein to form conjugate **26.** More efficient conjugation was achieved with the isothiocyanate coupling procedure. In this approach the aminophenyl glycoside **24** is treated with thiophosgene to afford the reactive isothiocyanate **27,** which reacts with the ε-amino groups of the lysine residues by forming a stable thiourea linkage in the conjugate **28.** Richter and Eby[78] found that the conjugates listed in Table I induced anti-dextran antibodies in rabbits when injected at weekly intervals up to four times, using 1 mg of the conjugate for each injection. In agreement with Kabat's[75] findings they demonstrated that a single glucosyl residue in not sufficient for inducing dextran-reactive antibodies in the rabbit model.[78] The formation was noted of antibody populations that are directed at distinct glucosyl epitopes having α-(1→2), α-(1→3), and α-(1→6) linkages. The failure of conjugate **21** to induce an antibody population to the α-(1→4) glucosyl epitope may be interpreted as a consequence of the presence of this linkage in glycogen, a self-polysaccharide of

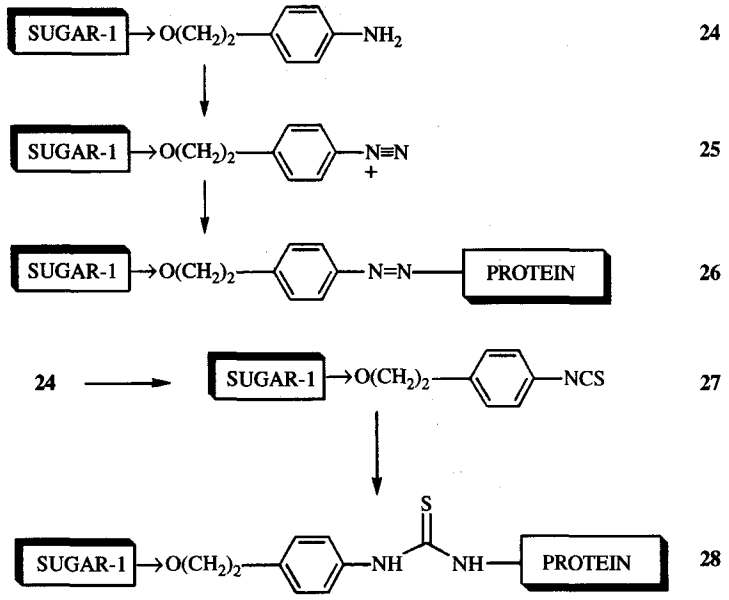
TABLE I

**Protein Conjugates of Synthetic Oligosaccharide Fragments of Dextrans**

| Conjugate Number | Oligosaccharide Structure | Coupling Method[a,b] | Carrier Protein | Saccharide/ Protein Ratio (mol/mol) |
|---|---|---|---|---|
| **13** | α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | Diazo | Edestin | 8.4 |
| **14** | α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | Diazo | Edestin | 10 |
| **15** | α-D-Glc*p*-(1 → 6)-[α-D-Glc*p*-(1 → 6)]$_2$-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | Diazo | Edestin | 7.5 |
| **16** | α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | Diazo | BSA | 4.4 |
| **17** | α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | Diazo | BSA | 2 |
| **18** | α-D-Glc*p*-(1 → 6)-[α-D-Glc*p*-(1 → 6)]$_2$-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | Diazo | BSA | 1 |
| **19** | α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | NCS | BSA | 7.8 |
| **20** | α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | NCS | BSA | 19 |
| **21** | α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | NCS | BSA | 18 |
| **22** | α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | NCS | BSA | 32.31 |
| **23** | α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1 → O-$(CH_2)_2C_6H_4NH_2$ | NCS | Hemocyanin | 800 |

[a] Diazo coupling; see ref. 25.

[b] For the NCS (isothiocyanate) coupling, see ref. 89.

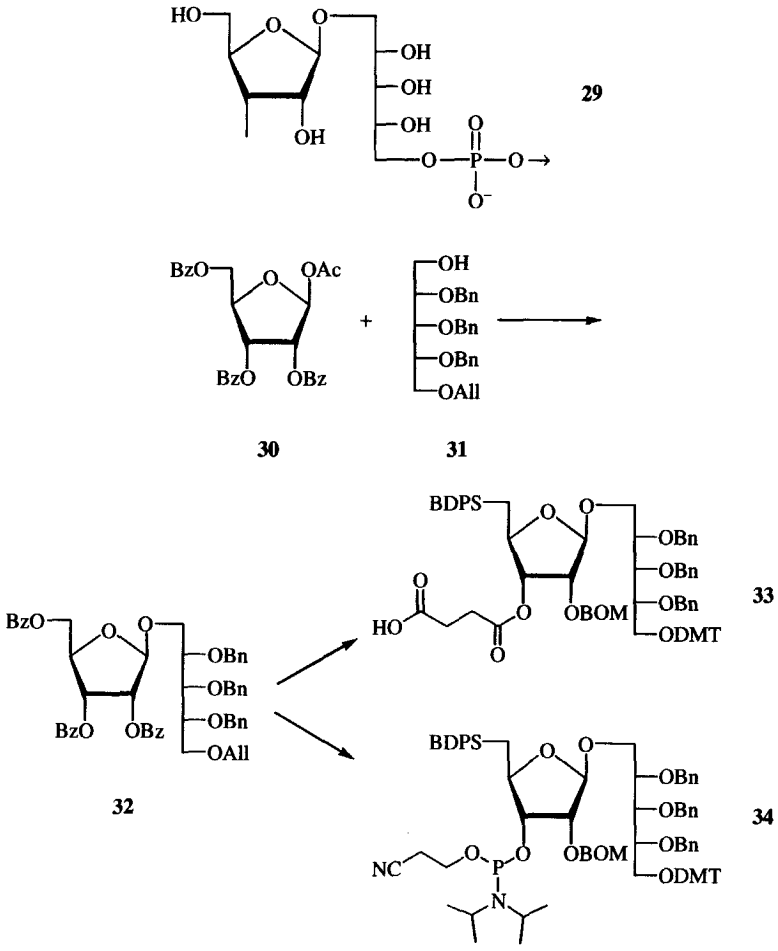



the host. Antiserum induced by conjugates **17** and **18,** which contain an average of two and one saccharide chains per conjugate, respectively, failed to induce immunoprecipitation when mixed with the homologous conjugates. However, conjugate **16,** having only four saccharide chains per BSA, was able to precipitate antidextran antibodies. This finding supports the hypothesis that immunoprecipitation occurs as a consequence of formation of a supramolecular chain between the antibody and the antigen moieties.[78] Interestingly, conjugate **22,** containing an average of 32 saccharide chains per BSA, was only weakly immunogenic. This observation, combined with the low immunogenicity of the conjugates having low saccharide loadings, led to the conclusion that 10–25 oligosaccharide chains per BSA are optimal for eliciting anti-carbohydrate antibodies.[78]

## 2. Protein Conjugates of Oligosaccharide Fragments of Capsular Polysaccharides

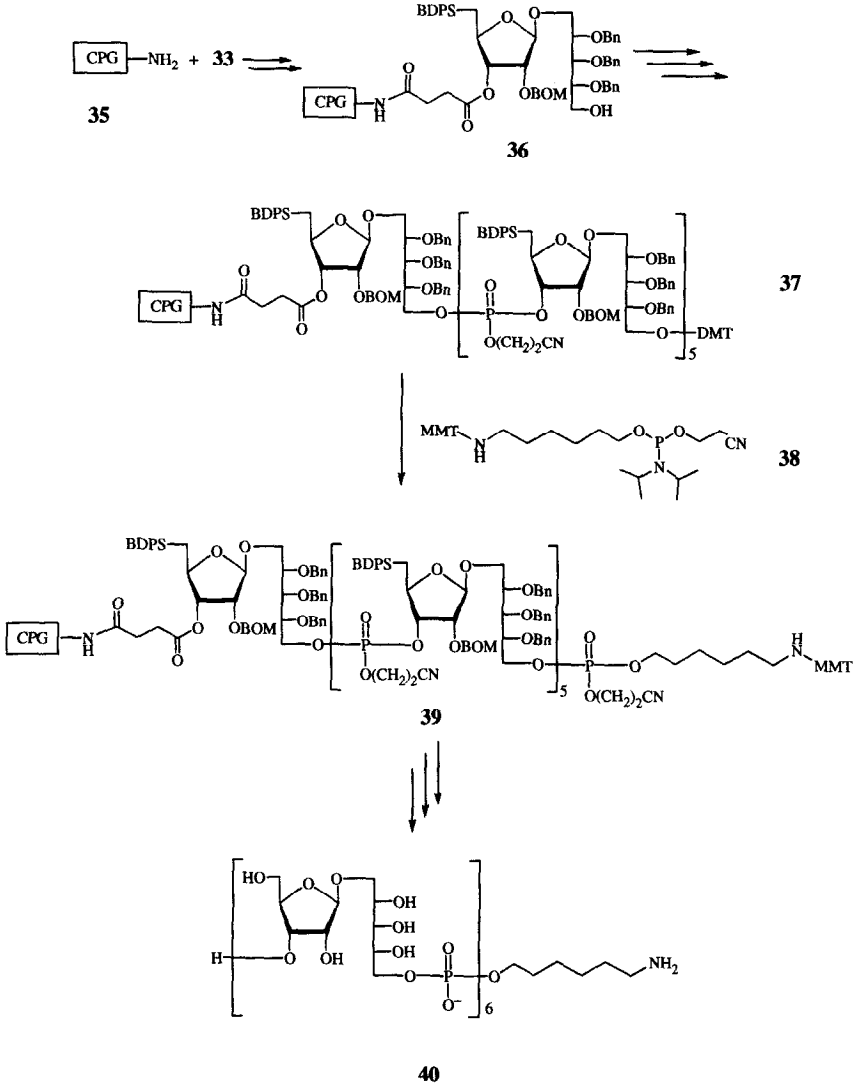
**a.** ***Haemophilus influenzae*—*H. influenzae*** **type b.**—There are six capsulated types of this organism,[87] of which type b (Hib) causes almost all systemic infections, the most dangerous of which is meningitis.[45] The capsular material of Hib is a polymer of the ribofuranose—ribitol—phosphate repeating unit **29** (polyribose—ribitol—phosphate, PRP). Monomers,[88,89] dimers,[90–96] trimers,[90,95–97] a tetramer,[96] pentamers,[94,96–99] hexamers,[94,97,100] a decamer,[101] and an analogue[102] of the pentamer of **29** have been synthesized. In van Boom's solid-phase

approach,[92,100] the key starting ribosyl—ribitol moiety **32** was assembled from compounds **30** and **31.** Next, the ribose–ribitol construct **32** was transformed to the succinylated derivative **33** and to the phosphoramidite **34.** Compound **33** was




immobilized to amino group-functionalized, controlled-pore glass (**35**), followed by the removal of the protecting group at O-5 of the ribitol moiety to afford **36.** Iterative, multistep chain-extension using the phosphoramidite moiety **34** afforded the hexamer (**37**) of the repeating unit. The synthesis was terminated by the attachment of the aminohexyl linker **38** (→**39**) that served as a spacer between the carbohydrate chain and the protein to minimize steric hindrance. Subsequent

release of the oligomers from the solid support and removal of the protecting groups afforded the hexamer of the repeating unit (**40**). Using a similar approach, Kandil[97] prepared oligomers of the Hib capsular material up to a hexamer of the repeating unit **29** and attached them to tetanus toxoid (TT) and to synthetic T-helper cell epitope peptide fragments **41–43** of the outer membrane proteins

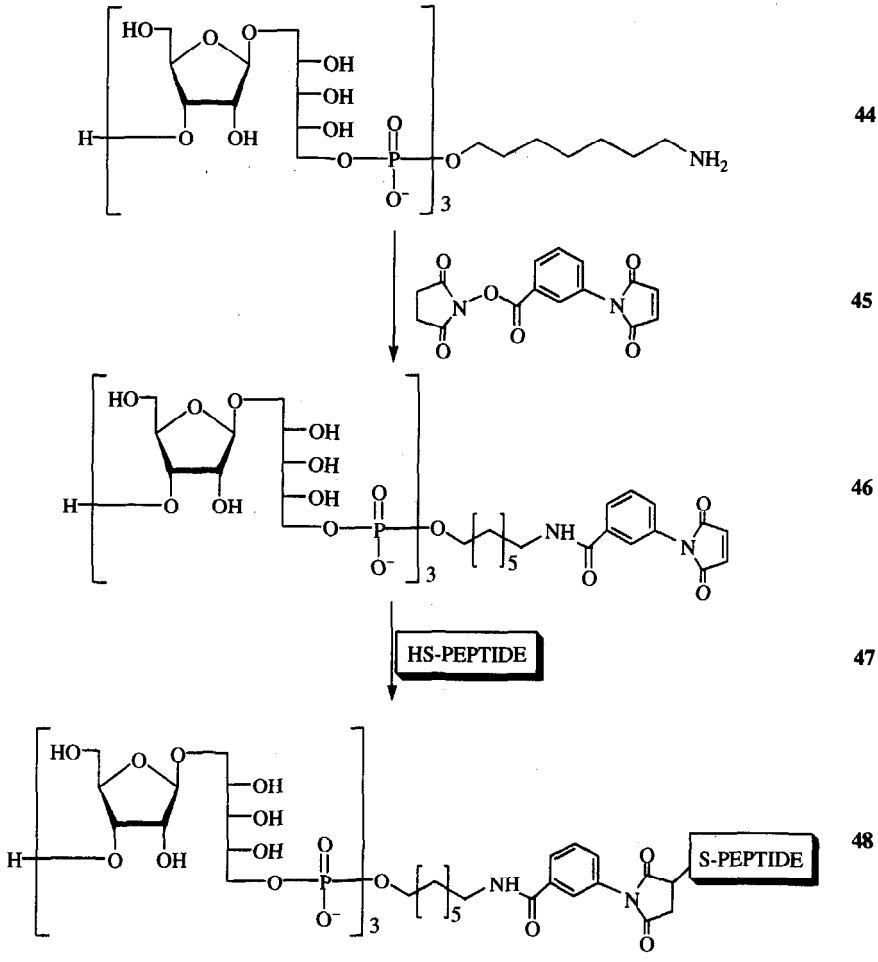


CYAKAQVERNAGLIADSVKDNQITSALSTQ **41**

CDIVAKIAYGRTNYKYNESDEHKQQLNG **42**

VKGYLAGYLAGKGVDAGKLGTVSYGC **43**

of Hib.[94] In the conjugation process, the saccharide–spacer construct **44** is treated with the heterobifunctional linker **45** to introduce a maleimidoyl group, through which the intermediate **46** so obtained is attached to the sulfhydryl group of immunogenic peptide or protein **47** to afford the conjugate **48.** In the rabbit model,




**44**

**45**

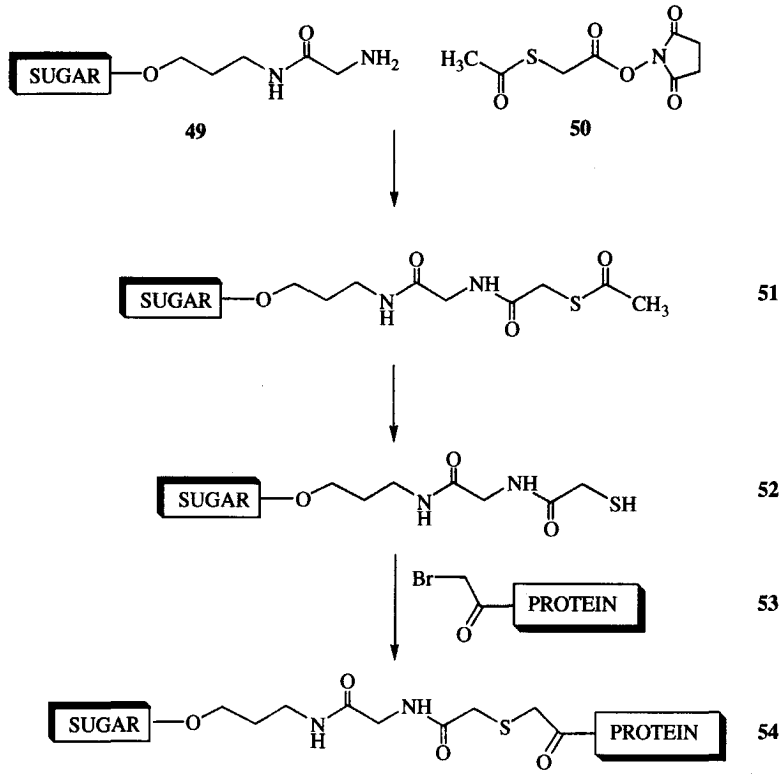
**46**

**47**

**48**

the TT conjugate of the trimer of **29** elicited antibody responses. The conjugate of the trimer with peptide **41** elicited protective antibody responses in rabbits comparable to those obtained with the first-generation commercial conjugate vaccine ProHibit (Connaught) when formulated with alum.[103] The immunogenicity of the saccharide–peptide conjugates was dependent on the site of attachment on the peptide: the anti-saccharide antibody responses were higher for conjugates in which the hapten was linked at the N-terminus (peptides **41** and **42**). This finding was interpreted in terms of the inability of the antigen-presenting cells to cleave the T-cell epitope peptide from the conjugates in which the saccharide components were attached to the C-terminus of the peptide. A similar observation was reported for T-cell epitope peptide conjugates of a pneumococcal polysaccharide.[104] In rabbits, the constructs containing the dimer of **29** attached to T-cell epitope peptides[103] or to proteins were weakly immunogenic.[105] In the range studied the highest antibody responses were observed with peptide conjugates of the synthetic trimer of **29.** Surprisingly, conjugates of the pentamer and the hexamer of **29** with the peptides **41–43** failed to elicited antibody levels higher than those observed with the conjugates of the trimer. This finding has been interpreted as being due to an adverse effect of the hapten moiety on the antigen processing and by its steric hindrance, which may mask the epitope determinants on the peptide.[103] The immunogenicity of the glycopeptide conjugates was enhanced in rabbits when multiple copies of the T-cell epitope peptide—saccharide constructs were presented on a polylysine backbone. The anti-polysaccharide IgG titers obtained with these constructs were comparable to or higher than those obtained with the commercial vaccine ProHibit.[103]

Diphtheria toxin and TT conjugates of synthetic di- to tetra-mers of the repeating unit **29** were prepared using either the thioether chemistry[105] or a random activation approach employing glutaric dialdehyde as the linker.[106] In the first phase of the thioether approach, a sulfhydryl group is introduced in hapten **49** by N-acylation with the active ester **50** (→**51**) followed by hydroxylamine treatment. *In situ* reaction of the thiol **52** so prepared with the N-bromoacetylated carrier protein **53** affords the conjugate **54,** having stable thioether and amide linkages between the sugar and the protein components. The composition of the Hib oligomer–protein conjugates prepared by the thioether approach is shown in Table II. In mice, the TT conjugate of the trimer prepared by the random activation approach induced higher primary and secondary responses than the conjugate of the dimer.[106] Similarly, the TT conjugate of the tetramer (**58**) made by the thioether chemistry elicited consistently higher IgG titers than the trimer conjugates **55** and **56** after both the first and second immunizations. In monkeys, the tetrameric conjugate **58** and the licensed vaccine HibTiter, which contains an average of 20 repeating units, were equally immunogenic, whereas the TT conjugate **56** containing the trimer was less




efficient, notwithstanding its higher saccharide/protein ratio.[105] Tetanus toxoid and meningococcal outer-membrane protein conjugates of a synthetic pentamer of **29** elicited anti-Hib polysaccharide antibodies in male mice at a level higher than did the Hib polysaccharide–diphtheria toxoid conjugate.[99] Using conjugates containing 4–12 repeating units of the Hib capsular material and a genetically toxoided mutant (CRM197, a cross-reacting mutant) of DTx, Anderson *et al.*[54] concluded that in humans the length of the hapten and its exposed terminus are less critical for anti-polysaccharide immunity than the extent of the hapten loading of the conjugate.

**b.** ***Streptococcus*—Group A.**—The cell-wall polysaccharide of the gram-positive group A *Streptococcus* is a polymer of the branched trisaccharide **59.** Pinto and others[107–113] reported chemical synthesis of a number of its di- to hexa-saccharide components with and without a linking moiety, as well as their protein conjugates. Typical features of these syntheses are illustrated in the construction of the pentasaccharide **70,** for which di- and a tri-saccharide blocks were first constructed.[109]

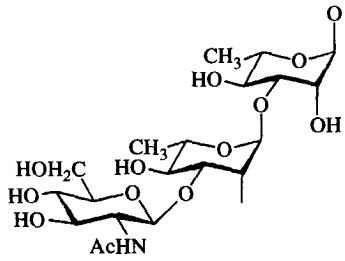
TABLE II

**Toxoid Conjugates of Trimers and Tetramers of the Hib Polysaccharide[105] 29**

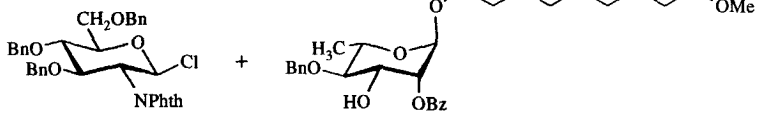
| Conjugate Number | Conjugate Composition[a] | Saccharide/Protein Ratio (mol/mol) |
|---|---|---|
| **55** | $(RRP)_3$-DTx | 9.9 |
| **56** | $(RRP)_3$-TT | 21 |
| **57** | $(RRP)_4$-DTx | 6.5 |
| **58** | $(RRP)_4$-TT | 5.3 |

[a] RPR, ribose–ribitol–phosphate; DTx, diphtheria toxin; TT, tetanus toxoid.

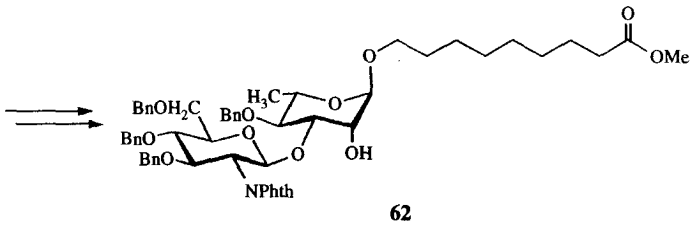
Thus, the O-benzylated glucosamine derivative **60** was condensed with the spacer-linked L-rhamnoside **61,** followed by the removal of the base-labile protecting group at the site of the chain extension to afford the disaccharide acceptor **62.** The trisaccharide block **67** was prepared in a similar approach. Thus, condensation of the glucosamine derivative **63** with rhamnoside **64** afforded the disaccharide




**59**

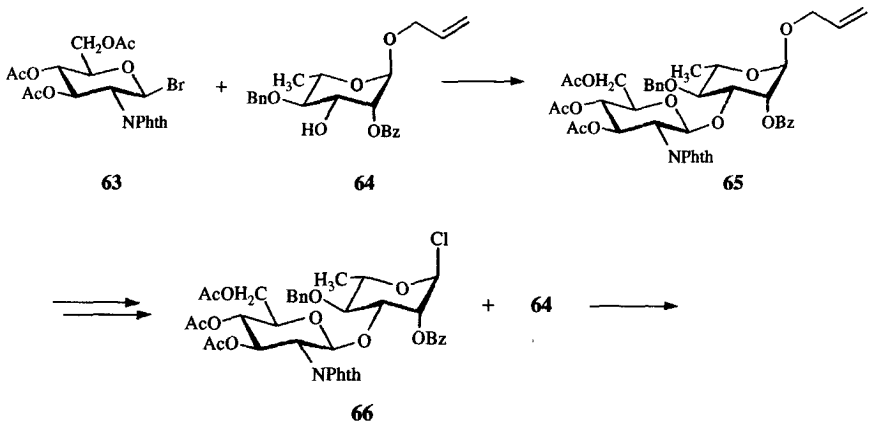



**60** **61**




**62**

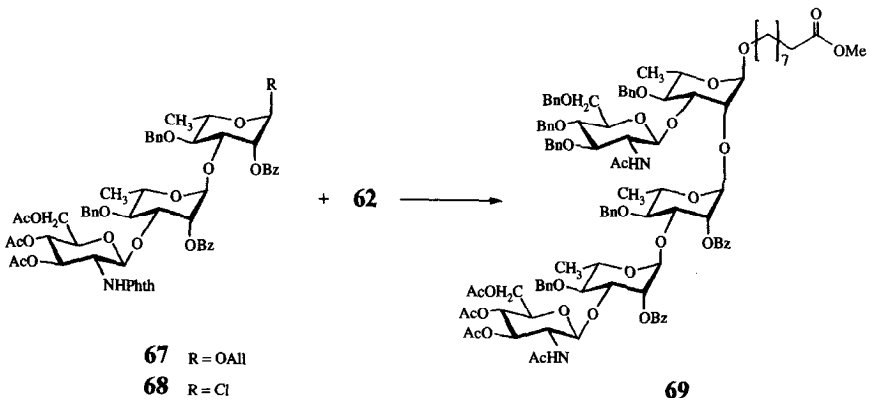
**65** in which the allyl group was replaced by chlorine to afford the glycosyl donor **66.** This was condensed with a second rhamnose unit **64** to provide the




trisaccharide **67.** Next, compound **67** was converted into the glycosyl chloride **68,** which was condensed with the disaccharide block **62** to afford the pentasaccharide **69,** and this was deprotected (→**70**) with subsequent conversion into hydrazide **71.** Covalent attachment of the pentasaccharide **71** to BSA was achieved by the acyl azide method[114] involving treatment of a hydrazide (**72**) with $N_2O_4$ to form an intermediate acyl azide (**73**), which is allowed to react *in situ* with BSA to afford glycoconjugate **74.** BSA conjugates of the frame-shifted trisaccharide repeating-units **75** and **76** were also prepared, having an average incorporation level of 8–18 mol of saccharides per mol of BSA.[115] Polyclonal antibodies, elicited in rabbits by repeated injections of the conjugates prepared in Freund's complete adjuvant, were highly specific for the homologous immunogen. Antibodies to the linear trisaccharide **75** were equally well recognized by the homologous trisaccharide **75** and by the pentasaccharide **71,** whereas they reacted poorly with the branched trisaccharide **76.** Because of the presence in the pentasaccharide **71** of both epitopes present in the trisaccharides **75** and **76,** polyclonal antibodies raised against the trisaccharide conjugates equally well recognized the branched pentasaccharide antigen. Only the antibodies raised against the pentasaccharide conjugates but not the trisaccharide conjugates recognized the native group A streptococcal polysaccharide, indicating the importance of both the size and the presence of the branching epitope that in this system appears to be essential for antibody recognition.

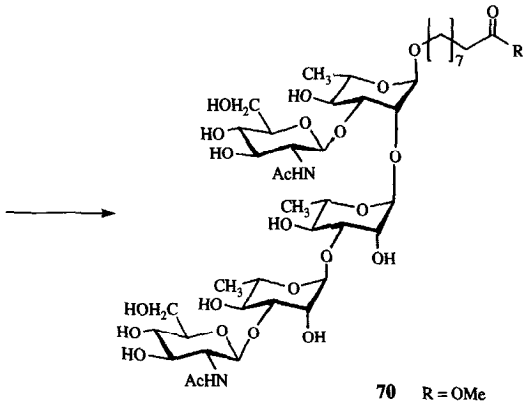
**c.** ***Streptococcus—Str. pneumoniae*** **Type 2.**—The capsular polysaccharide (**77**) of this bacterium contains α-linked glucuronic acid as the terminal residue of the

side-chain disaccharide.[116] When studying the structural requirements of disaccharides necessary for eliciting anti-type 3 pneumococcal antibodies, Goebel[117] synthesized the *p*-aminobenzyl glycoside of gentiobiuronic acid, a positional isomer of cellobiuronic acid whose presence in the type 3 pneumococcal

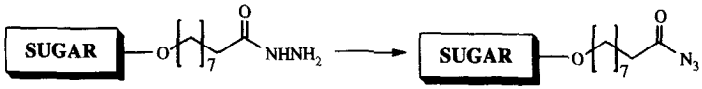



**67** R = OAll
**68** R = Cl

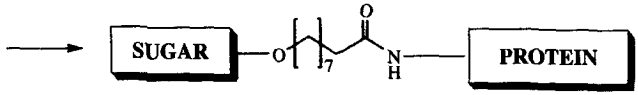
**69**



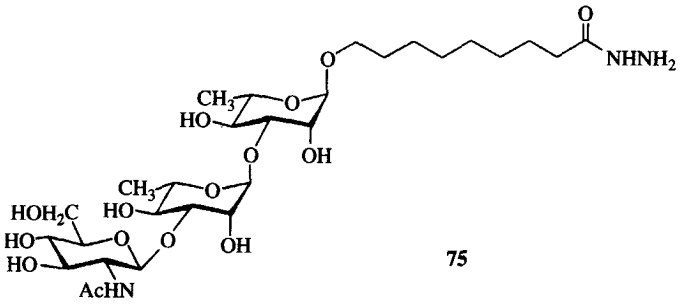

**70** R = OMe
**71** R = $NHNH_2$



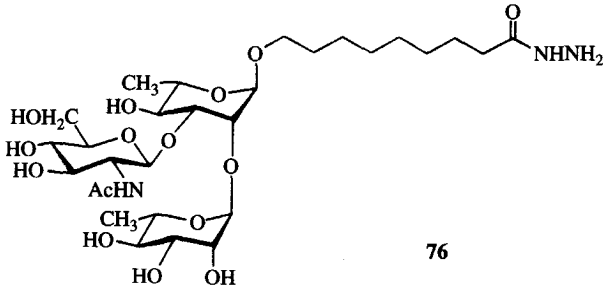

**72** **73**




**74**



75



76

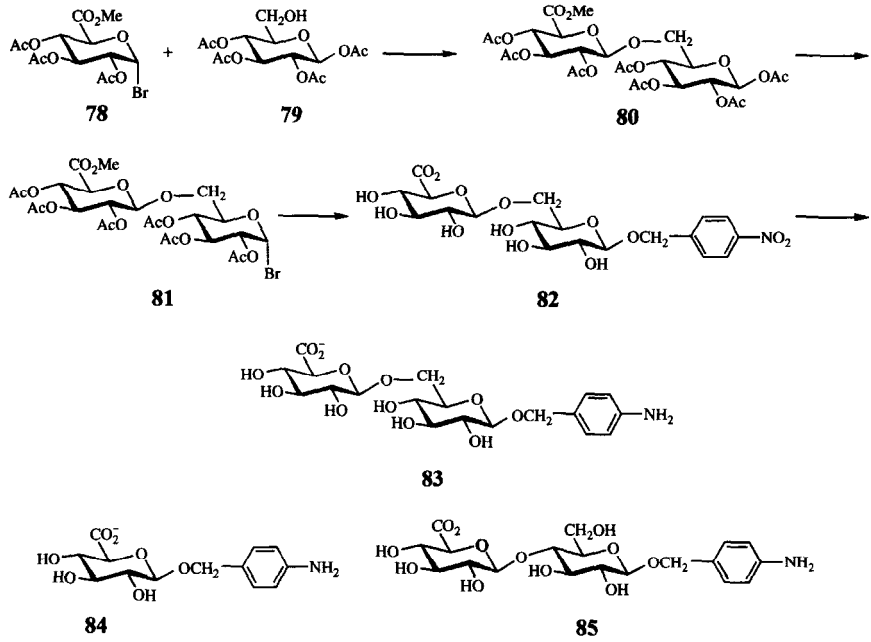
3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→

2
1

α-D-Glc*p*A-(1→6)-α-D-Glc*p*

77

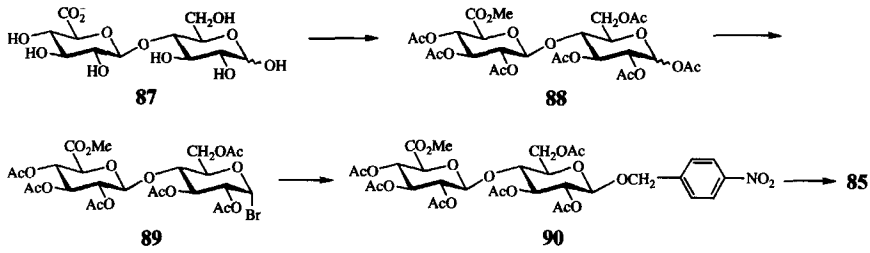
polysaccharide was already established at that time. Thus, condensation of the methyl ester **78** and the tetraacetate **79** afforded the disaccharide **80,** which was converted into the glycosyl bromide derivative **81.** Sequential reaction with *p*-nitrobenzyl alcohol followed by removal of the protecting groups yielded the disaccharide glycoside **82,** which was subjected to catalytic reduction to afford the *p*-aminobenzyl derivative **83.** Horse globulin conjugates of gentiobiuronic acid **83,** glucuronic acid **84,** and cellobiuronic acid **85** prepared by the diazo method elicited antibodies in rabbits (without an adjuvant) that precipitated the homologous antigens linked to an unrelated carrier protein.[117] Rabbit anti-gentiobiuronic acid sera conferred passive protection in mice against challenge by type 2 pneumococcus, despite the unnatural anomeric configuration of the uronic acid moiety in the conjugate. This remarkable observation was the first to show that even a monosaccharide can act as a hapten in a protein conjugate to elicit protective antibodies. Interestingly, no agglutinating activity was observed in the sera of rabbits immunized to the gentiobiuronic acid conjugate with either type 2 or type 3 pneumococci. This study



concluded that the protection is due to antibodies directed toward the glucuronic acid moiety in the conjugates, and correctly established the presence of a glucuronic acid residue in the type 2 pneumococcal polysaccharide 35 years before the structure of this polysaccharide was elucidated.

**d. *Streptococcus—Str. pneumoniae* Type 3.**—The first disaccharide conjugated to a protein was derived from the capsular polysaccharide of *Streptococcus pneumoniae* type 3, whose repeating unit is β-(1→3)-linked cellobiuronic acid[117–119] **86.** Controlled acid hydrolysis of the type 3 pneumococcal polysaccharide afforded the disaccharide **87,** which was converted into the methyl ester[120] **88** from which the glycosyl bromide derivative **89** was prepared.[121] Conversion into the *p*-nitrobenzyl glycoside **90** followed by deprotection and catalytic reduction of the nitro group afforded the aminobenzyl glycoside **85,** which was conjugated to horse globulin using the diazo method (p. 8).[118] This material elicited cellobiuronic acid-specific antibodies in rabbits that agglutinated type 3 pneumococcus and precipitated the capsular polysaccharide **86.** The specificity of this reaction was demonstrated by the failure of antibodies raised against horse globulin conjugates of cellobiose, glucose, or glucuronic acid to cross-react with the same polysaccharide.[118] Rabbits immunized with the artificial cellobiuronic acid immunogen developed resistance against challenge by the homologous organism,

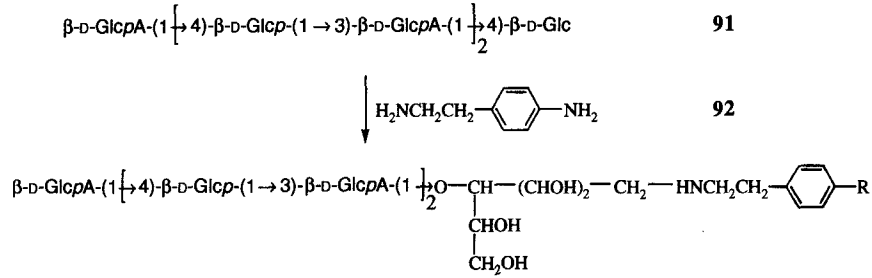
3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→ **86**



**87** **88** **89** **90** **85**

and the rabbit antisera provided passive protection in mice not only against type 3, but also against type 2 and type 8 pneumococci that bear a similar or an identical disaccharide moiety in their respective repeating units. Neither active nor passive immunity could be induced with the corresponding cellobiose conjugate or horse globulin conjugates containing glucuronic acid as the hapten.[119]

In order to study the role of multiple antigenic subunits in oligosaccharide-based conjugate vaccines, Snippe *et al.*[122] isolated a trimer (**91**) of cellobiuronic acid from the type 3 pneumococcal polysaccharide by acid-catalyzed partial depolymerization and condensed it with bovine serum albumin, keyhole limpet hemocyanin, and tetanus toxoid using the method of Svenson and Lindberg.[123] Thus, hexasaccharide **91** was reacted with the amine **92** in the presence of $NaCNBH_3$ to afford the pentasaccharide glycoside **93,** which was treated with thiophosgene. The resulting isothiocyanate **94** was reacted *in situ* with the proteins to yield BSA, KLH, and TT conjugates of the pentasaccharide **93** in which the molar ratio of the saccharide chains to the protein was 9, 6, and 16, respectively. Immunization of mice with the BSA and the KLH conjugates (without an adjuvant) elicited low amounts of



**91**

**92**

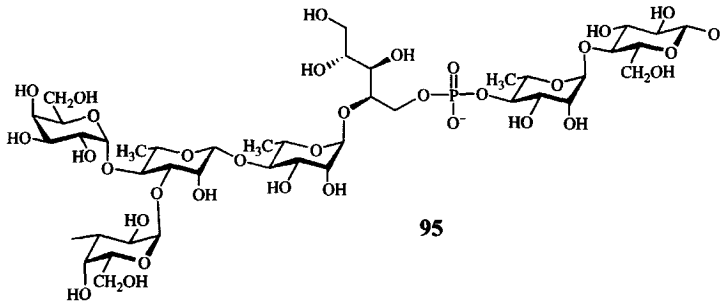
**93** R = $NH_2$

**94** R = NCS

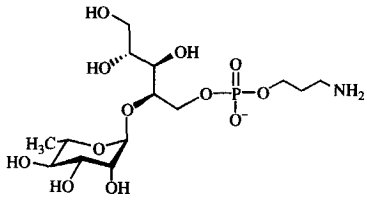
anti-type 3 polysaccharide specific IgM antibodies. The importance of the carrier protein was demonstrated by the observation that although one additional injection only was needed to elicit high amounts of IgG antibodies for the group treated with the KLH conjugate, repeated booster injections were necessary to augment the IgG levels with BSA conjugate. Antisera raised against the native polysaccharide had no detectable levels of IgG antibodies and repeated injections failed to increase the IgM antibody level. After only one injection, the BSA conjugate provided better protection in mice against challenge with live bacteria for 3 weeks after immunization than either the KLH or the TT conjugates. The protection could be enhanced by repeated injections. For example, a single booster injection of the KLH conjugate resulted in complete protection against challenge with 25-fold of mean lethal dose of live bacteria for at least 23 weeks. Passive protection in mice by antisera elicited by the KLH conjugate was superior to that provided by the antisera raised against the unconjugated polysaccharide. Because the antisera raised against the latter contained no or only very low levels of IgG, the passive-protection experiment demonstrated that antisera containing both IgM and IgG antibodies offer better protection than those lacking IgG.

**e. *Streptococcus—Str. pneumoniae* Type 17F.**—To study the immunogenicity of small oligosaccharide fragments of the capsular polysaccharide **95** of type 17F pneumococcus as part of carbohydrate–protein conjugates, Veeneman *et al.*[124] synthesized its overlapping di-, (**96**) tri-, (**97**) and tetra-saccharide fragments (**98**).

In the approach to **98,** condensation of the thiorhamnoside donor **99** with D-arabinitol **100** followed by the removal of the allyl group afforded the alcohol **101** that was phosphitylated with the reagent **102.** Reaction of the resulting phosphoramidite **103** with the disaccharide **104** afforded an intermediate phosphitetriester that was oxidized to afford the phosphodiester **105,** which was deprotected to give **98.** KLH conjugates containing 484, 1017, and 1654 chains of the di-, tri-, and tetrasaccharides, respectively, were prepared by the thioether approach (p. 11).[105] When injected in female mice without an adjuvant, only a low anti-polysaccharide IgG response was obtained. Inclusion of the adjuvant Quil A significantly enhanced the titers for the di- and the trisaccharide, but not for the tetrasaccharide conjugates: the di- and trisaccharide conjugates used with the adjuvant offered protection against challenge with 25-fold mean lethal doses of *Str. pneumoniae* type 17B, whereas no or little protection was provided by the tetrasaccharide conjugate. Surprisingly, the tetrasaccharide conjugate was not antigenic when tested with antibodies raised against the native polysaccharide **95.** This, and the failure of the tetrasaccharide conjugate to induce protecting antibodies was rationalized by a conformation for this unit that is distinctly different from the conformation of this



95



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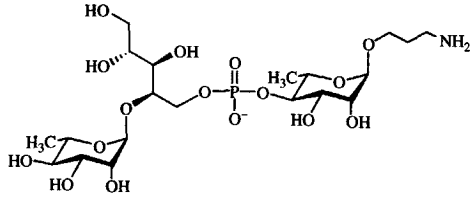

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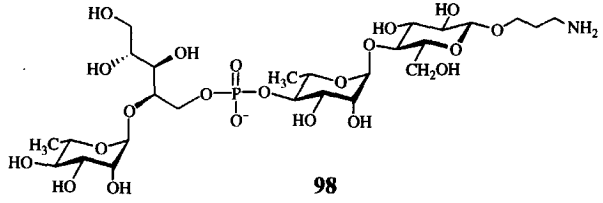
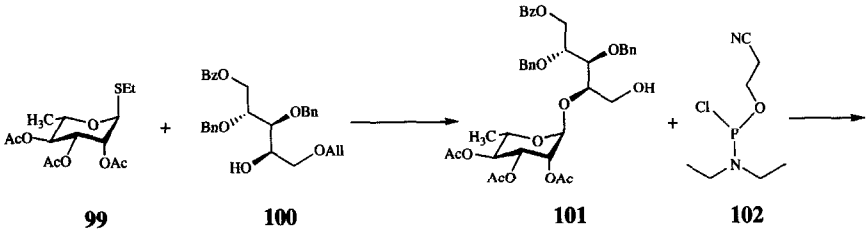


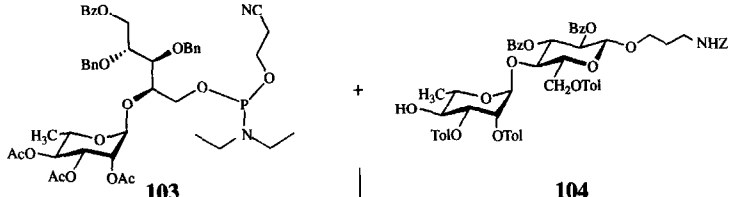
98

sequence in the native polysaccharide and from the partial conformations in its di- and trisaccharide fragments.

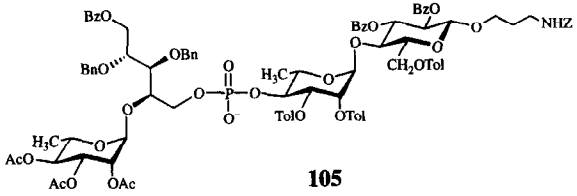
**f.** ***Streptococcus—Str. pneumoniae*** **Type 23F.**—In order to probe the epitope specificity of antibodies induced by oligosaccharide fragments of capsular polysaccharides, de Velasco *et al.*[125] studied the immunogenicity of synthetic tri- and tetra-saccharide fragments[126,127] **107, 108** of the type 23F pneumococcal polysaccharide **106** as part of KLH conjugates. In the synthesis of **108,** the key compound was the tetrasaccharide thioglycoside **113** that was assembled from the monosaccharide precursors **109–112.** Next, this intermediate was coupled with the spacer **114** followed by regioselective deprotection to afford the alcohol **115,** which was coupled with glycerol phosphonate **116.** Oxidation of the resulting intermediate afforded the phosphodiester **117,** which was subjected to deprotection steps to afford the aminopropyl glycoside[126] **108.** Covalent attachment of **107** and **108** to keyhole limpet hemocyanin was achieved by using thioether chemistry to afford conjugates that contained an average of 940 and 1163 chains of the tri- (**107**) and



**99** **100** **101** **102**



**103** **104**



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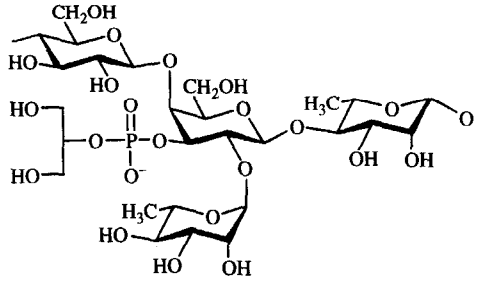
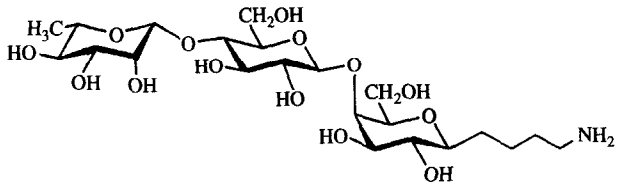
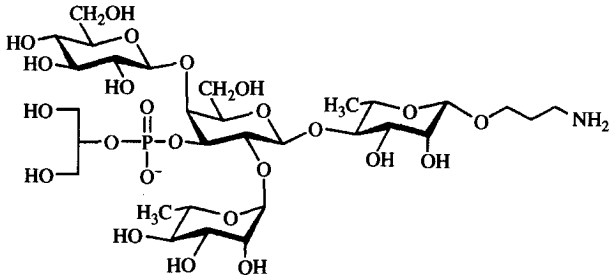


**106**



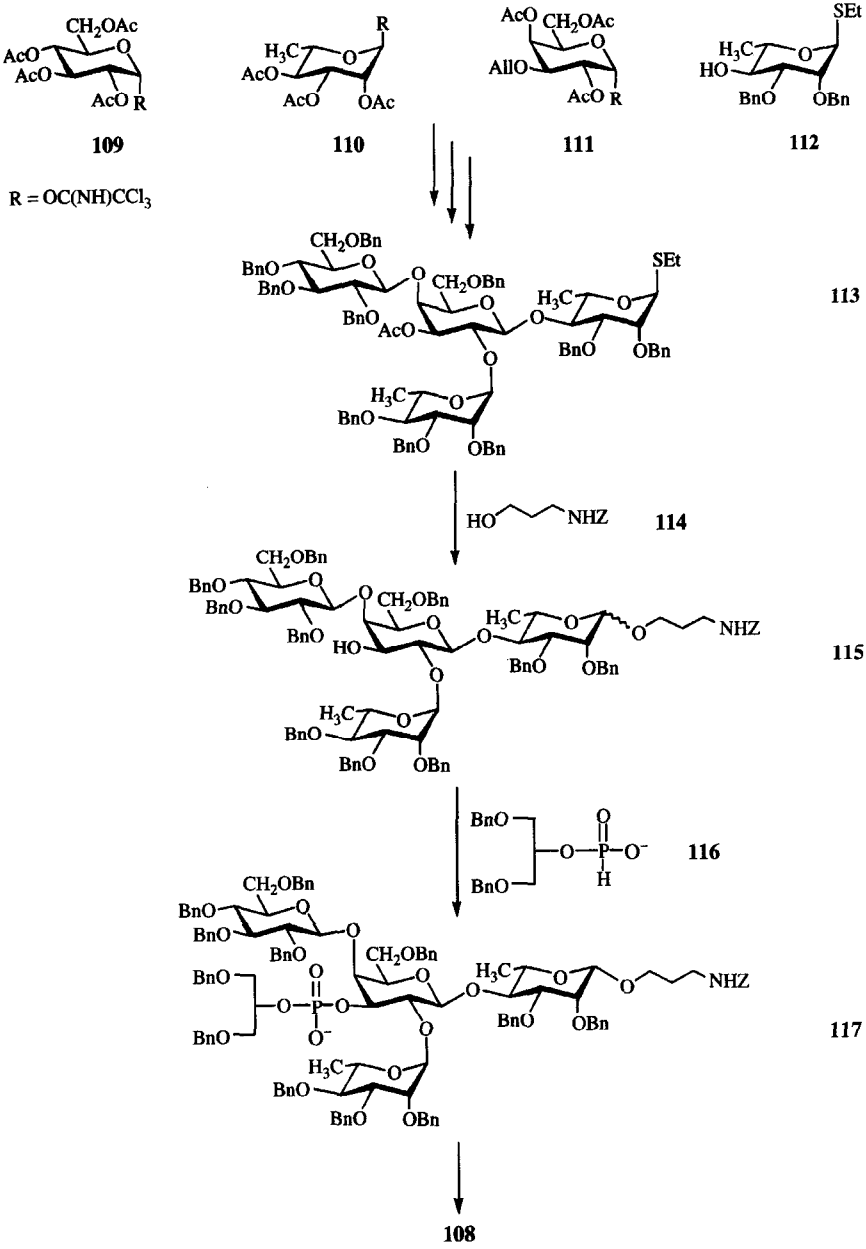
**107**

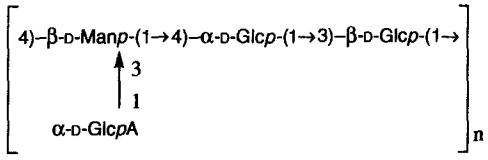


**108**

tetra-saccharide (**108**) per KLH molecule, respectively.[125] In rabbits, the tetrasaccharide conjugate elicited anti polysaccharide-specific antibodies when injected in Freund's complete adjuvant, whereas no antibody could be elicited in mice with the same conjugate. The weak immunogenicity of the trisaccharide conjugate in rabbits was attributed to the lack of the side-chain rhamnose epitope. That this unit was the immunodominant residue was demonstrated by inhibition experiments: The interaction between antibodies raised in rabbits against the native polysaccharide, its KLH conjugate, or the tetrasaccharide conjugate and the homologous polysaccharide were equally well inhibited by L-rhamnose regardless of the immunogen, indicating no major differences in the epitope specificity of antibodies raised against the native polysaccharide and the synthetic tetrasaccharide. In contrast to the rabbit immunesera, interaction between the native type 23F polysaccharide and human sera raised against this polysaccharide could only moderately be inhibited by oligosaccharides and not at all by monosaccharides, leading to the suggestion that human IgG possesses a larger antibody binding-site than the rabbit IgG. Alternatively, the human anti-polysaccharide antibody may recognize a conformational epitope present on the polymeric form of the repeating unit but not on its oligosaccharide fragments. This study was the first that documented the need for evaluating experimental vaccines in humans by demonstrating the differences in epitope specificity between the rabbit and the human anti-polysaccharide antibodies.[125]

**g. *Klebsiella*—Type 2.**—A series of monomer (**118**), dimer (**119**), and trimer (**120**) of the repeating unit of the capsular polysaccharide of type 2 *Klebsiella* were prepared by Geyer[128] by bacteriophage-associated *endo*-glycanase-catalyzed hydrolysis of the native polysaccharide. Oligosaccharides **118–120** were covalently linked to proteins by the diazo method (p. 8). The edestin conjugates so prepared contained an average of 40 tri-, 49 hepta-, and 10 undeca-saccharide chains per protein molecule, the reducing-end residue having been converted into a polyhydroxyalkyl moiety. In a similar manner the oligosaccharides were coupled to hemocyanin, yielding conjugates having 1700, 575, and 980 chains of **118, 119,** and **120,** respectively. Immunization of rabbits with the edestin conjugates in Freund's

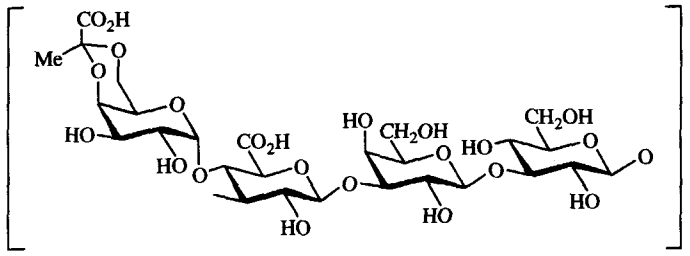

109
110
111
112
R = OC(NH)CCl3
113
114
115
116
117
108




**118** $n = 1$
**119** $n = 2$
**120** $n = 3$

complete adjuvant three times at monthly intervals elicited polysaccharide-specific antibodies, as determined in passive hemagglutination assays. Both the octa- and the dodeca-saccharide conjugates elicited high titers after the first vaccination. The initially weak response to the conjugate of the monomeric repeating-unit could be boosted by a second injection. Interestingly, the hemagglutination titers for all conjugates were of comparable magnitude during the final phase of the immunization course. A major difference was found between the agglutinating ability of the antibodies raised against the different oligosaccharides: whereas the antibodies elicited by the octa- and the dodecasaccharide conjugates agglutinated *Klebsiella* type 2 well, the sera raised against the tetrasaccharide conjugate caused only very slight agglutination. In addition, the precipitin reaction between the native polysaccharide and antibacterial antibodies could be completely inhibited by the octa- **119** and the dodeca-saccharide **120.** In contrast, the tetrasaccharide **118** caused <80% inhibition at most. Rabbit sera raised against the oligosaccharide–edestin conjugates conferred passive protection in mice: the protection achieved by the octa- and the dodeca-saccharide conjugates was higher than that of the tetrasaccharide conjugate.[129] Similar results were obtained in the active immunization experiments. The hemocyanin conjugates of the octa- **119** and the dodeca-saccharide **120** offered significantly higher protection against challenge by the homologous organism than did the tetrasaccharide conjugate. It was concluded that the minimum saccharide length for a "substantial representation" of the serological specificity of the *Klebsiella* B5055 capsular polysaccharide corresponds to two contiguous repeating units and that protein conjugates of oligosaccharide fragments of bacterial polysaccharides can be as potent immunogens as the whole bacterium or its isolated polysaccharide.[128,129]

**h. *Klebsiella*—Type 11.**—A tetra- (**121**) and an octa-saccharide (**122**) corresponding to one and two repeating units, respectively, of the capsular polysaccharide of *Klebsiella* serotype 11 were isolated by depolymerization of the latter by a bacteriophage-associated glycanase.[130] The octasaccharide **122** was twice as efficient an inhibitor of the precipitin reaction between the polysaccharide and anti-polysaccharide antibodies than was the tetrasaccharide **121.** This finding led to the conclusion that the octasaccharide more closely resembles the antigenic determinant of the polysaccharide than does the tetrasaccharide. The octasaccharide **122** was attached to BSA and KLH using the method of Svenson and Lindberg[123]



**121** $n = 1$

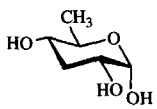
**122** $n = 2$

(p. 15) to afford conjugates with BSA and KLH containing 5 and 18 octasaccharide chains, respectively, linked to one molecule of the protein. These loadings correspond to similar saccharide/protein weight-ratios for the two conjugates. The BSA conjugate was used in Freund's complete adjuvant, and the KLH conjugate was injected in combination with bentonite. In BALB/c mice both conjugates induced anti-*Klebsiella* type 11 polysaccharide IgG and IgM antibodies after primary immunization that could be boosted by a second injection to similar levels at 9 weeks, independent of the carrier used. This study confirmed the thymus dependence of the octasaccharide–protein conjugates and demonstrated the importance of the carrier protein in mice whose B cells carry X chromosome-linked immunodeficiency. It was concluded that two contiguous repeating units of the native polysaccharide are necessary to express the serologic specificity of the polysaccharide.[130]

## 3. Protein Conjugates of Oligosaccharides Related to Lipopolysaccharides

### a. Oligosaccharide Fragments of O-Specific Polysaccharides.—

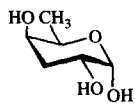
(i) ***Salmonella.***—The O-specific polysaccharide of numerous *Salmonella* serogroups feature dideoxyhexose moieties **123–127** as side-chain residues that are the immunodominant monosaccharides defining the serogroup specificity. Albumin conjugates of dideoxyhexoses **123, 124,** and **127** prepared as their *p*-aminophenyl glycosides[131] **128–130** (Table III) elicited antibodies in rabbits and



**123**

Paratose

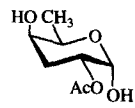
(Factor O:2)



**124**

Abequose

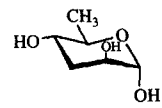
(Factor O:4)



**125**

2-*O*-Acetyl-abequose

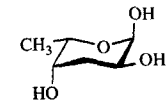
(Factor O:5)



**126**

Tyvelose

(Factor O:9)



**127**

Colitose

(Factor O:35,50)

goats specific to the haptenic residues.[132,133] The rabbit antibodies precipitated the synthetic immunogen against which they were raised, but not did not react with the homologous polysaccharide and lacked bactericidal activity. In contrast, the goat antibodies, although produced in low titers, agglutinated the homologous bacteria. Antisera raised in rabbits by the albumin conjugate of the 2-*O*-acetyl-abequose moiety **131** (O:5 specificity) were capable of precipitating the O-specific polysaccharide of *S. paratyphi* expressing the O:5 specificity. No cross-reaction was noted with the O:4 antigenic factor (abequose) that differs from the O:5 only by the absence of the acetyl group in the former. The rabbit anti-O:5 sera agglutinated *Salmonella* bacteria carrying the O:5 but not the O:4 factor.

**(ii) *Salmonella*.—*S. typhi* and *S. typhimurium*.**—Chemical synthesis of disaccharides (**132–135,** Table III) corresponding to the O-SPs of *S. typhi* (**136**) and *S. typhimurium* (**137**) and their conjugation to BSA were reported by several researchers.[43,86,135–141] The synthesis of the tyvelosyl-mannose disaccharide **133** employed the *xylo*-hexopyranosyl donor **138** and the *p*-nitrophenyl mannoside **139** as the acceptor.[86] Using $Hg(CN)_2$ as the promoter, the α-linked disaccharide **140** was obtained in a very high stereoselectivity, in spite of the participating group at O-2 of the donor. Removal of the protecting groups followed by reduction of the nitro function afforded the *p*-aminophenyl glycoside **133.** This and other disaccharide haptens listed in Table III were conjugated to BSA using the isothiocyanate procedure.[123] The conjugate of **133,** containing four disaccharide chains per conjugate molecule, elicited humoral antibodies specific to the O:9 determinant (**129**) of *S. typhi* in male mice when injected four times 1 week apart at 25 μg doses

TABLE III

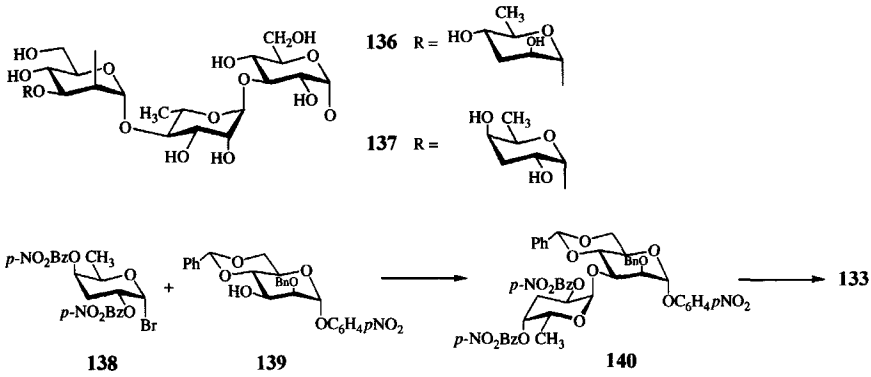
**Bovine Serum Albumin Conjugates of Mono- and Di-saccharides Corresponding to O-Specific Polysaccharides of *Salmonellae*[a]**

| Structure of Hapten[b] | Serogroup | Ref.[c] |
|---|---|---|
| α-Abe-(1 → O-$C_6H_4$*p*$NH_2$ (**128**) | O:4 | 131, *132, 133* |
| α-Tyv-(1 → O-$C_6H_4$*p*$NH_2$ (**129**) | O:9 | 131, *132, 133* |
| α-Col-(1 → O-$C_6H_4$*p*$NH_2$ (**130**) | O:35,50 | 131, *132, 133* |
| 2-*O*-Ac-α-Abe-(1 → O-$C_6H_4$*p*$NH_2$ (**131**) | O:5 | *137,* 142 |
| α-Abe-(1 → 3)-α-D-Man*p*-(1 → O-$C_6H_4$*p*$NH_2$ (**132**) | O:4 | 136–138, *143* |
| α-Tyv-(1 → 3)-α-D-Man*p*-(1 → O-$C_6H_4$*p*$NH_2$ (**133**) | O:9 | *42,* 86, 139, 140, *143* |
| α-Man*p*-(1 → 4)-α-L-Rha*p*-(1 → O-$C_6H_4$*p*$NH_2$ (**134**) | O:12 | 135, *143* |
| α-Par-(1 → 3)-α-D-Man*p*-(1 → O-$C_6H_4$*p*$NH_2$ (**135**) | O:2 | 140, 144, 145, *146* |

[a] Conjugations were performed by the isothiocyanate procedure.

[b] Abe = 3,6-dideoxy-D-*xylo*-hexopyranosyl; Col = 3,6-dideoxy-L-*xylo*-hexopyranosyl; Par = 3,6-dideoxy-D-*ribo*-hexopyranosyl; Tyv = 3,6-dideoxy-D-*arabino*-hexopyranosyl.

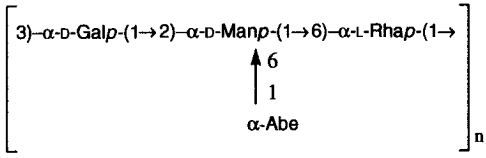
[c] References in roman type are for synthesis; references in italic type are for conjugation.




of the antigen, together with Freund's complete adjuvant.[40] Bactericidal activity was detected already 1 week after the initial immunization and reached a maximum in the sera taken 17 days after the last immunization. In a double-challenge experiment using the O:4 and O:9 strains having equal virulence, the growth of the homologous strain (O:9 specificity) was suppressed in comparison to the organism expressing the isomeric O:4-specific polysaccharide **137.** This study was the first to show that antibodies to a disaccharide component of an enteric bacterium may display specific bactericidal activity. In another study, the BSA conjugate of the Abe-Man disaccharide **132** expressing the O:4 specificity failed to induce detectable levels of anti-carbohydrate antibodies in mice.[147,148]

Rabbits immunized with BSA conjugates of disaccharides **132** and **134** (saccharide loading: 21 disaccharides per BSA in each) in Freund's complete adjuvant three times (10 μg of the synthetic antigen each time), elicited predominantly IgG antibodies against the haptenic disaccharides.[143] These sera had high complement-mediated bactericidal titers with no cross-reactivity detectable between serogroups O:4 and O:9. Although the anti-*Salmonella* specificity of the sera produced by the these immunogens exceeded that of those obtained after immunization with heat-killed bacteria, the serum hemagglutination and bactericidal titers elicited by the synthetic conjugates were lower than those obtained by whole bacteria. The immune response to the carrier protein BSA was approximately five-fold higher that the anti-hapten titer.[143] The high specificity of the antisera obtained after immunization with the synthetic immunogens has been used for the identification of *Salmonella* bacteria by immunofluorescence.[141,143,146]

A series of tetra- (**141**), octa- (**142**), and dodeca-saccharide fragments (**143**) of the O-specific polysaccharide (**139**) of *S. typhimurium* were prepared by partial degradation of the polysaccharide, using bacteriophage-associated endo-α-L-rhamnosidases.[123,149,150] BSA conjugates of the *S. typhimurium* octasaccharide




**141** $n = 1$

**142** $n = 2$

**143** $n = 3$

**142** were prepared by both the aldonate[151] and the isothiocyanate techniques (p. 9).[86] The former differs from the variation proposed by Kabat,[75] (p. 8) in that activation of the carboxyl group is achieved by a water-soluble carbodiimide. The octasaccharide conjugates, when injected in Freund's complete adjuvant in rabbits, elicited antibodies having O:4 (Abe-Man disaccharide) or O:12 (Man-Rha disaccharide) specificities. The titer for the latter was ~35 times higher than that of the O:4 specificity.[123,149] This is in contrast to the relative ratios of the O:4 and O:12 specificities of the serum raised against killed whole bacteria, for which the anti-O:4 titer was 1.6-fold higher than the O:12 titer.[149]

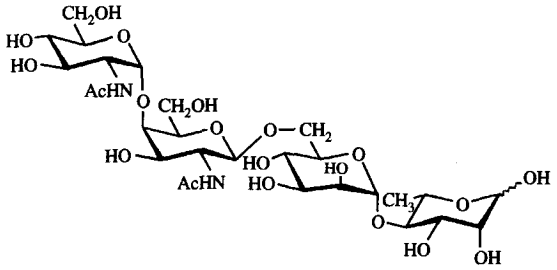
Precipitation of the *S. typhimurium* O-specific polysaccharide by homologous *Salmonella* antisera raised against the whole organism was inhibited by the *S. typhimurium*-derived octa- (**142**) and dodeca-saccharides, (**143**) but not by the tetrasaccharide **141,** indicating that the size of the polysaccharide determinant is larger than a tetrasaccharide and equal to or smaller than the octasaccharide **142.**[152] The specificities of the rabbit antibodies elicited by the octasaccharide conjugate were similar to those elicited by whole bacteria, whereas those elicited by the BSA conjugate of the disaccharide **132** carrying the immunodeterminant abequose residue (O:4 specificity) were different.[123] Reaction between the O-specific polysaccharide of *S. typhimurium* carrying the O:4 determinant and the antibodies against the O:4 determinant Abe-Man disaccharide **125** was more efficiently inhibited by the homologous Abe-Man disaccharide than by the octasaccharide **142.** This finding led to the assumption that the combining site of the antibodies raised against the synthetic disaccharide also accommodates the linking arm or portions thereof, which are absent in the native polysaccharide.[152]

In order to assess the effect of the protein carrier on anti-hapten immunogenicity, conjugates of the *S. typhimurium* octasaccharide **142** with diphtheria toxin (ds 13), edestin (ds 120), and the outer-membrane protein complex (porin) of *S. typhimurium* (ds 15) were prepared and evaluated in both mice and rabbits, with or without Freund's complete adjuvant.[153] The highest titers against either the native LPS or the octasaccharide **142** were obtained with the diphtheria toxin conjugate. The synthesis of this conjugate represents the first example of conjugational toxoiding whereby the toxic properties of a bacterial toxin are diminished by the modification of the lysine residues.[123] The resulting conjugate is immunogenic, and is capable of evoking anti-carbohydrate- and anti-toxin-specific antibodies,

of which the latter can neutralize the native diphtheria toxin. When administered in Freund's complete adjuvant, all conjugates offered active protection in mice with a >10-fold increase in the $LD_{50}$ value. The protective effect of the anti-octasaccharide antibodies was directly demonstrated by the finding that both the anti-octasaccharide–porin and the anti-octasaccharide–diphtheria toxin antibodies raised in rabbits conferred passive protection in mice against *S. typhimurium* challenge at up to 20-fold $LD_{50}$ doses.[153]

A correlation between the oligosaccharide length and anti-saccharide immunogenicity was demonstrated by the finding that, in rabbits, BSA conjugates of the octa- (**142**) and the dodeca-saccharide (**143**) were about 20- and 200-fold more immunogenic, respectively, than the conjugate of the tetrasaccharide **141** conjugate when administered in Freund's complete adjuvant and assayed against the native LPS.[42] A corresponding trend in antibody response was also observed in mice. It should be noted that the actual size of the intact saccharide chain in the conjugates is shorter by one residue, because of the loss of the reducing-end terminus as a pyranose moiety during the conjugation process. This study also showed the importance of the saccharide loading on the immune response. Increase of the molar ratio of the octasaccharide **142** to BSA from 7 to 23 resulted in a 15- to 22-fold increase in the anti-carbohydrate antibody response in mice. Passive immunization experiments showed that the rabbit antiserum against the octasaccharide–BSA conjugate fully protected mice against challenge by *S. typhimurium* up to 100 times the $LD_{50}$, adding further support to the hypothesis that "humoral antibodies are important for protection."[42] It was demonstrated in both active and passive immunization experiments in mice and rabbits that the octa- and the dodeca-saccharide conjugates elicited opsonizing antibodies that enhance phagocytosis of O-antigenetically homologous organisms. A correlation of the bacterial clearance rate and the O-antigen-specific antibody titer was also established.[148] It was concluded that an artificial immunogen corresponding to the *S. typhimurium* O-antigen should contain about 20 octa- (**142**) or dodeca-saccharide (**143**) haptens per conjugate molecule.[42] The finding that a human serum albumin conjugate of the dodecasaccharide **143** was able to induce O-antigen-specific pyrogenic tolerance to the native LPS represents a further mechanism by which O-specific polysaccharide-based neoglycoproteins may contribute to antibacterial immunity.[154]

**(iii)** ***Salmonella—S. illinois.***—The tetrasaccharide **144** corresponding to a complete chemical repeating unit of the O-specific polysaccharide of this bacterium was isolated by controlled acid hydrolysis of the O-specific polysaccharide, exploiting the weaker anomeric linkage of the rhamnose residue relative to the other interglycosidic linkages in this polysaccharide.[155] Coupling to edestin by the azo method[79] afforded a conjugate (**145**) in which the reducing-end rhamnose residues became a part of the linking moiety. According to the Kauffmann–White



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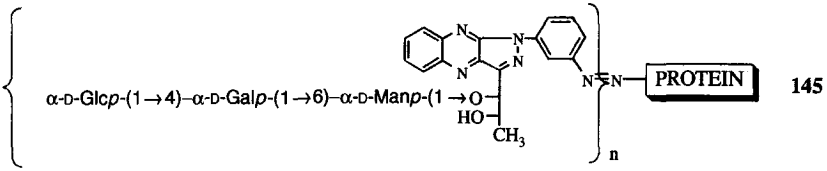
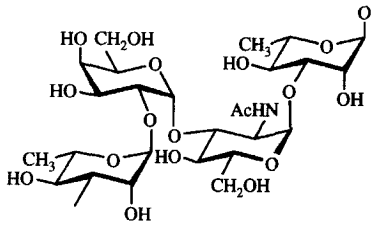



145

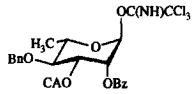
scheme of the classification of *Salmonellae, S. illinois* carries O-specificities 3, 15, and 34. In the antisera obtained by immunization of rabbits with 250 μg of the conjugate **145** in Freund's complete adjuvant most of the O-specificity was directed against the Glc—Gal—Man trisaccharide (O-factor 34), and only slightly against the Gal—Man (O-factor 15) and Man—Rha (O-factor 3) disaccharides. The high specificity of the antibodies was demonstrated by the lack of cross reaction with O-factor $12_2$, which differs from O-factor 34 in the anomeric configuration of the Gal residue. This is the first report suggesting the use of protein conjugates of O-polysaccharide fragments for immunization against gram-negative pathogens.[155]

**(iv)** ***Shigella dysenteriae*—*Sh. dysenteriae* Type 1.**—The O-specific polysaccharide of *Sh. dysenteriae* type 1 consists of ~27 tetrasaccharide repeating-units **146.** Syntheses of oligosaccharide fragments corresponding up to six contiguous repeating-units with and without a spacer and their covalent attachment to human serum albumin was reported by several researchers.[156–164] The key intermediate in the synthesis of the spacer-equipped hexadecasaccharide **167** and its fragments



146

**164, 165,** and **166** was the thioglycoside building block **151,** which was assembled from four appropriately protected and activated monosaccharide precursors **147–150.**[162] The tetrasaccharide thioglycoside **151** was converted into the more reactive imidate **152,** which was linked to the spacer **153** to afford the intermediate **154.** Subsequent removal of the monochloroacetyl group of the rhamnose residue at the non-reducing-end afforded the acceptor **155.** Coupling of the tetrasaccharide donor **152** with the alcohol **155** furnished the dimeric repeating unit **156.** Two iterations of the deprotection–glycosylation sequence afforded the dodeca- (**157**) and hexadeca-saccharides (**158**). In the final stages of the synthesis, the protecting groups were removed followed by hydrazinolysis to afford the oligosaccharides **159–162** corresponding to 1 to 4 consecutive repeating-units of **146.** In order to increase the length of the connecting moiety, the oligosaccharide hydrazides were condensed with a heterobifunctional secondary spacer (**164**) that also introduced an aldehydo moiety in the saccharide constructs to afford constructs **164–167.** These were attached to human serum albumin using reductive amination.[165] The conjugates so prepared (Table IV) differ in two variables of oligosaccharide–protein vaccines: (*i*) the length of the saccharides and (*ii*) the saccharide/protein ratio.[163] The saccharide content of these conjugates was assayed by using MALDI-TOF mass spectrometry, which has a higher degree of accuracy than colorimetric methods. Except for the tetrasaccharide conjugate, all conjugates in Table IV were immunogenic in young outbred mice when injected subcutaneously three times at biweekly intervals at a dose of 2.5 μg of saccharide in the conjugates without an adjuvant.[166] Although the antibody response was poor 1 week after the first injection, almost all animals responded with antibody synthesis after the second injection and a booster response after the third. The anti O-SP IgG levels elicited by the synthetic oligosaccharide–HSA conjugates were significantly higher than those elicited by the conjugate of the native O-specific polysaccharide. If confirmed by clinical trials, it indicates a new approach for manufacturing saccharide-based vaccines. The highest anti-O-SP IgG levels were achieved by the hexadecasaccharide conjugate that contains nine saccharide chains per HSA (Fig. 1). Lower responses were obtained when the loading was either lower (item no. 7, 4 chains) or higher (item no. 9, 19 chains). A similar relationship between saccharide loading and immunogenicity was found for the dodecasaccharide conjugates (items nos. 4–6). The octasaccharide conjugate containing 20 chains (item no. 3) in contrast, was more immunogenic than the conjugate containing 11 chains (item no. 2) (Fig. 1). The complex nature of the influence on immunogenicity of the saccharide length and the loading was revealed by the finding that, at a higher loading, the octasaccharide conjugate containing 20 chains induced higher anti-polysaccharide IgG titers than did the dodecasaccharide conjugate at a lower loading (8 chains). It was concluded that at low levels of saccharide loading, receptor clustering



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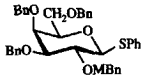

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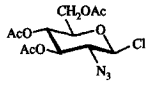

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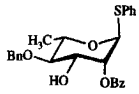

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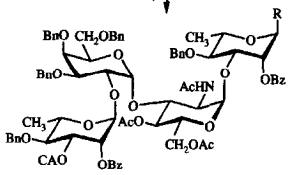
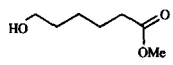


151 R = SPh

152 R = OC(NH)CCl$_3$



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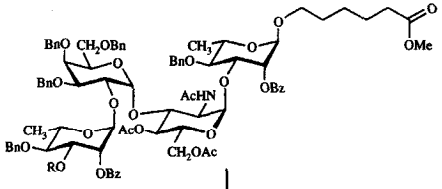
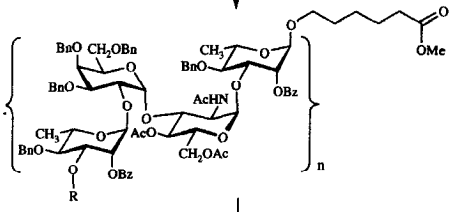


154 R = CA

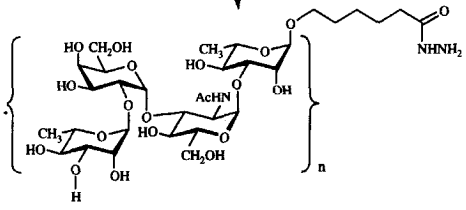
155 R = H



156 $n$ = 2 R = CA

157 $n$ = 3 R = CA

158 $n$ = 4 R = CA

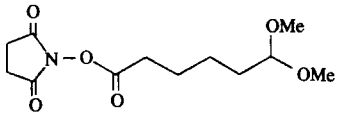


159 $n$ = 1

160 $n$ = 2

161 $n$ = 3

162 $n$ = 4



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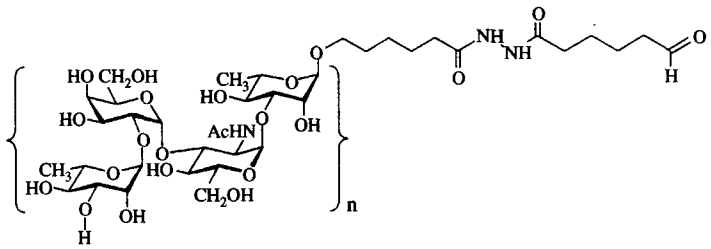


**164** $n = 1$
**165** $n = 2$
**166** $n = 3$
**167** $n = 4$

is insufficient for optimal immunogenicity, whereas at high saccharide loading, the T-cell epitopes of the carrier protein are shielded by the saccharide from antigen processing. Optimum immunogenicity of oligosaccharide–protein conjugates can thus be achieved at an intermediate loading defined by the saccharide length.[166]

**(v) *Escherichia coli—E. coli* Type O8.**—A heptasaccharide (**168**) was obtained from the O-specific polysaccharide of *E. coli* serotype O8 by bacteriophage-associated *endo*-mannosidase-mediated cleavage[167] and was attached to BSA by

TABLE IV

**Human Serum Albumin Conjugates of Tetra-, Octa-, Dodeca-, and Hexadeca-saccharide Fragments of the O-Specific Polysaccharide of *Shigella dysenteriae* Type 1[163]**

| Item | Conjugate[a] | MW (Da) | Saccharide–Protein Ratio (mol/mol) |
|---|---|---|---|
| 1 | **IV**-HSA | 77,970 | 13 |
| 2 | **VIII**-HSA | 83,160 | 11 |
| 3 | **VIII**-HSA | 98,000 | 20 |
| 4 | **XII**-HSA | 83,340 | 8 |
| 5 | **XII**-HSA | 89,200 | 10 |
| 6 | **XII**-HSA | 118,000 | 23 |
| 7 | **XVI**-HSA | 78,000 | 4 |
| 8 | **XVI**-HSA | 92,400 | 9 |
| 9 | **XVI**-HSA | 120,000 | 19 |

[a] The numbers **IV, VIII, XII,** and **XVI** stand for tetra-, octa, dodeca-, and hexadeca-saccharide, respectively.

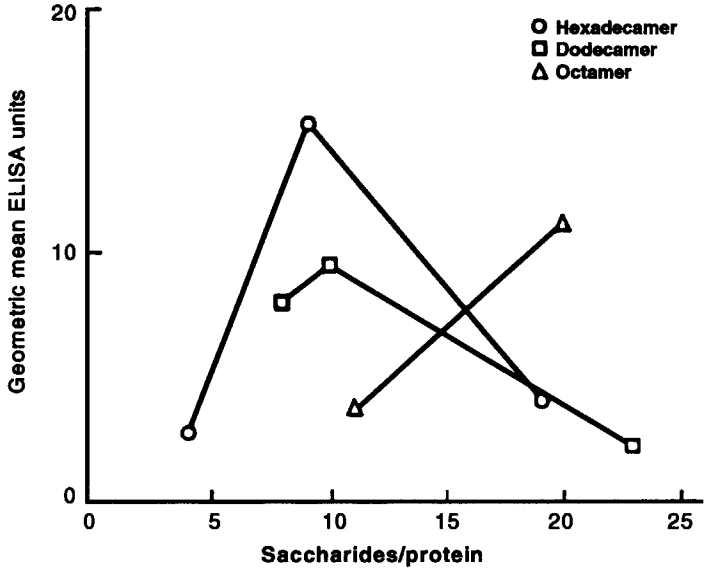



FIG. 1. Geometric mean of serum IgG anti-LPS antibodies (in ELISA units) elicited by HSA conjugates of synthetic oligosaccharide fragments of the O-SP of *Sh. dysenteriae* type 1 in mice 7 days after the their s.c. injection, 2 weeks apart, each injection containing 2.5 μg of saccharides. (Reproduced from *Proc. Natl. Acad. Sci. U.S.A.*, 96 (1999) 5194–5197, V. Pozsgay, C. Chu, L. Pannell, J. Wolfe, J. B. Robbins, and R. Schneerson, with permission from the publisher.)

the method of Svenson and Lindberg[123] to give a conjugate with an average of 20 saccharide chains per protein molecule.[168] When injected in rabbits in Freund's complete adjuvant at a dose of 20 μg of saccharide, the conjugate elicited anti-LPS and anti-oligosaccharide **168** IgG antibodies that could be boosted by repeated inoculations on days 14 and 21. Anti-polysaccharide IgG antibodies were elicited in rhesus monkeys when vaccinated with the conjugate without any adjuvant (100 μg) at monthly intervals. When an arbitrary titer of anti-polysaccharide IgG antibody was reached, the monkeys were infected by *E. coli* O8 inoculated in the kidney. Compared to the controls, the degree of renal scarring was significantly decreased in the vaccinated animals, although the duration of bacteruria was the same. It was concluded that the decreased renal damage was due to interference of the polysaccharide-specific IgG antibodies with the endotoxin presentation to the host.[168]

α-D-Man*p*3Me-(1→[3)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1-]$_2$

**b. Oligosaccharide Fragments of the Core Region—Meningococcal oligosaccharides.**—In contrast to the O-antigens that are specific for the different types of meningococcal bacteria, the inner core regions of the lipopolysaccharides are the same or similar for most immunotypes of *N. meningitidis.* This observation led to the assumption that antibodies directed against the core region might offer protection against several types simultaneously.[169] In order to identify the minimal oligosaccharide structures required for the induction of antibodies against the common meningococcal epitopes, Boons *et al.*[170–172] synthesized di- (**169, 170**) and tri-saccharides (**171, 172**) related to the inner core region of the most prevalent

β-D-Glc*p*-(1→4)-L-α-D-Hep*p*-(1→$OCH_2CH_2CH_2NH_2$ **169**

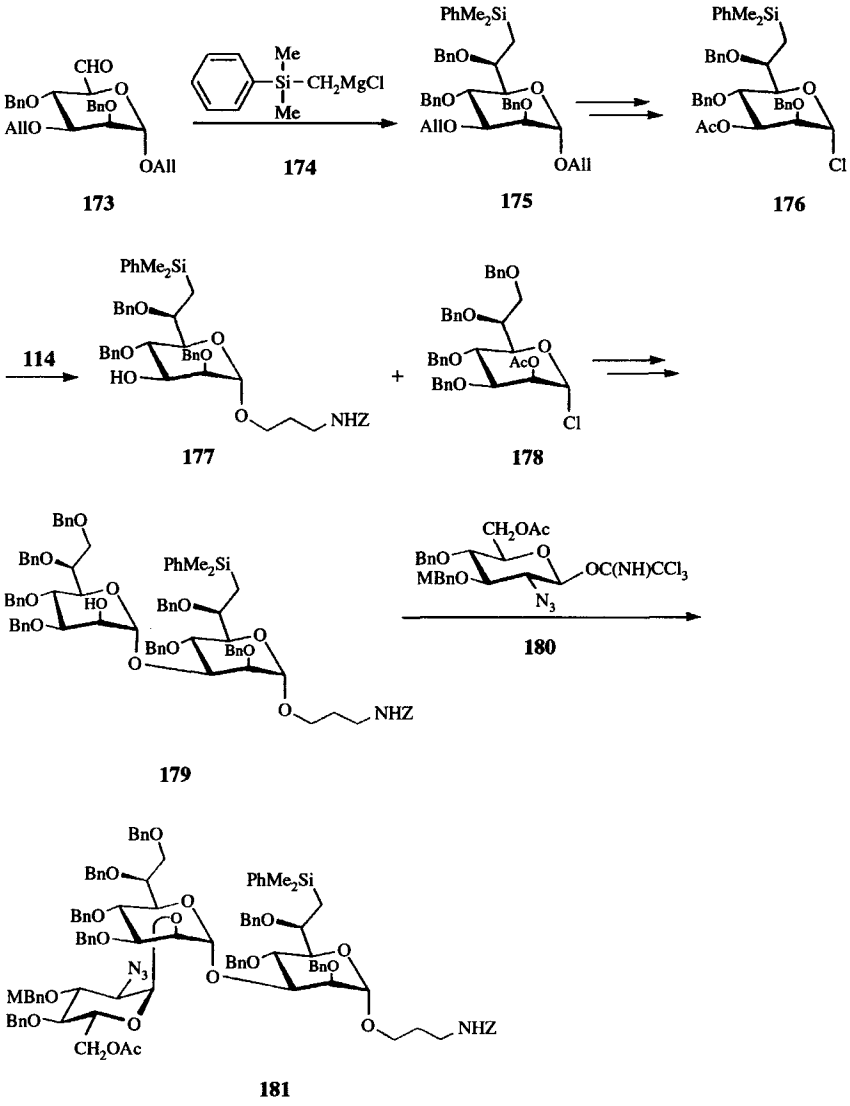
L-α-D-Hep*p*-(1→3)-L-α-D-Hep*p*-(1→$OCH_2CH_2CH_2NH_2$ **170**

α-D-GlcNAc*p*-(1→2)-L-α-D-Hep*p*-(1→3)-L-α-D-Hep*p*-(1→$OCH_2CH_2CH_2NH_2$ **171**

β-D-Glc*p*-(1→4)-[L-α-D-Hep*p*-(1→3)]-L-α-D-Hep*p*-(1→$OCH_2CH_2CH_2NH_2$ **172**

serotypes L1, L2, and L3,7,9. Starting material for the trisaccharide **171** was the *manno*-dialdehyde **173** that upon treatment with the reagent **174** afforded the silylated derivative **175** in which the silyl group is a masked hydroxyl group-equivalent.[170] Replacement of the allyloxy group by chlorine afforded the heptose donor **176** that was combined with the spacer **114** followed by selective deprotection at the site of the chain extension to afford the glycoside **177.** The latter was glycosylated with the heptosyl chloride **178** followed by deacetylation (→**179**). Subsequent stereoselective glycosylation with the glucosamine donor **180** furnished the intermediate **181,** from which the trisaccharide **171** was obtained after deprotection and functional group manipulations. Conjugation of the aminopropyl spacer-equipped saccharides **169–170** to the protein tetanus toxoid was achieved by the thioether method (p. 13).[105] The incorporation levels for the di- (**169** and **170**) and the trisaccharides (**171** and **172**) were 13, 26, 22, and 35 saccharide chains, respectively, per tetanus toxoid molecule.[169] Phosphoethanolamine-containing oligosaccharides **182, 183,** and **184** were isolated from the native polysaccharides of immunotypes L2 and L3,7,9, respectively. Compounds **183** and **184** were thiolated, and then coupled to bromoacetylated tetanus toxoid through a thioether linkage to afford conjugates having 20 and 15 chains of the L2 (**183**) and the L3,7,9 oligosaccharide (**184**) per tetanus toxoid, respectively.

In rabbits, all conjugates elicited substantial IgG antibody levels after repeated inoculations, while the IgM levels were low. The conjugate of the L2 immunotype-specific oligosaccharide **183** elicited both L2- and L-3,7,9-specific antibodies but not the L1 structure **182,** and the conjugate of the L3,7,9 oligosaccharide **184** induced antibodies that recognized the L1, L2, and the L3,7,9 structures. Thus,



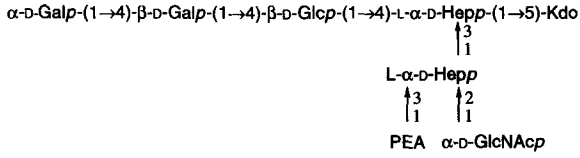

173 174 175 176

177 178

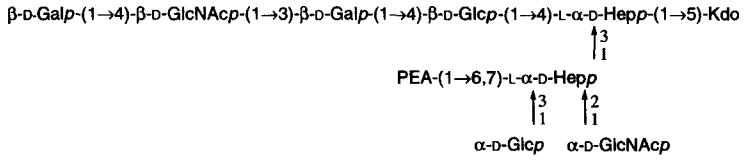
179 180

181

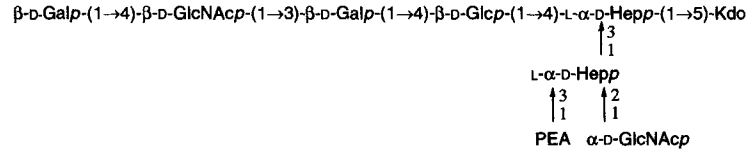
common epitopes are shared by the L2 and L3,7,9 types and by the L1 and L3,7,9 types, but not by the L1 and L2 serotypes. The finding that the conjugate of the branched trisaccharide **172,** but not the linear structures **170** and **171** were able to elicit IgG antibodies that recognized determinants of the L1 and L3,7,9 immunotypes led to the conclusion that the branched trisaccharide **172** represents the minimal structure necessary for inducing cross-reactive antibodies against




**182** (L1)




**183** (L2)




**184** (L3,7,9)

several meningococcal immunotypes. Antibodies elicited by the conjugates of the oligosaccharides **169, 170,** and **171** were specific for the L2 immunotype.[169] In mice, the tetanus toxoid conjugates of the L2 (**183**) and the L3,7,9 oligosaccharide (**184**) elicited IgG antibodies only when added together with an adjuvant. These antibodies, although able to recognize epitopes on meningococcal lipopolysaccharide, were not bactericidal for group B meningococcal organisms, leading to the conclusion that it "will be difficult, if not impossible" to induce protective antibodies against multiple meningococcal serotypes simultaneously.[173]

**c. Lipopolysaccharides of *Chlamydia*.**—The genus-specific lipopolysaccharide of the intracellular gram-negative bacterium *Chlamydia* contains the unique oligosaccharide epitope **185** that is shared by all Chlamydiae.[174] In order to further prove the structure of the lipopolysaccharide and to study the epitope specificity of antibodies raised against the chlamydial lipopolysaccharide, Kosma synthesized di- (**186, 187**), tri- (**188**), tetra- (**189**), and penta-saccharides (**190**) related to **185** as allyl glycosides and conjugated to BSA.[175–178] A key precursor in the trisaccharide synthesis was the Kdo donor **191** that was condensed with the triol (**192**) to give, predominantly, the 6-*O*-linked disaccharide **193.** Subsequent transformations furnished the diol **194,** in which the HO-3 hydroxyl group was selectively glycosylated with the Kdo donor **191** to afford the intermediate **195.** Removal of the protecting group provided the trisaccharide **188.** Conjugation of the oligosaccharide allyl glycosides (**186–190**) to BSA was achieved by Lee's protocol.[179] Accordingly, ligands having the general formula **196** were combined with cysteamine (**197**) to afford amine **198** that was converted into the

α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo-(2→6)-α-D-Glc*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→ **185**

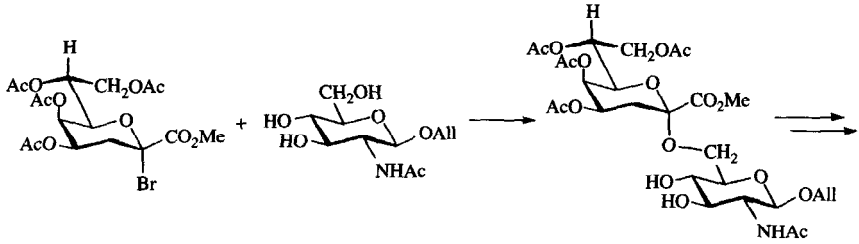
α-Kdo-(2→4)-α-Kdo-(1→ $OCH_2CH=CH_2$ **186**

α-Kdo-(2→8)-α-Kdo-(1→ $OCH_2CH=CH_2$ **187**

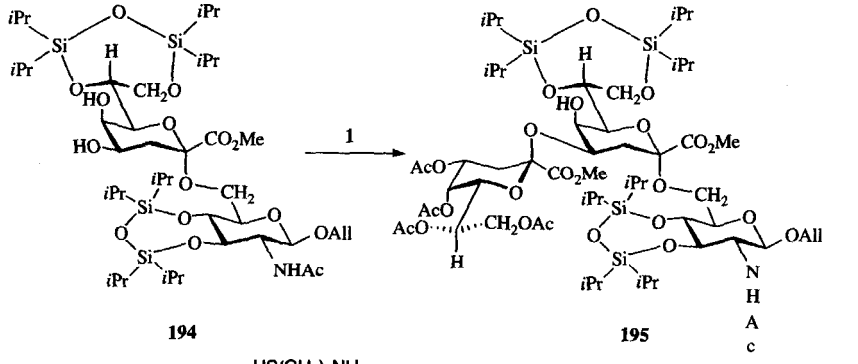
α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo-(1→ $OCH_2CH=CH_2$ **188**

α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo-(2→6)-β-D-Glc*p*NAc-(1→ $OCH_2CH=CH_2$ **189**

α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo-(2→6)-α-D-Glc*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→ $OCH_2CH=CH_2$ **190**



**191** **192** **193**



**194** **195**

SUGAR-(1→$OCH_2CH=CH_2$ $\xrightarrow[\textbf{197}]{HS(CH_2)_2NH_2}$ SUGAR-(1→$O(CH_2)_3S(CH_2)_2NH_2$

**196** **198**

→ SUGAR-(1→ $O(CH_2)_3S(CH_2)_2NCS$

**199**

isothiocyanate-derivative **199** by reaction with thiophosgene followed by *in situ* coupling with BSA. The resulting conjugates contained an average of 2.5 to 6 oligo-saccharide chains per BSA molecule.[178] Both the tetra- (**189**) and the penta-saccharide (**190**) conjugate elicited lipopolysaccharide-specific antibodies

in mice when injected with Freund's complete adjuvant.[178,180] Selected monoclonal IgG and IgM antibodies against the tetrasaccharide conjugate, prepared by the hybridoma technology, only recognized the complete Kdo–trisaccharide sequence, whereas others exhibited a more diverse antigen recognition pattern that also included Kdo monomer and Kdo-disaccharides.[178] Monoclonal antibodies against the BSA conjugate of the pentasaccharide **190** showed an absolute requirement for the terminal Kdo–trisaccharide segment.[180] One group of these conjugate-induced monoclonal antibodies exhibited 100-fold higher affinity toward chlamydia-specific recombinant lipopolysaccharide as compared to the antibodies elicited by chlamydial elementary bodies. This finding led to the conclusion that synthetic oligosaccharide-containing immunogens may be superior to their natural counterparts.[174]

## III. Conclusion

The recognition that surface carbohydrates on the bacterial cell may be utilized as protective antigens made these macromolecules a target for vaccine developments many years ago. Current developments in carbohydrate chemistry may provide access to well-defined fragments of bacterial cell-surface polysaccharides either by specific degradation of the native polysaccharides or by chemical synthesis. As shown by the studies reviewed here, oligosaccharides related to bacterial extracellular polysaccharides hold great promise for the development of vaccines against infectious diseases. If successful in human trials, the synthetic oligosaccharide–protein conjugates may eliminate many of the problems associated with the use of native bacterial polysaccharides or their degradation products, including product heterogeneity and a potential for biological contamination, and may offer the advantage of improved immunogenicity. It has been demonstrated that protein conjugates of oligosaccharide fragments of cell-surface polysaccharides of pathogenic bacteria can elicit antibodies that are recognized by the native polysaccharide. In has also been shown that the IgG antibodies elicited by the oligosaccharide—protein conjugates may offer protection against the homologous organisms in animal models, even if the conjugates are used without an adjuvant.

A study on the *Shigella dysenteriae* type 1 system established the optimum range of saccharide loading in the conjugates. These and other studies have also demonstrated that oligosaccharides as small as a dimer or a timer of the repeating unit of O-specific or capsular polysaccharides might be sufficient for optimum antibody production in animal models. Although the requirements may show some variance for each individual polysaccharide, the findings may be regarded as the basis for the design of oligosaccharide-based conjugate antibacterial vaccines and are all the more encouraging because chemical synthesis of such fragments is already within the scope of numerous laboratories.

Although the efficacy of polysaccharide–protein conjugates in preventing enteric and respiratory infections has been established, it remains to be seen whether the antibody populations elicited by protein conjugates of oligosaccharide fragments of such polysaccharides share the same protective efficacy. It is expected that increasing availability of the experimental vaccine candidates will answer this question in the near future. Because of the lack of reliable animal models for most bacteria pathogenic to humans, human trials will be necessary in the future to verify the efficacy of synthetic carbohydrate—protein conjugates as vaccines. However, conclusions drawn from immunogenicity experiments in which dosage, adjuvant, and route of administration are not clinically acceptable msut be tempered when the experimental vaccines are considered for clinical use.

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