concerted fashion, allowed by orbital symmetry considerations via an excited state rearrangement.

Because the observed kinetics have the same form observed for other radio frequency plasma processes, it seems appropriate to consider these rearrangements as part of the paradigm[1] which invokes electron impact induced electronic excitation and major product formation via neutral species. However, because the yields are low one cannot be sure of mechanistic conclusions based on product structures or yields. Indeed, in this case it is possible that ionic intermediates are involved or that the light generated as a product from some other plasma process gives indirect photolytic conversions.

A comparison with the work of Kropp and Manning[10] is appropriate and reveals some important differences. In both photolysis and plasmolysis, allylcyclopropanes are the major isomers formed from 1,5-dienes. The products from the heptadienes **9** and **10** are, however, not the same. Photolysis gives mixtures in which [1,2] allyl shift not [1,4] vinyl shift products are present in larger amounts. Furthermore, the photoproducts from **1**[9,10] are entirely different from the plasma products. Perhaps there would be a closer correspondence of products if the photolyses were performed in the gas phase at low wavelength (see footnote 16 of ref 10).

1,3-Dienes. The acyclic 1,3-dienes simply undergo allylic isomerization, interconverting the various 1,3-hexadienes. These reactions are reminiscent of the 2-butene to 1-butene radio frequency isomerization.[5] *cis,cis*-1,3-Cycloocadiene (**18**) gives quite different chemistry which is reminiscent of the UV photolysis result. It is recognized, of course, that allylic isomerization of **18** may be occurring undetected and that the 2 + 2 cycloaddition process is distinctly favored by the s-cis conformation of **18.**

Summary. The radio frequency chemistry of alkenes and dienes forms a consistent set. Linear $\log A/A_0$ vs. Pr^{-1} relationships are usually found, and isomerizations, fragmentations, and polymerizations are dominant. Isomerizations are more prevalent in cooler (low Pr^{-1}) plasmas. Simple alkenes and 1,3-dienes isomerize by cis, trans and allylic-type rearrangements; 1,4-dienes give vinylcyclopropanes and 1,5-dienes give allylcyclopropanes. There are correlations in many cases with photochemistry, and it seems possible that photolysis in the gas phase at low wavelength and at the same pressure used for radio frequency chemistry would give an even more thorough correlation.

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Registry No. 1, 111-78-4; **2**, 45531-46-2; **3**, 3760-14-3; **4**, 103240-53-5; **5**, 103240-54-6; **6**, 592-42-7; **7**, 4663-23-4; **8**, 627-58-7; **9**, 76589-01-0; **10**, 1541-33-9; **11**, 76588-97-1; **12**, 76588-95-9; **13**, 1541-23-7; **14**, 76588-96-0; **15**, 76588-99-3; **16**, 76588-98-2; **18**, 1700-10-3; **19**, 616-10-4; **20**, 592-48-3; **21**, 5194-51-4; **22**, 5194-50-3; **23**, 6108-61-8.

Bimolecular Chemistry of Macromolecules: Synthesis of Bacterial Polysaccharide Conjugates with *Neisseria meningitidis* Membrane Protein

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Abstract: Covalently linked macromolecules of biological importance (bacterial-polysaccharide conjugates) are prepared by a general, multistep synthetic method employing a bigeneric spacer that is an oligopeptide spacer molecule whose parts are derived from each of the two modified macromolecules to be covalently linked. The strengths of this synthetic approach are (a) the covalency of the resulting conjugate is readily and absolutely determined and (b) analysis for the bigeneric spacer defines the purification of the conjugate and facilitates the identification of separation procedures that remove contaminating, unconjugated macromolecules. The multistep bigeneric spacer method includes the following steps: (1) covalent modification of one macromolecule to attach basic amine groups that are then bromoacetylated; (2) covalent modification of the second macromolecule with *N*-acetylhomocysteine thiolactone to attach pendant thiol groups; (3) coupling of these macromolecules under mildly basic conditions; (4) determination of the covalency ratio by amino acid analysis of *S*-(carboxymethyl)homocysteine, formed by reaction of bromoacetyl and homocysteinyl moieties, relative to a standard endogenous amino acid; (5) purification of the mixture to maximize the covalency ratio. By this method several conjugates of polydisperse bacterial polysaccharides with an immunogenic *Neisseria meningitidis* membrane protein were prepared and characterized by NMR and chemical analysis.

It has become clear that biological macromolecules have different domains that are independently responsible for such diverse functions as receptor recognition, cell entry, effector control, and effector activity. Moreover, these domains are often physically separable, and appreciation of this fact has generated an increasing body of research devoted to preparing hybrid macromolecules that combine particular properties in a desired way. Thus, the so-called immunotoxins or "magic bullets" seek to destroy cancer cells with high specificity by combining the recognition power of antibodies with highly toxic effector molecules (toxins).[1] A synthetic blood substitute has been prepared by binding an altered hemoglobin molecule to the polysaccharide inulin.[2] Modified hormonal responses have been obtained by coupling insulin to macromolecules

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0002-7863/86/1508-5282$01.50/0

Table I. Polysaccharide Structures:[a] Monomeric Units

-3)-β-D-Ribf-(1-1)-D-ribitol-(5-O-P(=O)(OH)-O-	*H. influenzae* type b
-2)-α-D-Galp-(1-3)-α-D-Glcp-(1-3)-α-L-Rhap-(1-4)-D-ribitol-(5-O-P(=O)(OH)-O-	*S. pneumoniae* type 6B
-4)-β-D-Glcp-(1-6)-β-D-GlcpNAc-(1-3)-β-D-Galp-(1- (4 → β-D-Galp)	*S. pneumoniae* type 14
-4)-β-D-ManpNAc-(1-4)-α-D-Glcp-(1-2)-α-L-Rhap-(1-O-P(=O)(OH)-O-	*S. pneumoniae* type 19F
-4)-β-D-Glcp-(1-4)-β-D-Galp-(1-4)-L-Rhap-(1- (Glcp 6-PO$_3$H; Rhap 2 → α-L-Rhap)	*S. pneumoniae* type 23F

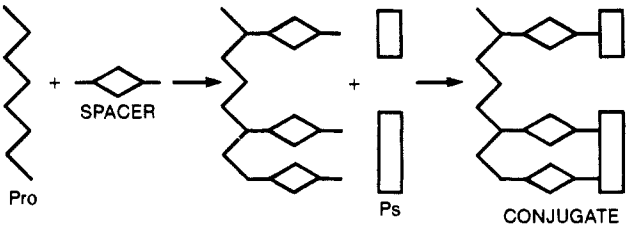
[a] Reference 20.

with particular binding properties.[3] In the area of immunology, an attempt has been made to change the isotype response to tetanus toxoid by coupling it to the polysaccharide pullulan.[4] Another application involves the enhancement of immunogenicity of various antigens, notably bacterial polysaccharides[5] and synthetic polypeptides. The word "conjugate" is most commonly used to describe these linked macromolecules, and it will be used in this paper.

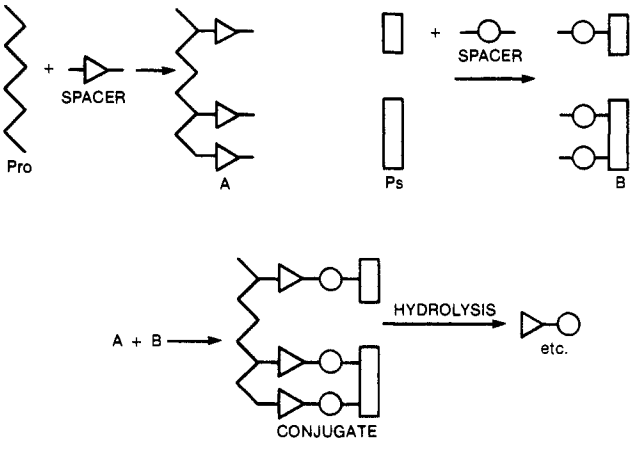
In the course of an effort to prepare conjugates of polysaccharides (Ps) and an immunogenic protein (Pro), we have addressed a number of interrelated problems. They involve the conjugation strategy, the chemistry used to effect that strategy, and the analytical approach to the structural and constitutional definition of the product. There are obvious complexities that arise when a polyfunctional Ps of polydisperse molecular weight (ca. M_r 5×10^5–1.5×10^6) is conjugated to a polyfunctional Pro (M_r 3×10^7). For instance, it is clear that *one* covalent bond between the two reaction partners will be very difficult to detect by conventional means, yet such a situation will produce an aggregate product similar to one where multiple covalent bonds are involved. Ambiguities also arise when several low molecular weight Ps molecules are conjugated to a given Pro vis-à-vis fewer high molecular weight Ps molecules bonded to the same Pro. It was therefore felt that the degree of conjugation is an important parameter to define especially if one wants to correlate biological results with chemical constitution. Another problem is that the constitutions of the substrates under consideration make them soluble only in water, which severely limits the available conjugation chemistry. It thus became clear that solubilization of one of these, the Ps, in a nonhydroxylic solvent was important for the chemical solution to the problem. A third problem involves the separation of unconjugated material from the conjugate, a situation again complicated by the polyfunctionality and polydispersity of the system. Such separations can sometimes be effected by size-exclusion chromatography when the products and the reactants differ significantly in their molecular weights and hydrodynamic properties. However, small percentage increases in molecular weight that obtain when a few Ps molecules (M_r 10^5) are added to a Pro (M_r 10^7) do not allow this approach. Our solution to these problems is the subject of this paper.

Our system evolved from the need to conjugate the capsular Ps of *Haemophilus influenzae* type b (PRP; Table I) with a protein carrier so that the polysaccharide would become immunogenic (i.e., elicit an immune response to its antigenic surface)

Scheme I. Monogeneric Spacer Conjugate



Scheme II. Bigeneric Spacer Conjugate



in children under 1 year of age. Since such infants do not respond to the unconjugated polysaccharide, an effective conjugate might provide a vaccine that would protect against *H. influenzae* type b caused meningitis.[6] The highly immunogenic serotype 2 outer membrane protein of *N. meningitidis*[7] was chosen as the protein partner for the conjugation. Ultimately, this protein, with our new methodologies, was conjugated to the streptococcal polysaccharides (vide infra) with the view that an infant otitis media vaccine might result. Useful immune responses have been afforded by all conjugates, the details of which will be published elsewhere.

Results and Discussion

A. Covalency and Degree of Conjugation. There are currently two published protein conjugates of PRP. One[8] is prepared by reducing, with $NaBH_3CN$, the Schiff base formed between the amino groups of the protein (a nontoxic variant of diphtheria toxin) and the reducing end of PRP. The product was not separated in a controlled way from unconjugated Ps and Pro, and one cannot

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(6) (a) Schneerson, R.; Barrera, O.; Sutton, A.; Robbins, J. *J. Exp. Med.* **1980**, *152*, 361–376. (b) Robbins, J. S.; Schneerson, R.; Pittman, M. *Bacterial Vaccines*; Germanier; R., Ed.; Academic: Orlando, FL, 1984; pp 290–316.

(7) Frasch, C. E.; Robbins, J. D. *J. Exp. Med.* **1978**, *147*, 629–644.

(8) Anderson, P. *Infect. Immun.* **1983**, *39*, 233–238.

Table II. Characteristics of Polysaccharides and Their Derivatives

Ps	deriv	K_D[e]	rate nephelometry[a]	% N calcd	% N found
PRP		0.05 – 0.43[d]			
	Bu_4N			2.39	2.29
	BuA_2	0.68 ± 0.05	123 ± 25[b]		
	BuA_2–BrAc	0.67 ± 0.10	105 ± 30[b]		
6B		0.33			
	Bu_4N			8.02	8.19
	BuA_2	0.43	136		
	BuA_2–BrAc	0.35	64		
14		0.07			
	BuA_2	0.57	153		
	BuA_2–BrAc	0.79	120		
19F		0.17 – 0.58[d]			
	Bu_4Na			3.36	3.09
	BuA_2	0.49	142		
	BuA_2–BrAc				
23F[c]		0.26			
	Bu_4N			1.49	1.53
	BuA_2	0.23	97		

[a] Percent of native Ps. [b] Average of three separate preparations. [c] The bromoacetyl derivative was not isolated because of its insolubility. [d] Bimodal elution. [e] PRP was determined on Sepharose Cl 4B; all other, on Sepharose Cl 2B.

rigorously conclude that the product was covalent. Assuming the putative chemistry was effected, the conjugate could be considered to be branches of Ps chains attached at their ends to a central axis of protein. A second approach[6] involves coupling the protein (diphtheria toxin, tetanus toxoid) to one end of the bifunctional molecule adipic acid dihydrazide. This is accomplished with carbodiimide and presumably forms a diacylated hydrazine with the pendant glutamic or aspartic carboxyl groups. This product is then reacted with a cyanogen bromide activated Ps, presumably forming an *O*-alkyl-4-acylated isosemicarbazide. Characterization and purification proceeded via size-exclusion chromatography, and covalency was inferred from an aggregate molecular weight. However, control experiments that exclude electrostatically bonded complexes were not reported. We suggest the term "monogeneric spacer" conjugate to describe this product and represent this scenario in Scheme I.

Our approach, which results in a "bigeneric spacer" product (Scheme II) involves functionalizing each reaction partner with a bifunctional "spacer" molecule. Each is chosen so that the remaining valency can react with that of its opposite partner to form a stable covalent bond. Furthermore, the system is designed so that the bond formed in the conjugation step is stable to conditions that degrade (e.g., hydrolyze) the final conjugation product and can form in that process a readily assayable molecule. This molecule then unambiguously represents a covalent bond between the two macromolecules, providing that the bonds formed in the initial steps (i.e., functionalizing the Ps and Pro with their respective bifunctional spacer) and can be established as covalent. In our system we can assert this from NMR evidence (Ps) and separation experiments (Pro). The system is schematically represented in Scheme II.

B. Chemistry. Our first concern was to develop chemistry that would allow us to functionalize our polysaccharides (Table I) in water. Hitherto, the most effective way of functionalizing a polyhydroxy compound in water was to activate it with cyanogen bromide, a reagent that presumably acquires some rate advantage when reacting with *vic*-diols. This chemistry is not uniformly effective (unpublished results), and the functionalization yields are low, typically 75–100 nmol/mg. It was felt that the best way to circumvent competition with H_2O was to work in a nonaqueous system. In the case of acidic polysaccharides such as the capsular coats of *H. influenzae* type b (PRP) and *Streptococcus pneumoniae* types 6B, 19F, and 23F (Table I), which contain as part of their monomeric unit a phosphodiester function, this is accomplished by replacing the remaining acidic hydrogen by a hydrophobic cation such as tetra- or tributylammonium. The resulting products (**1**) can be characterized by their elemental analyses, molecular weight distribution (K_d),[9] and topologies (rate nephelometry units)[10] (Table II). For a nonacidic Ps such as *S. pneumoniae*, solubilization was effected by molecular weight reduction via a short aqueous hydrazine or pH 10 treatment. All of these products were adequately soluble in either dimethylformamide (DMF) or dimethyl sulfoxide (Me_2SO). Although this concept has been applied before embodying an extensive hydrolysis of PRP for preparing NMR solutions,[11] in our hands those procedures afforded products of greatly reduced antigenicities and were not appropriate to our purposes.

Once in nonaqueous solutions, the polysaccharides may be reacted with a bis electrophile such as carbonyldiimidazole[12] ($COIm_2$) to form, putatively, an imidazolylurethane (**2**). **2** is then susceptible to nucleophile substitution, in aqueous systems if necessary, with bis nucleophiles such as diamines. Under appropriate circumstances (e.g., diamine excess), a stable covalent urethane with pendant amine is produced. Using 1,4-butanediamine and using a fluorescamine assay[13] to quantify the amine titer, we prepared **3** and used it for a variety of aqueous-based chemistries.

$$\text{PsOH} + \text{Dowex 50 } (Bu_4N^+ \text{ form}) \longrightarrow \underset{\textbf{1 (a, Ps = PRP)}}{\text{PsOH–}Bu_4N^+ \text{ salt}} \quad \text{(a)}$$

$$\textbf{1} + COIm_2 \xrightarrow{\text{DMF}} \underset{\textbf{2}}{\text{PsO}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{Im}} \quad \text{(b)}$$

$$\textbf{2} + H_2N(CH_2)_4NH_2 \xrightarrow{aq} \underset{\textbf{3 (a, Ps = PRP)}}{\text{PsO}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{NH}(CH_2)_4NH_2} \quad \text{(c)}$$

The amine levels can be readily controlled by the level of $COIm_2$ and have reached a titer of 700 nmol/mg (**3a**), which may be compared to the cyanogen bromide chemistry (vide supra). It should be noted that this level of substitution yields a product with significantly different antigenicity from the reactant, and for our purposes lower degrees of substitution were appropriate. The evidence for a carbamate functionality in **3** can be inferred from the ^{13}C and ^1H NMR spectra (vide infra).

As shown in Scheme II, one of the conjugation partners is a protein that can be analyzed, after acidic hydrolysis, for its constituent amino acids. Therefore, generation of a new, unique amino acid in the process of forming the bimacromolecular bond would afford a readily assayable small molecule, assuming it appeared in "window" in the amino acid spectrum. Moreover, since this new amino acid could be related in the same assay to one of the constituent amino acids of the protein, an internal standard is available, and the titer of the new amino acid need not be based on other assays of the conjugate. This obviates the problems of purification, drying, standards, etc. A thioether bond seemed appropriate for this strategy since it is readily formed in aqueous systems by alkylation of thiols with halides[14] and is stable to the conditions of the amino acid analysis. After a survey of several amino acids containing a thioether linkage, it was found that *S*-(carboxymethyl)homocysteine (**8**, SCMHC) is detected in a window in the analytical amino acid "spectrum" of the constituent acids of proteins. It was also found to be stable to the conditions of the hydrolysis and chromatography even in the presence of the polysaccharide. It was therefore decided to use conjugation reactions that would result in the generation of **8** at

(9) McCauley, J. A.; Mancinelli, R. J.; Downing, G. V.; Robbins, J. B. *J. Biol. Standard.* **1981**, *9*, 461–468.
(10) Steinberg, J. C. *Clin. Chem.* (*Winston-Salem, N.C.*) **1977**, *23*, 1456–1464.
(11) Egan, W.; Schneerson, R.; Werner, K. E.; Zon, G. *J. Am. Chem. Soc.* **1982**, *104*, 2898–2910.
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(13) Udenfriend, S.; Stein, S.; Bohlen, P.; Dairman, W.; Leimgruber, W.; Weigele, M. *Science* (*Washington, D.C.*) **1972**, *178*, 871–872.
(14) Rowley, G. L.; Leung, D.; Singh, P. U.S. Patent 4328311, 1982.

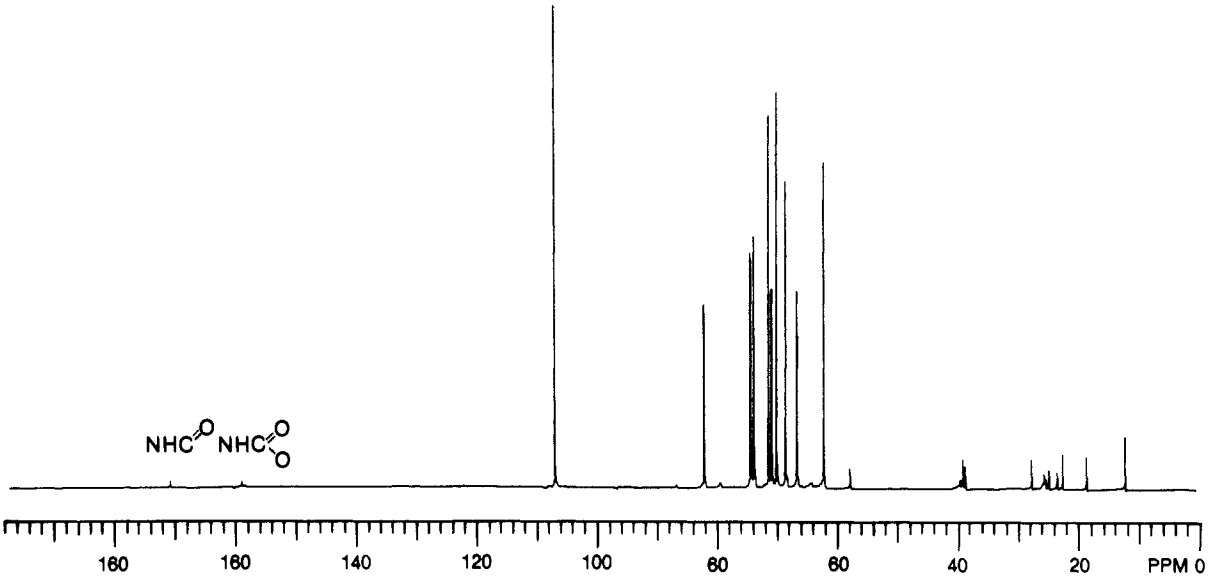


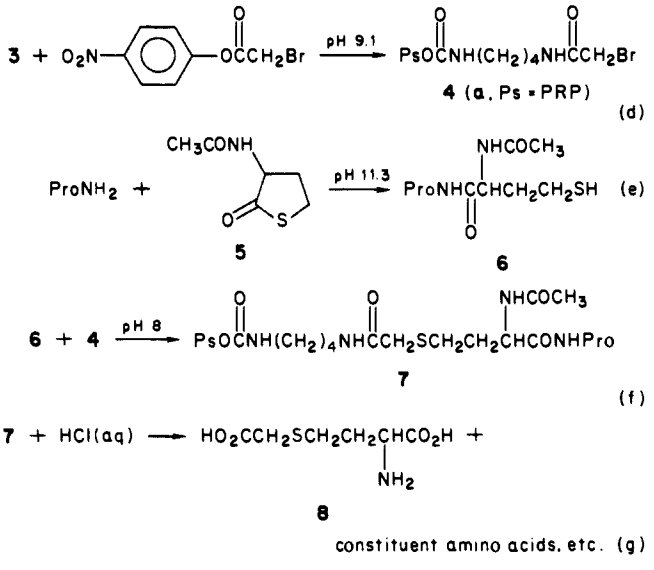
Figure 1. ^{13}C NMR of PRP-BuA_2-BrAc.

Table III. Ps Conjugates of NMP[a]

Ps	Δ[b]	Ps/Pro[c]	SCMHC/ Lys	recovery,[d] wt % Ps based	recovery,[d] wt % Pro based
6B	138	0.66	0.084	12	15.8
14	146	0.37	0.128	8.2	19.7
19F	125	0.39	0.027	23.8	43
	130	0.20	0.032	6.6	35
23F	136	0.31	0.057	5.9	19
PRP					
column	340	0.09	0.019	3.6	33
uncapped	300	0.33	0.060	12.8	39.8
capped[e]	300	0.26	0.035	12.5	48

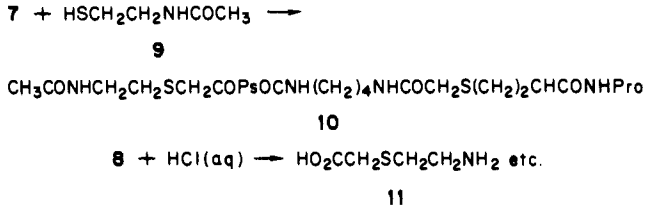
[a] Prepared by centrifugation method. [b] Δ, fluorescamine titer of **3** minus titer of **4**. [c] Determined as in the Experimental Section. [d] Based on assay of final product solutions/weight of starting material × 100. [e] *S*-(Carboxymethyl)cysteamine/Lys = 0.197.

the site of junction. The following reactions were used to effect this strategy:



The bromoacetylation product **4** is assayed for residual amine by fluorescamine, and the difference between its titer and that of **3** defines a value Δ, which is indicative of the bromoacetyl (BrAc) level in **4**. It should be noted that alkylation of nitrogen in **3** is very unlikely considering that the reaction of *p*-nitrophenyl bromoacetate with 6-aminohexanol results only in acylation and no detectable bromide ion.

From the Spinco amino acid analysis of **7** (after hydrolysis) on can obtain a ratio of moles of **8**/mole of lysine (any protein constituent amino acid would be appropriate if it is stable to the hydrolysis conditions). This value (SCMHC/Lys) reflects the degree of covalency between the two macromolecules and may be likened to the number of rungs between the rails of a ladder. From the data it was generally clear that not all bromoacetamide moieties in **7** were reacted, and it was desired for reasons of vaccine safety that their alkylating potential be eliminated. Therefore, a "capping" procedure was developed that utilizes *N*-acetylcysteamine (**9**) and affords a capped conjugate **10**. On hydrolysis **10** affords another unique, determinable amino acid, *S*-(carboxymethyl)cysteamine (**11**, SCMCA). The sum of **8** and **11** is found as expected to be equal to the number of bromoacetyl groups established by the value of Δ.



The outer membrane protein of *N. meningitidis* described by Helting[7] was chosen as the protein partner since it is a very effective inducer of the immune response and was expected to elicit antibodies to the polysaccharides (Table I) when conjugated to the latter. This protein is a multimer of a 40 000-Da unit with molecular weights in the (3–5) × 10^7 range. It is isolated as a milky colloidal suspension and will not resuspend if it is lyophilized to a dry state. However, ultracentrifugation pellets the protein in a form that can be resuspended by homogenization with a Dounce tissue homogenizer. Derivatives of the protein (i.e., **4**) and the polysaccharide conjugates share this property, and this allows a convenient way of separating unconjugated polysaccharides and their derivatives (i.e., **2** and **3**) from the conjugates. All nonconjugated materials in these systems were shown *not* to pellet under the same centrifugation conditions. These separations are important since the vaccine dosage is based on the polysaccharide titer of the conjugate, but available assays can only determine total polysaccharides. The protein is readily acylated by *N*-acetylhomocysteine thiolactone (**5**), liberating a thiol group whose titer can be determined by Ellman assay.[15] The level

(15) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.

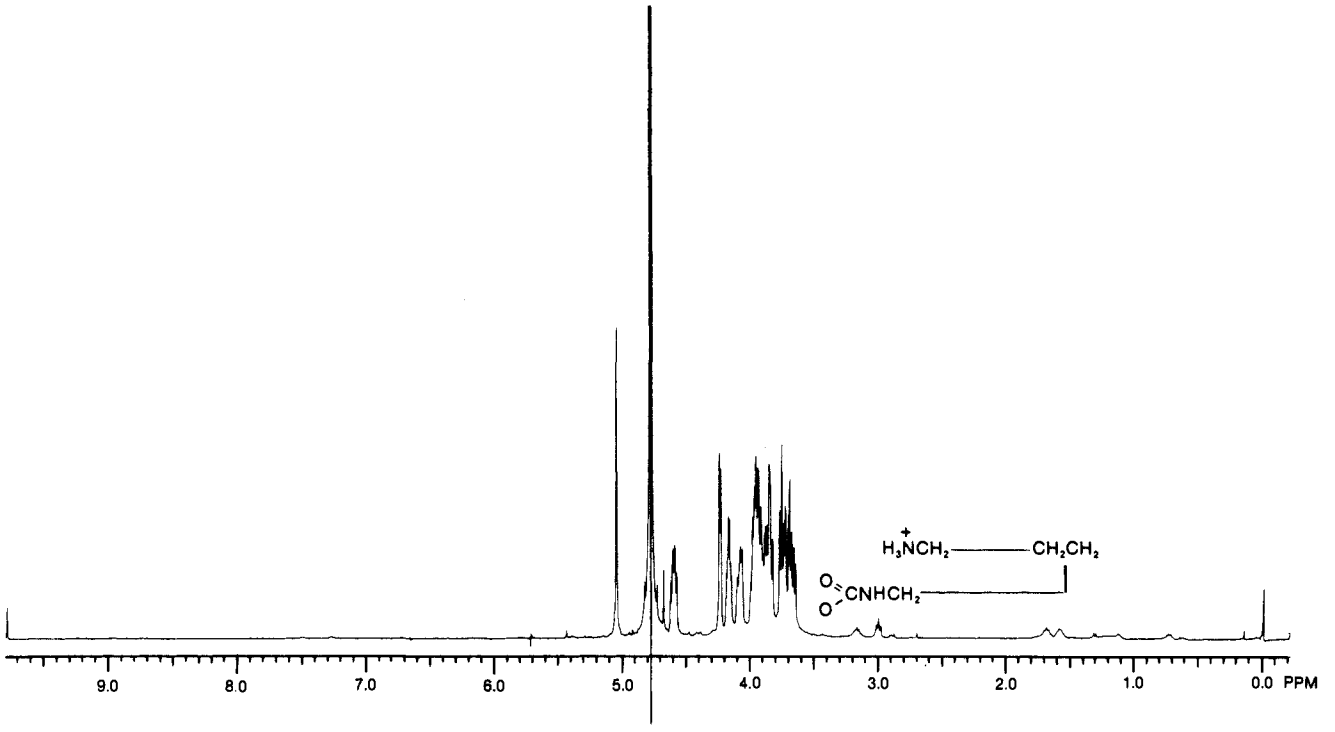


Figure 2. 400-MHz ^{1}H NMR spectrum of PRP–BuP$_2$ (**3a**).

corresponds to about 25% of the number of lysines in the protein that are presumed to be the sites of acylation. All efforts to increase this percentage have been unsuccessful. The bigeneric spacer analysis, to be meaningful, requires scrupulous removal of any free homocysteine or thiolactone, a consideration that also applies to subsequent chemistry and analysis. This can be accomplished by Sephadex G-25 chromatography, but this is accompanied by unacceptably large losses of protein that stick to the column. More efficiently, the separation can be effected by ultracentrifugation. No more than 4% of thiols found in the pellet are small molecules as shown by the control experiment where rethiolation is done with an iodoacetamide-capped protein. This 4% may also be due to an incremental thiolation of the protein. Considering the separation and thiol titer of the product, one can infer that reaction with thiolactone had produced a covalent product.

The final conjugate **10** is then characterized by its SCMHC/Lys ratio, its SCMCA/Lys ratio, and its polysaccharide/protein ratio as determined by conventional assays (Table III). Additionally, the values of K_d (Table II) of the intermediate derivatives afford another parameter that can be varied and correlated with the biological and other properties of the conjugate.

C. NMR. In order to argue that **7** is a covalent conjugate, it is important to establish that derivatives **3** and **4** are covalent. A nondialyzable amine titer does not necessarily imply covalency since the acidic Ps could bind amine electrostatically in the same way as a cation-exchange resin. Utilizing NMR spectroscopy, we established our structural formulation of **3a** and **4a** in accord with the following arguments. ^{13}C NMR signals (Figures 1) at 159.1 and 170.7 ppm are consistent with carbamate and amide carbonyl carbons, respectively. The latter signal was not present in PRP or **3a**, and the former was absent in PRP. The ^{1}H NMR spectrum is also supportive of our contention.

In Figure 2, the signals between 3.6 and 5.1 ppm represent the Ps protons, while those at higher field are attributable to the 1,4-butanediamine methylene protons and some resonances due to residual tetrabutylammonium ion. The peaks at 3.0 and 3.2 ppm are typical chemical shifts for methylene protons bonded to nonbasic or protonated nitrogen, and these two types may be distinguished by comparing D_2O spectra with those obtained at higher pH. Removal of a positive charge normally results in a 0.3–0.5 ppm upfield shift of the adjacent methylene protons, whereas the α-methylene protons in a nonbasic (i.e., carbamate) nitrogen compound were unaffected. It can be inferred from the spectrum after spiking with 1,4-butanediamine dihydrochloride that the 3.0 ppm signal, which is overlayed by added compounds, is attributable to $CH_2NH_3^+$ and the 3.2 ppm signal to the CH_2NHCO_2. This view was confirmed when the spectrum obtained after addition of deuterated NH_3 revealed that the 3.0 ppm peak but not the 3.2 ppm peak was displaced upfield. The spectrum of **3a** in which the remaining traces of tetrabutylammonium ion had been removed by additional dialysis shows four separate signals for the 1,4-butanediamine methylenes. This indicates that the two nitrogens are nonequivalent, which supports our contention that one is acylated and one is a basic amine. Their comparable areas indicate that little, if any, cross-linking of Ps has occurred.

Experimental Section

Preparation of the Tetra-*n*-butylammonium Salt of *H. influenzae* Type b Polysaccharide (1a). A 250-mL round-bottom flask fitted with a magnetic stirrer was charged with 3.29 g of PRP and 84 mL of water. The mixture was stirred for 20 min, and then an additional 15 mL of water was added. Stirring was continued for an additional 0.5 h until all PRP was in solution. The PRP solution was then applied to 150 mL of Dowex 50X8 (200–400 mesh; tetrabutylammonium form) in a 45–270-mm column. Ten milliliters of water was used as a rinse. The column was topped with water, and pressure was applied with a hand pump. Fractions of 50 mL were collected, and each tube was assayed for organic material by spotting on a silica gel plate, spraying with 1% $Ce^{IV}SO_4/H_2SO_4$, and heating on a hot plate. A total of 190 mL of organic-containing eluent was lyophilized, affording 3.56 g of **1a**. The product was stored in vacuo over P_2O_5 at −20 °C.

Preparation of 3a. 1,4-Butanediamine dihydrochloride (1.46 g) was dissolved in 58 mL of H_2O and the pH adjusted to 10.35 with 5 mL of 2.5 N NaOH. To a flask containing 0.64 g of **1a** was added 17.5 mL of DMF, and the mixture was stirred at room temperature for 25 min at which point almost all the material appeared to be in solution. Eighty milligrams of $COIm_2$ was then added in one portion to the DMF solution. The flask was capped, and the solution was stirred at room temperature for 35 min. The solution was then added to 32 mL of the ice-cooled 1,4-butanediamine solution, and the resultant solution was stirred in an ice bath for 15 min and at room temperature for an additional 17 min. The solution was then transferred to 17-in Spectropor 2 dialysis tubing (cylinder volume 0.21 mL/mm), was dialyzed in a 4 °C room vs. 8 L of 0.01 M pH 7.0 phosphate buffer for 5 h, and then was dialyzed twice vs. a fresh 8 L of phosphate buffer first for 5 h and then for 11 h. Finally, the solution was dialyzed vs. 18 L of water for 6 h. The solution was then

lyophilized, and there was obtained 0.48 g of **3a**. The fluorescamine assay indicated 468 nmol of NH_2/mg.

Preparation of 4a. To 380 mg of **3a** dissolved in 37 mL of a pH 9.15 buffer was added 346 mg of *p*-nitrophenyl bromoacetate in 9 mL of acetonitrile, and the mixture was stirred at 4 °C for 24 h and then transferred to 18 in. of dialysis tubing (Spectropor 2). This solution was then dialyzed vs. 18 L of H_2O for 5.25 h and then vs. a fresh 18 L of H_2O for 17.25 h at 4 °C. After lyophilization, 0.25 g of **4a** was obtained, which had a titer of 128 nmol of NH_2/mg, resulting in a Δ of 340 nmol of BrAc groups/mg.

Conjugation of 4a to Functionalized *N. meningitidis* Membrane Protein (NMP). Column Method. Forty-two milligrams of ethylenediaminetetraacetic acid disodium salt and 8 mg of dithiothreitol were added to 5 mL of a pH 11.3 borate buffer. Then, 3.8 mL of this solution and 11.5 mL of an NMP solution (5.5 mg/mL) were mixed, and the pH was adjusted to 11.39 with 0.05 mL of 2.5 N NaOH. After the solution was degassed and the air replaced by N_2, 53 mg of *N*-acetylhomocysteine thiolactone was added in an N_2 box and the resultant solution aged at room temperature for 16.7 h. It was then applied to a 120-mL Sephadex G-25 (fine) column (in the N_2 box). The column had been equilibrated and was eluted with 0.1 M pH 8 phosphate buffer. Fractions of 5 mL were collected, and base-line separation of high from low molecular weight thiols, as assayed by Ellman reagent, was effected. The high molecular weight fractions were combined and added to 0.06 g of **4a** and aged for 6 h at room temperature in N_2. The solution was then twice dialyzed vs. 18 L of H_2O for 15 and 25 h. The dialysate (32 mL) was centrifuged in polycarbonate tubes at 37 000 rpm for 2 h in a Ti 60 rotor. After resuspension of the pellets in H_2O and recentrifugation with the same conditions, the second pellets were resuspended with a Dounce homogenizer in 15 mL of H_2O. Found: Pro, 1800 μg/mL; PRP, 166 μg/mL; SCMHC/Lys, 0.027.

Conjugation–Centrifugation Method. All centrifugations were performed in polycarbonate tubes and unless otherwise noted at 43 000 rpm for 2 h at 4 °C in a Beckman Ti 75 rotor. All manipulations of thiolated material were done in an N_2 box. The unfunctionalized NMP (20 mL, 5.5 mg/mL) was centrifuged, and the pellets were resuspended with a Dounce homogenizer in 8 mL of a thiolation solution (126 mg of ethylenediaminetetraacetic acid disodium salt, 24 mg of dithiothreitol in 15 mL of pH 11 borate buffer). After the solution was degassed and the air replaced with N_2, 110 mg of *N*-acetylhomocysteine thiolactone was added in an N_2 box. The solution was aged for 18.7 h at room temperature and then diluted with 5 mL of 1 M KH_2PO_4 and 5.2 mL of 0.1 M pH 8 phosphate buffer (the resultant pH is 7.15). This solution was centrifuged, and the pellets were resuspended in 20 mL of the pH 8 buffer and recentrifuged. The pellets from this second centrifugation were resuspended with a Dounce homogenizer in 9 mL of pH 8 buffer. An Ellman assay indicated a total of 9 μmol of SH. To this resuspended thiolated NMP solution was added 112 mg of **4a** (Δ = 300) and the solution was degassed and aged for 17.8 h. Then, 11 mL of H_2O was added and the solution centrifuged. The pellets were resuspended in 20 mL of a "capping" solution (212 mg of *N*-acetylcysteamine/30 mL of pH 8 buffer) and aged under N_2 for 18 h. The solution was then centrifuged, the pellets were resuspended in 20 mL of H_2O, and the solution was recentrifuged. The final pellets were resuspended (Dounce) in 50 mL of H_2O, affording the "capped" PRP–NMP conjugate. Found: Pro, 905 μg/mL; PRP, 233 μg/mL; SCMHC/Lys, 0.035; SCMCA/Lys, 0.197.

Control Experiment Concerning Thiol Content of Centrifugation Pellet. Ten milliliters of a solution containing 59 mg of NMP was centrifuged and then "thiolated" with 54 mg of *N*-acetylhomocysteine thiolactone exactly as in the above example except that a Ti 80 rotor was used in all centrifugations. The pH of the reaction solution was adjusted to 8.5, the solution centrifuged, the pellet resuspended at pH 8.0, and the solution centrifuged a second time. The resultant pellet, resuspended in 10 mL of pH 8 buffer, was assayed and found to contain a total of 6.0 μmol of thiol. Fifty milligrams of iodoacetamide was added to this solution, which was then aged for 18 h. The resultant reaction mixture was centrifuged, the pellet resuspended in 10 mL of H_2O, and the solution was recentrifuged. The resulting capped pellet was thiolated exactly as above (using 54 mg of *N*-acetylhomocysteine thiolactone/19 h). The reaction solution was centrifuged, the pellet resuspended (pH 8 buffer), and the solution recentrifuged. The second pellet was resuspended in 6.5 mL of pH 8 buffer, assayed, and found to have a total of 0.26 μmol of thiol corresponding to 4% of the first thiolation.

General Procedures. Ribose assays were done by the method of Dische and Schwarz,[16] methylpentose assays by the method of Dische and Shettles,[17] and hexose assays by the method of Scott and Melvin.[18] Protein assays were done by the method of Lowry,[19] and amino acid analyses were performed on Beckman 121 M13 and Beckman System 6300 amino acid analyzers.

A general concept, the bigeneric spacer concept, has been conceived, which allows the unambiguous assignment of the level of covalency of protein–polysaccharide conjugates. A general method has been devised, transforming acidic polysaccharides from water-soluble to organic-soluble entities by converting them to their tetrabutylammonium salts. This allows a variety of chemistries not asscessible in aqueous systems. Both of these have allowed the preparation of unique Ps–Pro conjugates, which are useful as vaccines.

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Registry No. $COIm_2$, 530-62-1; $NH_2(CH_2)_4NH_2$, 110-60-1.

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