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^{31}P- AND ^{13}C-N.M.R.-SPECTRAL AND CHEMICAL CHARACTERIZATION OF THE END-GROUP AND REPEATING-UNIT COMPONENTS OF OLIGOSACCHARIDES DERIVED BY ACID HYDROLYSIS OF *Haemophilus influenzae* TYPE b CAPSULAR POLYSACCHARIDE

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ABSTRACT

Haemophilus influenzae type b capsular polysaccharide [repeating unit, →3)-β-D-Rib*f*-(1→1)-D-Ribol-5-(PO_2H→] was partially hydrolyzed with HCl to give oligosaccharides that were isolated by size-exclusion chromatography, and then characterized by ^{31}P- and ^{13}C-n.m.r.-spectral and chemical methods, in order to determine the end-group composition and, hence, the number-average chain-length ($\bar{L}$). The ratio (~17:8) of monophosphate end-groups to D-ribofuranose end-groups revealed the relative rates of hydrolysis of the phosphoric diester linkage and the glycosidic linkage in the repeating-unit structure. Cleavage of the phosphoric diester linkage was ~92% regioselective, as indicated by the ~12:1 ratio of D-ribofuranose monophosphate end-groups to D-ribitol monophosphate end-groups. The n.m.r. spectra of the oligosaccharide repeating-unit provided evidence for partial stereomutation (~3–8%) that involved rearrangement of the D-ribofuranose phosphoric diester linkage and anomerization at C-1 of D-ribofuranose. Variously sized oligosaccharides (L = 4, 7, and 12) that had D-ribofuranose end-groups reacted with bovine serum albumin that had an average of ~9 adipyl hydrazide functionalities, to give, within experimental error, quantitative yields of the corresponding, hydrazone-linked, oligosaccharide–protein conjugates.

INTRODUCTION

We have recently employed a combination of n.m.r.-spectroscopic, chemical, and physical methods to study the base-catalyzed depolymerization of phosphoric diester-linked capsular polysaccharides isolated from *Haemophilus influenzae*[1] and *Streptococcus pneumoniae*[2]. In view of the pathogenicity of the *H. influenzae* type b organism[3], and considering the widespread interest in immunogenic, carbohydrate–protein conjugates[4–14], it was desirable to investigate further the chemistry of the

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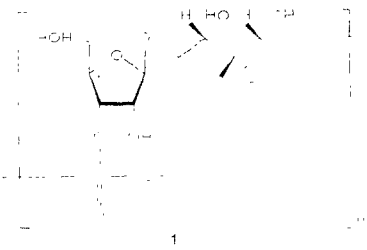


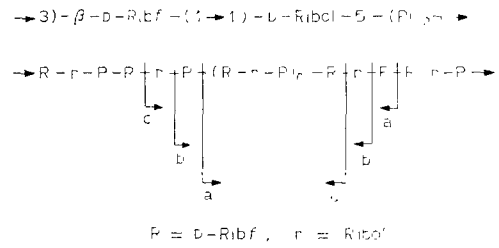
Repeating-unit structure of *H. influenzae* type b capsular polysaccharide (HIB), shown in its protonated form, →3)-β-D-Rib*f*-(1→1)-D-Ribol-5-(PO_2H^-).

H. influenzae type b capsular polysaccharide (HIB; see formula **1**) with the aim of preparing structurally defined oligosaccharides for conjugation with proteins. The present study of the oligosaccharides (hib_n) derived by acid-catalyzed depolymerization of HIB reports methodology for a *complete* end-group and chain-length analysis of hib_n, together with n.m.r.-spectral evidence for stereomutated, repeating-unit linkages in these oligosaccharides, and the use of D-ribofuranose end-groups as the specific site for one-step attachment of hib_n to a modified protein. This conjugation chemistry was based upon several factors: (*i*) the availability[4] of derivatized bovine serum albumin bearing adipyl hydrazide groups, (*ii*) the "supernucleophilicity"[15] of such hydrazides, and (*iii*) the chemical stability of *aldehydo*-glycohydrazones[16].

RESULTS AND DISCUSSION

Preliminary considerations of HIB hydrolysis, hib_n characterization, and conjugation. — In contrast to the alkaline depolymerization of HIB, which occurs mainly by cleavage of phosphoric diester linkages[1], the acid hydrolysis of HIB should cleave phosphoric diester bonds and the glycosidic linkage, due to oxygen stabilization of the incipient carbonium ion at C-1 of the D-ribosyl residue[17]. These proton-mediated, hydrolysis modes are depicted in Scheme 1 by the letters "a" and "b" for the two bonds to phosphorus, and the letter "c" for the glycosidic linkage; a corresponding set of primed letters is included, in order to account for the fact that each hydrolysis mode results in the formation of two non-equivalent end-groups. Accordingly, the nine combinations of unprimed and primed letters designate all of the possible oligosaccharide products, which differ by virtue of their pair-wise combinations of end-groups. Oligomers of types 3, 6, and 9 have D-ribo*furanose* termini (e.g., PO_2H→3-β-D-Rib*f*) for coupling to a protein, whereas the D-ribo*furanosidic* termini [β-D-Rib*f*-(1→1)-D-Ribol,] in oligomer-types 1–3 would be comparatively unreactive.

Equal rate-constants for linkage hydrolysis ($k_a = k_b = k_c$) would thus give a 33% yield of "usable" material, *i.e.*, oligosaccharides having a D-ribofuranose end-group; however, with mechanistic bias, the yield of "usable" oligosaccharides could vary from ~100% ($k_c \gg k_a, k_b$) to ~0% ($k_c \ll k_a, k_b$). To evaluate these kinetic factors unambiguously, we planned to use a ^{31}P-n.m.r.-spectral method[1] for quantifying the relative amounts of D-ribofuranosidic 3-monophosphate termini (PO_3H_2→3-β-D-Rib*f*-) and D-ribitol 5-monophosphate termini (e.g., D-Ribol-5→PO_3H_2); more-



P = D-Ribf , r = Ribo'

Oligomer type	Cleavage modes	Oligomer end-groups with additional residues
1	a, a'	β-D-Ribf-(1→1)-D-Ribol , [illegible]
2	a, b'	β-D-Ribf-(1→1)-D-Ribol , [illegible]
3	a, c'	β-D-Ribf-(1→1)-D-Ribol , PO_2H→3-D-Ribf
4	b, a'	PO_3H_2→3-β-D-Rib , [illegible]
5	b, b'	PO_3H_2→3-β-D-Ribf , [illegible]
6	b, c'	PO_3H_2→3-β-D-Ribf , PO_2H→3-D-Ribf
7	c, a'	D-Ribol-5→$(PO_2H$, [illegible]
8	c, b'	D-Ribol-5→$(PO_2H$, β-D-Ribf-(1→1)-D-Ribol
9	c, c'	D-Ribol-5→$(PO_2H$, PO_2H→3-D-Ribf

Scheme 1. Line formula for the repeating-unit structure of HIB, and a schematic representation of the hydrolytic cleavage modes for HIB, where R, r, and P respectively symbolize the D-ribosyl and D-ribitol residues, and phosphoric diester groups in the repeating unit. The lettered, vertical lines designate the cleavage sites and the corresponding end-groups that were conceptually associated with the (R-r-P)$_n$ oligomer products. These hib$_n$ products are designated types 1–9.

conventional procedures[2,18] could be used to measure the relative amount of D-ribofuranose termini in the oligosaccharide mixture. This trio of end-groups results from hydrolysis modes b, a', and c', respectively, whereas hydrolysis modes b', a, and c lead to the remaining three classes of end-groups that are designated ...D-Rib*f*-(1→1)-D-Ribol, β-D-Rib*f*-(1→1)-D-Ribol..., and D-Ribol-5→$(PO_2H$..., respectively. The latter three classes of end-groups would be automatically quantified as a result of $k_a = k_{a'}$, $k_b = k_{b'}$, and $k_c = k_{c'}$. Thus, the proportion of the *directly* measured trio of end groups is sufficient to define the number-average chain length (n) for hib$_n$. Moreover, the measurements proposed would establish both the regioselectivity for phosphoric diester-linkage hydrolysis ($k_{a'}/k_b$) and the chemoselectivity for hydrolysis at the phosphorus *vs.* anomeric reaction-centers [$(k_{a'} + k_b)$ *vs.* $k_{c'}$].

Preparation and characterization of hib$_n$. — The ^{31}P-n.m.r. spectrum (see Fig. 1A) of a sample of HIB at pH 7 consisted of an intense signal at 1.03 p.p.m., and three relatively weak signals, at 4.09, 18.92, and 20.90 p.p.m., that had previously been assigned[1] to the repeating-unit phosphoric diester linkage (1.03 p.p.m.), terminal monophosphates (4.09 p.p.m.), D-ribitol 4,5-cyclophosphate (18.92 p.p.m.) end-groups, and D-ribose 2,3-cyclophosphate (20.90 p.p.m.) end-groups. The relative, integrated intensities of these signals (97.77, 0.24, 0.54, and 1.45%, respectively) indicated that $\bar{L} = \sim 44$. ^{31}P-N.m.r. analysis of a second sample of HIB gave $\bar{L} = \sim 84$. These capsular-polysaccharide starting-materials were therefore designated

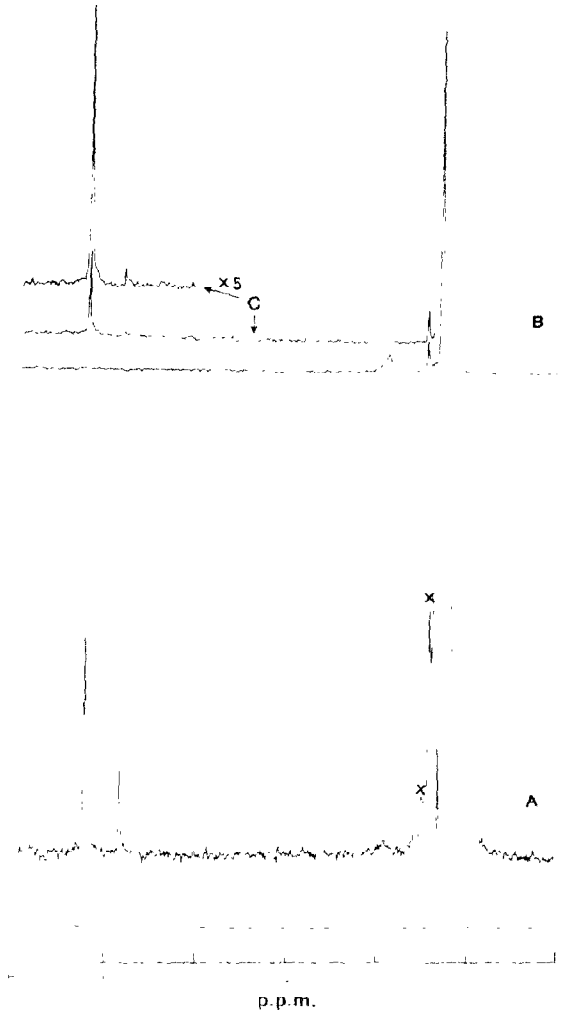


Fig. 1. $^{31}P\{^1H\}$-N.m.r. spectra (40.25 MHz) recorded for solutions in imidazole buffer [20% (v/v) of D_2O] at pH 7 and ~20°; see text for signal identifications. [A. A sample of HIB_{11} prior to acid hydrolysis; the lower and upper traces have relative Y-gains of 1 and 64, respectively; "x" refers to a spinning side-band. B. A sample of hib_{11} that was derived by hydrolysis of HIB_{11} in 0.1M HCl for 15 min at 60°. C. The same sample of hib_{11} after reaction with DEAE.]

HIB_{44} and HIB_{84}, respectively. The combined, pseudo-first-order rate-constant for hydrolysis of the repeating-unit phosphoric diester linkages ($k' = k_a + k_b = k_{a'} + k_{b'}$) in 0.1M HCl at 60° could not be measured accurately by ^{31}P-n.m.r. spectroscopy, due to overlapping absorption-signals for phosphoric diester and product monophosphate; however, the initial rate of disappearance of the phosphoric diester signal

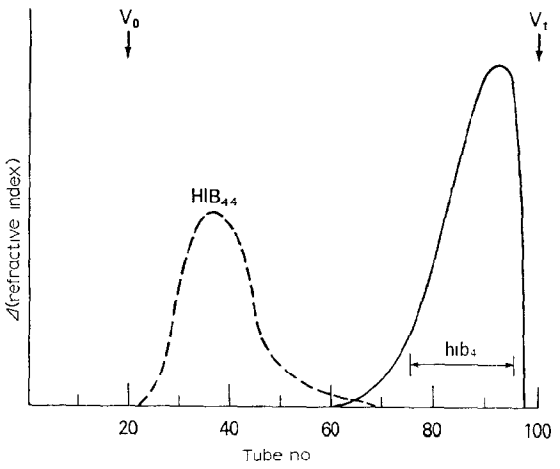


Fig. 2. Traces of the differential-refractometer readings during size-exclusion chromatography using Sephadex G-100 that was equilibrated and eluted with 0.2M NaCl. [The dashed trace was obtained with HIB$_{44}$ prior to hydrolysis in 0.1M HCl for 15 min at 60°, which gave the hydrolyzate used to obtain the solid-line trace; fractions 75–92 were pooled, to give the sample of hib$_n$ oligosaccharide.]

gave $\tau_{1/2} \leqslant \sim 60$ min. The corresponding half-life for acid hydrolysis of the glycosidic linkage of the repeating unit was not measured. Application of a statistical theory[2,19], of depolymerization indicated that <5 to 10% of random cleavage of the hydrolytically labile linkages in either HIB$_{44}$ or HIB$_{84}$ would lead to a substantial fraction of the desired, relatively short, oligosaccharide fragments ($\bar{L} < 20$). Based on these kinetic measurements and calculations, it was possible to select appropriate hydrolysis periods for depolymerization of the HIB samples.

Hydrolysis of HIB$_{44}$ in 0.1M HCl for 15 min at 60° followed by size-exclusion chromatography (see Fig. 2) afforded oligosaccharide material that gave, at pH 7, a ^{31}P-n.m.r. spectrum (see Fig. 1B) in which the repeating-unit signal at 1.03 p.p.m. was accompanied by overlapping monophosphate signals at ~4 p.p.m. and a new signal at 1.79 p.p.m. There were no downfield absorptions indicative of 5-membered-ring, cyclophosphate end-groups[1,2], in accord with their (well known) acid lability[20]. The ^{31}P-n.m.r. spectrum (not shown) at pH 4 confirmed the assignment of the monophosphate signals, which were now shifted upfield by[1,21] ~3–4 p.p.m. The spectrum at pH 4 also demonstrated that the signal at 1.79 p.p.m. was unaffected by increased acidity. Because ^{31}P chemical-shifts for phosphoric diesters are pH-independent over the range[21] of pH ~2–12, the absorption at 1.79 p.p.m. was tentatively attributed to rearranged phosphoric diester linkages that accounted for 8% of the total number of phosphoric diester bonds, based on the relative signal-intensities at 1.79 and 1.03 p.p.m. The relative, integrated intensities of the monophosphate absorptions (~4 p.p.m.) and both types of phosphoric diester linkages (1.03 and 1.79 p.p.m.) indicated that the oligosaccharide sample had 16.7 monophosphate end-groups/100 repeating-units. Reaction of this sample with 3-(3-dimethylaminopropyl)-

TABLE I

END-GROUP COMPOSITION OF hib_n SAMPLES DERIVED FROM HYDROLYSIS[a] OF HIB

End group	hib_1	hib_7	hib_{12}
	End groups/100 repeating-units		
$PO_3H_2 \rightarrow 3$-β-D-Rib*f*...[b,c]	16.8	10.0	5.0
...D-Ribol-5-$\rightarrow PO_3H_2$[b,d]	1.0	1.2	0.4
	17.8 (16.7)	11.2 (8.6)	5.4 (6.0)
..$PO_2H \rightarrow 3$-β-D-Rib*f*[e]	9.3, 7.2	4.0, 4.3	n.d.[f], 2.5
Total[g]	25.4	14.0	8.2

[a]Refers to 0.1M HCl at 60°; HIB_{11} afforded hib_1 and hib_7 after 15-min and 7-min hydrolyses, respectively; HIB_{81} afforded hib_{12} after 3-min hydrolysis. [b]Determined by ^{31}P-n.m.r. signal integrations (±5–10%) before and after cyclization with DEAC (total cyclophosphate composition is given in parentheses); see Experimental section for details. [c]The alternative monophosphate location is C-2 of the terminal D-ribose residue. [d]The alternative monophosphate location is C-4 of the terminal D-ribitol residue. [e]For each hib_n sample, the first and second entries respectively refer to values determined by ^{13}C-n.m.r. signal integrations (±5–10%) and a reducing sugar assay (±5%); see Experimental section for details. [f]Not determined by ^{13}C-n.m.r. spectroscopy. [g]Total proportion of the three types of end-groups that defined the value of *n*. For each hib_n sample, the various end-group determinations were averaged, *e.g.*, for hib_1, [(17.8 + 16.7)/2] + [(9.3 + 7.2)/2] = 25.4.

1-ethylcarbodiimide hydrochloride (DEAC), at pH 7 and 25°, transformed (see Fig. 1C) the monophosphate end-groups into their corresponding cyclophosphate termini[1,2], *viz.*, D-ribose 3-monophosphate into D-ribose 2,3-cyclophosphate (20.90 p.p.m.), and D-ribitol 5-monophosphate into D-ribitol 4,5-cyclophosphate (18.92 p.p.m.)*.

The relative, integrated signal-intensities indicated the presence of 16.8 D-ribose 2,3-cyclophosphate end-groups/100 repeating-units (1.03 and 1.79 p.p.m.), and 1.0 D-ribitol 4,5-cyclophosphate end-groups/100 repeating-units. The total proportion of these cyclophosphate end-groups was ~7% higher than the proportion of monophosphate end-groups that was measured prior to the reaction with DEAC; however, this relatively small difference was within the estimated error limits (±5–10%) for the n.m.r. integrations. The proportions of phosphorus-containing end-groups measured before and after the reaction with DEAC were, therefore, averaged, to give a value of 17.2 ±0.6 monophosphate termini/100 repeating-units of the original, oligosaccharide sample. The signal at 1.79 p.p.m. was unaffected by the DEAC treatment, and thus provided further evidence for its assignment to a re-arranged phosphoric diester linkage.

^{31}P-N.m.r. data (see Table I) that were obtained for the oligosaccharide material derived from similar hydrolysis of HIB_{44} for 7 min gave an average value

*The same cyclophosphates would be respectively formed from D-ribose 2-monophosphate and D-ribitol 4-monophosphate termini that may arise either from phosphate migration or non-selective ring-opening of cyclophosphate intermediates[3] during the acid hydrolysis of HIB

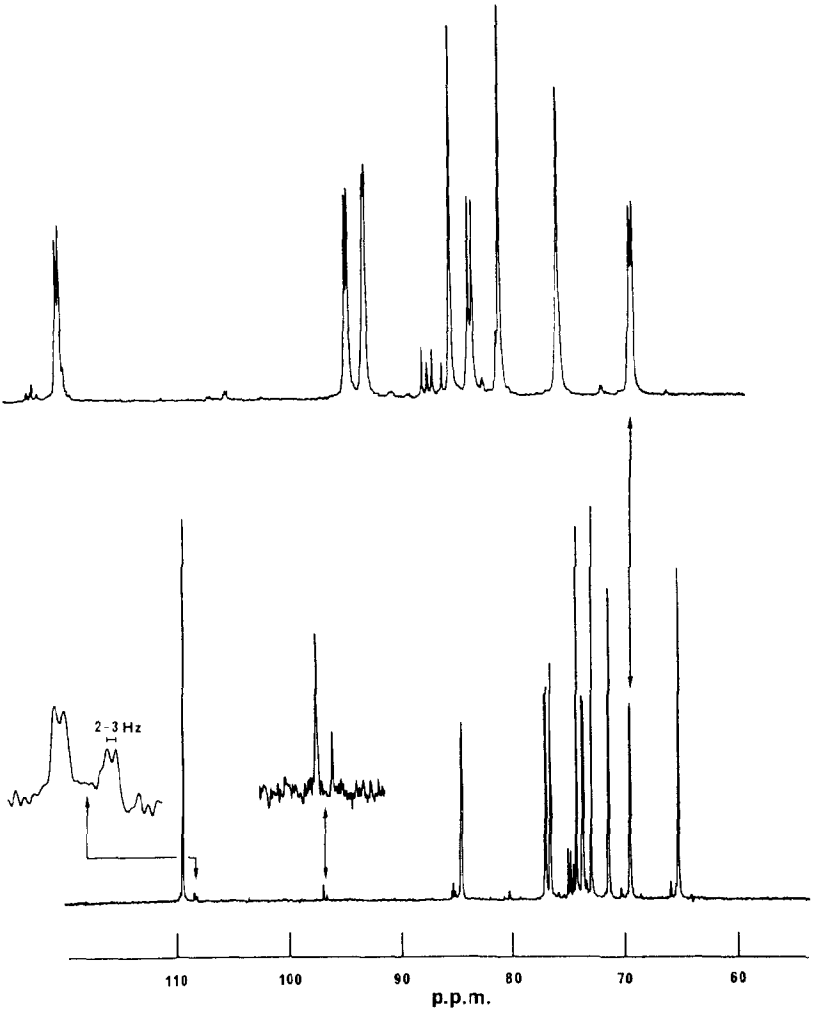
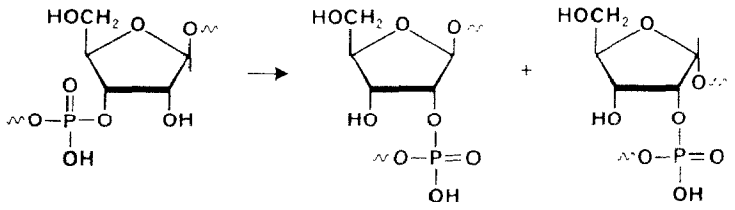


Fig. 3. $^{13}C\{^1H\}$-N.m.r. spectra (75.47 MHz) recorded for solutions in water [5% (v/v) of D_2O] at ~20°; see text for signal identifications. [The sample of hib_n was derived by hydrolysis of HIB_{44} in 0.1M HCl for 7 min at 60°. The lower trace shows the entire spectrum, whereas the remaining inserts are expanded displays of selected regions indicated by the connecting arrows.]

of 9.9 ±1.3 monophosphate termini/100 repeating-units, and 3.5 ±0.5% of phosphoric diester rearrangement. The oligosaccharide obtained on hydrolysis of HIB_{84} for 3 min had an average value of 5.7 ±0.3 monophosphate termini/100 repeating-units, and contained ~2% of rearranged-phosphoric diester linkages. In contrast to this proportionality between the degree of depolymerization and the extent of phosphoric diester rearrangement, the partitioning associated with phosphoric diester bond cleavage, to give D-ribose and D-ribitol monophosphates, was apparently independent of the reaction time, as evidenced by the roughly 10:1 ratio (see Table I)



Scheme 2. Partial structures of hib$_n$ that show, on the left, the original connectivity and stereochemistry of the D-ribose residue and, on the right, two possible, rearranged D-ribose residues.

for generation of D-ribose monophosphate end-groups under each of the aforementioned reaction-conditions.

Previous studies[22] had shown that the ten carbon atoms in the HIB repeating unit (structure **1**) give rise to separate resonance-signals. There is observable ^{13}C–^{31}P coupling (J, Hz) for the D-ribose C-2 (77.00 p.p.m., J 5.2 Hz), C-3 (76.51 p.p.m., J 2.8 Hz), and C-4 (84.64 p.p.m., J 6.7 Hz), as well as the D-ribitol C-5 (69.43 p.p.m., J 7.9 Hz), whereas the downfield-shifted D-ribose C-1 signal, at 109.4 p.p.m., and the remaining carbon signals appear as sharp singlets. Each of the aforementioned, oligosaccharide samples exhibited these ten carbon absorptions, and also showed a set of additional signals that had relatively low intensity. The new signals for the 15-min hydrolyzate (see Fig. 3) had roughly twice the intensity of their counterparts in the spectrum (not shown) of the 7-min hydrolyzate, and were therefore indicative of oligosaccharide end-groups. In the studies now reported, the D-ribose C-1 nuclei were of primary importance for the following reasons. The expanded, spectral display (see Fig. 3) recorded for the 7-min hydrolyzate revealed that the D-ribofuranosic C-1 *singlet* at 109.4 p.p.m. was accompanied by *two doublets* at 108.4 (J ~2–3 Hz) and 108.2 p.p.m. (J ~2–3 Hz) that had a *combined* intensity of ~3%, relative to the signal at 109.4 p.p.m. The relative intensity of these minor signals was comparable to the extent of phosphoric diester linkage-arrangement (~3–4%) that was deduced from the corresponding ^{31}P-n.m.r. spectrum. Moreover, the chemical shifts[22,23] and coupling constants[24] for these two ^{13}C doublets were consistent with migration of phosphoric diester, from D-ribose C-3 to D-ribose C-2, that was accompanied by anomerization at D-ribose C-1 to give α (108.2 p.p.m.) and β (108.4 p.p.m.) stereochemistries, as shown in Scheme 2. The ~1:2 ratio of the signals at 108.2 and 108.4 p.p.m. was also observed in the expanded, ^{13}C-n.m.r. spectrum (not shown) of the 15-min hydrolyzate; however, in this case, the combined intensity of the absorptions at 108.2 and 108.4 p.p.m. indicated ~8% rearrangement of phosphoric diester linkage, which agreed with the corresponding ^{31}P-n.m.r.-derived value. Both of the oligosaccharide samples gave rise to an ~1:3 ratio of low-intensity *singlets*, at 96.57 and 96.87 p.p.m. (see Fig. 3) that were respectively assigned to α- and β-D-ribofuranose end-groups, based on the spectrum (not shown) of D-ribose, wherein the resonances of the α and β anomeric (C-1) atoms appeared as two signals that had virtually the same chemical shifts and relative intensities as those seen in the oligosaccharide spectra. These spectral similarities, and the fact that the 96.57- and

96.87-p.p.m. absorptions gave no evidence for significant ($\geqslant$ 2 Hz) ^{13}C–^{31}P coupling, indicated[24] that the D-ribofuranose end-groups had C-3 (rather than C-2) attachments to the phosphoric diester unit. Additional support for this conclusion was obtained by reactions of the 7-min hydrolyzate with $H_2NOH \cdot HCl$ and with adipic dihydrazide at pH 7 and 25°. In each case, the oligosaccharide product gave a ^{13}C-n.m.r. spectrum that was identical to the spectrum recorded for the starting material, except for the absence of absorptions at 96–97 p.p.m., and the appearance of new signals that had chemical shifts indicative of the expected imino carbon (HC=N), *viz.*, 154.1 p.p.m. for the oxime[15], and 153.6 and 158.9 p.p.m. for the *E*- and *Z*-hydrazones[16]. The reaction with adipic dihydrazide also provided ^{13}C-n.m.r. evidence against the formation of adducts of the pyranosylamine type[25] and the occurrence of Amadori-type rearrangement[26] after coupling of the oligosaccharide with the adipyl hydrazide groups.

Based on the foregoing identification of key signals in the ^{13}C-n.m.r. spectra of the oligosaccharide products, the molar ratio of D-ribofuranose end-groups to repeating units was given by the sum of the peak heights at 96.57 and 96.87 p.p.m. divided by the sum of the peak heights at 108.2, 108.4, and 109.4 p.p.m. The 7-min and 15-min hydrolyzates obtained from HIB_{44} were thus found to have 4.0 $\pm$0.1 and 9.3 $\pm$0.3 D-ribofuranose end-groups per 100 repeating-units, respectively. These molar ratios were subsequently estimated by an extension of the Park–Johnson[27] colorimetric assay for reducing sugars that used D-ribose as the calibration standard*. The estimated values of 4.3 $\pm$0.2 and 7.2 $\pm$0.4 D-ribofuranose end-groups per 100 repeating-units agreed reasonably well with the corresponding values determined

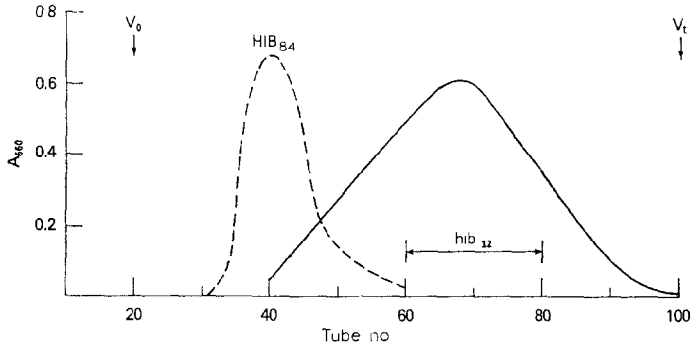


Fig. 4. Size-exclusion chromatography using Sephadex G-100 that was equilibrated and eluted with 0.2M NaCl; elution was monitored by the orcinol assay for D-ribose. [The dashed trace was obtained with HIB_{84} prior to hydrolysis in 0.1M HCl for 3 min at 60°, which gave the hydrolyzate used to obtain the solid-line trace; fractions 60–80 were pooled, to give the sample of hib_n oligosaccharide.]

*We had previously determined[2] that equimolar amounts of D-ribose, D-glucose, D-galactose, and L-rhamnose have significantly different color-forming capacities ($\pm$5 to 65% variance) in the Park–Johnson[27] assay. As the D-ribose calibration standard used in the work now reported is not chemically identical with the D-ribofuranose end-groups in hib_n, the assay can only provide an approximate value for the molar equivalents of these end groups.

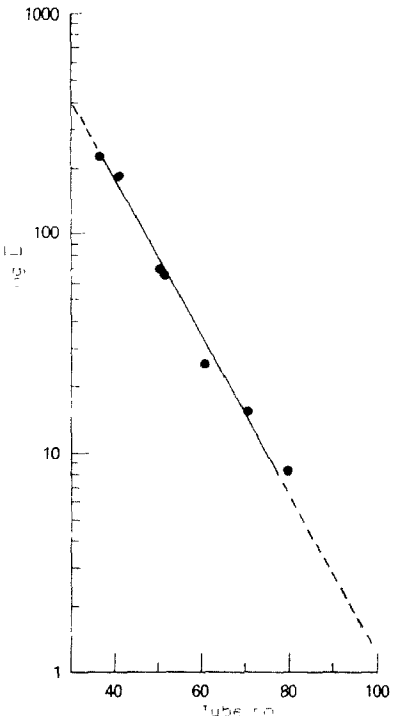


Fig. 5. Plot of log $\bar{L}$ *vs.* elution volume. [The data points were generated from the following information. For HIB_{81}: fractions 35–37, $\bar{L}$ = 230; fraction 40, $\bar{L}$ = 187; fractions 50–52, $\bar{L}$ = 66; for the hydrolyzate: fraction 50, $\bar{L}$ = 70; fraction 60, $\bar{L}$ = 25; fraction 70, $\bar{L}$ = 16; fraction 80, $\bar{L}$ = 8. For HIB_{84}, the values of $\bar{L}$ were determined by ^{31}P-n.m.r. spectroscopy. For the hydrolyzate, the values of $\bar{L}$ were determined by a combination of ^{31}P-n.m.r. spectroscopy and an assay for content of D-ribose end-group.]

by ^{13}C-n.m.r. spectroscopy and, consequently, the two sets of data were averaged. The total number of monophosphate end-groups and D-ribofuranose end-groups per 100 repeating-units (see Table I) gave oligosaccharide, number-average chain-lengths ($L = n$) that were rounded off to integer values: hib_4 and hib_7 for 15-min and 7-min hydrolyses of HIB_{44}, respectively.

The elution profiles (see Fig. 2) that were obtained for HIB_{44} and its oligosaccharide products suggested broad size-distributions for these materials. The *range* of chain lengths for products hib_4 and hib_7, which were isolated from relatively large pool-volumes, was therefore assessed in the following way. Samples of HIB_{84} and its hydrolyzate (0.1M HCl for 3 min at 60) were each subjected to size-exclusion chromatography, and the L values for various fractions were determined by ^{31}P-n.m.r. spectroscopy in conjunction with the D-ribofuranose end-group assay. The findings, summarized in Figs. 4 and 5, were in accord with the expected[28] linear relationship between log L and the elution volume, and further indicated that hib_4 and hib_7 were comprised of oligosaccharides that had chain-length ranges of ~2.5-

9.5 and ~2.0–23.0, respectively. Pooled fractions from the HIB_{84} hydrolyzate gave a sample of hib_{12} that had a chain-length range of ~8.3–25.4.

Preparation and characterization of adipyl hydrazide-functionalized bovine serum albumin (BSA-AH). — The general procedures developed by Robbins and co-workers[4] were used for the reaction of bovine serum albumin (BSA) with adipic acid dihydrazide (AAD) in the presence of DEAC. The extent of functionalization of BSA with nucleophilic adipyl hydrazide groups was estimated by measurements of protein content and $RNHNH_2$ content by amino acid analysis and the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay[29,30], respectively, which gave 9.7 ±0.4 mol. equiv. of $RNHNH_2$/mol. of BSA.

^{1}H-N.m.r. spectroscopy was briefly investigated as an alternative method for determining the degree of functionalization; however, the proton absorptions for AAD were not discernible in a surrogate sample that contained a 9.7:1.0 molar ratio of AAD:BSA. Consequently, doubly ^{13}C-labeled adipic acid (90 atom-% $^{13}CO_2H$) was converted into its dihydrazide derivative (AAD-$^{13}C_2$) for DEAC-mediated coupling with BSA. The 25.00-MHz, ^{13}C-n.m.r. spectra recorded (not shown) for the isolated product (BSA-AH-$^{13}C_2$) and for a surrogate sample that contained AAD-$^{13}C_2$ and BSA gave a molar ratio of AH-$^{13}C_2$ to BSA that was ~25% higher than the ratio determined by the aforementioned chemical methods, which was considered to be reasonably good agreement in view of the various experimental errors. The 67.9-MHz, ^{13}C-n.m.r. spectrum (not shown) of BSA-AH-$^{13}C_2$ at pH 7.4 and 25° appeared as a superposition of absorption signals centered ~0.2 p.p.m. upfield from the symmetrical peak that was recorded for the mixture of AAD-$^{13}C_2$ and BSA. These findings were consistent with exclusively covalent attachment of AAD to BSA *via* DEAC-mediated condensation.

The composition of the resultant BSA-AH product and its ability to conjugate with D-ribofuranose end-groups in hib_n were examined by parallel reactions of BSA and BSA-AH with D-ribose. It was assumed that D-ribose would couple with the pendant hydrazide functionalities in a 1:1 stoichiometry, and that D-ribose would also serve as a simple, model compound for roughly gauging the reactivity of the oligosaccharide D-ribofuranose end-groups of interest. The results obtained for 0.2M NaCl solutions that contained 100 mg of protein/mL and an ~200-fold molar excess of D-ribose indicated that only 0.25 mol of D-ribose was incorporated by 1 mol of BSA, whereas an additional 8.2 mol of D-ribose was incorporated by 1 mol of BSA-AH. The extent of uptake of D-ribose by BSA-AH hydrazide groups was, within experimental error, comparable to the TNBS-derived value of 9.7 ±0.4 mol.equiv. of $RNHNH_2$/mol of BSA, and the results were averaged to give a final value of 9.0 ±0.8 mol.equiv. of $RNHNH_2$/mol of BSA. It was thus assumed that each molecule of $BSA\text{-}AH_9$ could accommodate ~9 oligosaccharide chains that had D-ribofuranose end-groups.

Preparation and characterization of BSA-$[AH\text{-}hib_n]_9$ conjugates. — Hydrazone-linkage formation between $BSA\text{-}AH_9$ and hib_4 was facilitated by the use of a relatively high concentration of $BSA\text{-}AH_9$ (100 mg/mL) and amounts of hib_4 (1, 2, and 3

TABLE II

DATA FOR CONJUGATION[a] OF *hib*$_n$ SAMPLES WITH BSA-AH$_6$

Expt. no.	n	*Solvent*	*Reactant ratio*[b]	D-*Ribose*[c]	*Protein*[d]	*Yield* (%)[e]
				(*μg/mg of conjugate*)		
1	4	0.2M NaCl	1.5	35.6	850	48
2	4	0.2M NaCl	3.0	86.0	797	124
3	4	0.2M NaCl	4.5	89.5	1010	101
4	4	H_2O	1.5	37.6	738	58
5	4	H_2O	3.0	91.0	736	142
6	7	0.2M NaCl	3.0	108	783	90
7	12	0.2M NaCl	3.0	202	1171	66

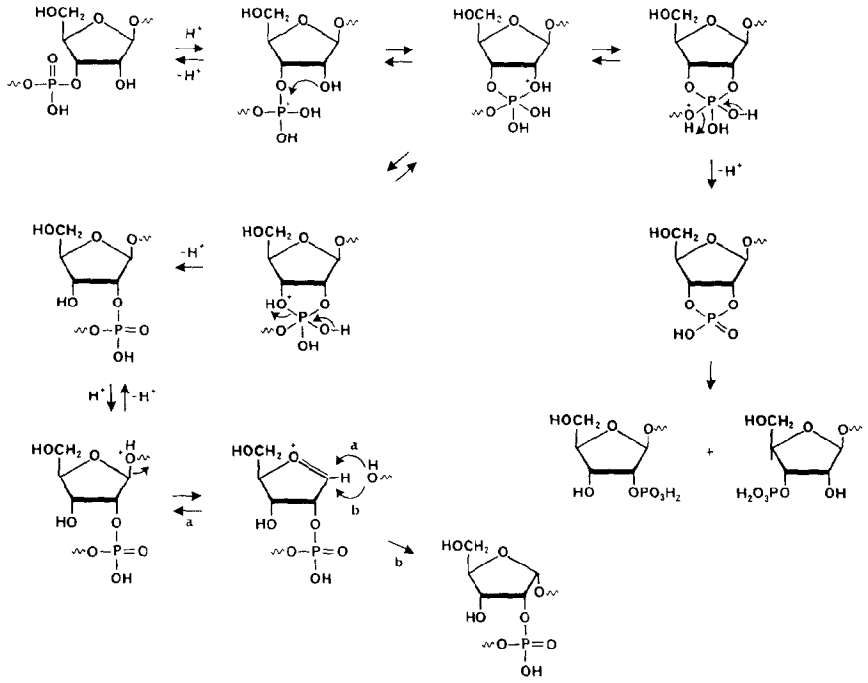
[a]Each experiment refers to the reaction of 10 mg of BSA-AH$_6$ in 0.1 mL of solvent for 20 h at 20–25°; [$RNHNH_2$] ~ 13mM. [b]Molar ratio of D-ribofuranose end-groups:$RNHNH_2$. [c]Total D-ribose content (± 5%) that was determined colorimetrically. [d]Estimated from u.v. absorbance (A_{280}) using BSA-AH$_6$ as the calibration standard. [e]Defined as [(D-ribose/protein)$_{found}$/(D-ribose/protein)$_{calc}$] × 100, wherein the calculated ratio (w/w) of D-ribose/protein was based on a product structure given by the formula BSA-[AH-hib$_n$]$_6$ with n = 4, 7, or 12.

mg/mg of BSA-AH$_6$) that provided 1.5, 3, and 4.5-molar excesses of the D-ribofuranose end-groups, relative to the $RNHNH_2$ functionalities in BSA-AH$_6$. The viscous reaction-mixtures, which thus contained 13mM $RNHNH_2$ and 19–56 mM D-ribofuranose end-groups, were stirred for 20 h at 20–25°. Size-exclusion chromatography with Sephadex G-100 and 0.2M NaCl was used to separate the comparatively small, unreacted oligosaccharides from the product molecules that had the D-ribose and protein compositions given in Table II. The data for similar reactions of BSA-AH$_6$ with hib$_4$ in water are also summarized in Table II, together with the information for conjugation of BSA-AH$_6$ with hib$_7$ and hib$_{12}$ in 0.2M NaCl. The coupling yields listed in Table II were derived by comparing the measured D-ribose:protein ratios with the appropriate D-ribose:protein ratio that was calculated for the fully "loaded" conjugate, BSA-[AH-hib$_n$]$_6$, wherein n = 4, 7, and 12. In view of the substantial limits of error that attended the calculated D-ribose:protein ratios, the coupling yields could only provide a *rough* indication of the extent of conjugation. On the other hand, the 48, 124, and 101% yields obtained in experiments 1–3 were consistent with complete conjugation of BSA-AH$_6$ with hib$_4$ when 3 or more mol. equiv. of D-ribofuranose end-groups were present (expts 2 and 3). A similar trend was apparent from the results obtained in expts 4 and 5, namely, 58 and 142% yields for 1.5 and 3 mol.equiv. of D-ribofuranose termini, respectively. These findings for expts 1, 2, 4, and 5 also indicated that there was no significant difference between 0.2M NaCl and water as the reaction medium. The yields obtained for expts 6 and 7 with hib$_7$ (90%) and hib$_{12}$ (66%) implied that the coupling efficiency was not significantly influenced by increases in the oligosaccharide chain-lengths.

The selectivity of the aforementioned, conjugation reactions and the existence of hydrazone linkages were supported by control experiments wherein BSA-AH

and HIB_{44} failed to form a detectable conjugate, and hib_4 that was "capped" by oxime derivatization of the D-ribofuranose end-groups failed to give a detectable conjugate with BSA-AH.

Summary. — The applications here reported of ^{31}P- and ^{13}C-n.m.r. spectroscopy provided a complete analysis of the end groups in oligosaccharides that were derived from the HCl-catalyzed hydrolysis of HIB. The relative ratio of monophosphate : D-ribofuranose end-groups in hib_4 and hib_7 revealed that ~30% of these two oligosaccharide mixtures had chains terminating in D-ribofuranose. This percentage was, within experimental error, equal to the proportion (33%) of "usable" oligosaccharide that results from equal rates of cleavage of the three hydrolytically labile linkages in the HIB repeating-unit structure. Consequently, under the stated reaction-conditions, the depolymerization of HIB occurred with no significant chemoselectivity. By contrast, the high regioselectivity (92 ±2%) for HCl-catalyzed cleavage of the phosphoric diester linkages in HIB was comparable, in both magnitude and direction, to the regioselectivity previously found[1] for the depolymerization of HIB under alkaline conditions. This finding suggested mechanistic analogy between the acid- and base-catalyzed degradation processes. By extension of the alkaline depolymerization mechanism[1], the preponderance of D-ribose 3(and 2)-monophosphate end-groups in hib_n was rationalized by more-effective, neighboring-group



Scheme 3. A mechanistic scheme for rationalizing the HCl-catalyzed depolymerization of HIB leading to, *inter alia*, D-ribose 3- and 2-monophosphate end-groups, O-3-linked D-ribose end-groups, and rearranged linkages to D-ribose residues in hib_n.

participation (see Scheme 3) of the 2-hydroxyl group of D-ribose, which is necessarily cisoid to the phosphoric diester linkage and, hence, is a better nucleophile by comparison with the 4-hydroxyl group of D-ribitol. Such nucleophilic substitutions at phosphorus generally (but not always[31]) proceed *via* pentacoordinate phosphoranes[20]. Intermediate phosphoranes have been implicated[1] as the source of linkage-rearrangement during the alkaline hydrolysis of the capsular polysaccharide from *H. influenzae* type a, and similar species (see Scheme 3) could account for the relatively small fraction (~4–8%) of stereomutated phosphoric diester linkages found* in hib_4 and hib_7. The mechanistic rationale depicted in Scheme 3 also invokes the *reversible* formation of an oxonium ion to account for epimerization of the D-ribofuranosidic C-1 linkage. In view of the fact that there was no ^{13}C-n.m.r. evidence for D-ribose components having C-3 linkages to phosphorus *and* inverted stereochemistry at C-1, the phosphoric diester linkage-rearrangement proposed may be a prerequisite for C-1 stereomutation.

It is important to emphasize that the connectivity difference represented by phosphoric diester linkages to C-3 and C-2 of the D-ribose components in hib_n is similar to that between the type-6A and type-6B pneumococcal polysaccharide antigens[2], which are immunochemically distinct. Stereomutated phosphoric diester linkages in hib_n, as well as anomerization at C-1, thus constitute potential, immunochemical determinants that could complicate comparative studies of HIB, hib_n, and their respective protein conjugates. With this caveat in mind, it would be advisable to prepare immunogenic oligosaccharides by methods that demonstrably bypass stereomutation of the residual, repeating-unit structure. The present studies have shown that n.m.r. spectroscopy is especially useful for investigating the stereochemical integrity of such oligosaccharides.

EXPERIMENTAL

N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded by using quadrature phase-detection at 22.49, 25.00, or 75.47 MHz with JEOL FX-90Q, JEOL FX-100, or Bruker WM-300 spectrometers, respectively, and aqueous samples that contained 5% (v/v) of D_2O and sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate as the internal chemical-shift (p.p.m.) reference. ^{31}P-N.m.r. spectra were similarly recorded at either 40.25 (JEOL FX-100) or 36.23 MHz (JEOL FX-90Q); chemical shifts, which were relative to an external solution of 25% (v/v) H_3PO_4 in D_2O, were measured at 40.25 MHz by using a coaxial capillary tube that was positioned with a vortex plug. The n.m.r. sampling-conditions have been described[1,2]. The "pH" values recorded for n.m.r. sample-solutions that contained D_2O were not corrected

*It should be emphasized that the stereomutation mechanisms given in Scheme 3 are heuristic. To underscore the complexity of these processes, we found that the percentage of rearranged, phosphoric diester linkages increased in a non-linear fashion with decreasing values of $\bar{L}$ for oligosaccharides that were fractionated by size-exclusion chromatography. For example, with $\bar{L}$ = 9.0, 4.2, and 1.4, the extent of rearrangement was 2.6, 6.8, and 21.5%, respectively.

for isotope effects, and refer to digital readings obtained with a precalibrated, Radiometer PHM 64 instrument equipped with an Ingold combination electrode that was inserted into the n.m.r. tube.

Assay methods. — Moisture content was determined as described[32]. D-Ribose was measured (±5%) by a procedure[33] that used orcinol as a color-forming reagent for optical absorbance (A) readings at 660 nm. The mol.equiv. of D-ribofuranose end-groups/mg of hib_n was estimated (±5%) by a previously described[2] modification of the Park–Johnson[27] assay. In this assay, it was assumed that the D-ribofuranose end-groups and the D-ribose calibration-standard had approximately equivalent color-forming capacities, on a molar basis. The validity of this assumption was checked by ^{13}C-n.m.r. analysis of hib_n (*vide infra*). The adipyl hydrazide content of derivatized BSA was estimated (±5%) as described[4] and used 2,4,6-trinitrobenzenesulfonic acid (TNBS) as the color-forming reagent[29,30]. The accuracy of this TNBS assay was checked by ^{13}C-n.m.r. measurements using ^{13}C-enriched materials (*vide infra*). The protein content in samples of BSA-AH-hib_n was estimated by comparison of A_{280} values with those measured for BSA-AH, which served as the calibration standard.

Size-exclusion chromatography, and dialyses. — Size-exclusion chromatography was performed in a column (3 × 115 cm) of Sephadex G-100 (Pharmacia Fine Chem.) that was equilibrated, and eluted, with 0.2M NaCl [containing 0.05% (w/v) of sodium azide as a preservative] at a flow rate of 2.1 mL/min; 5.0- to 5.5-mL fractions were collected, and the column effluent was continuously monitored by using a differential refractometer (Waters Assoc., Model R403). The collected fractions were subsequently assayed for protein (A_{280}), or D-ribose[33], or both of these components, as needed. Dialyses were conducted by using tubing having molecular-weight cutoff values of either 10,000 or 3,500, and employed de-ionized water (6 L, twice) at 5°.

Kinetics of hydrolysis of HIB phosphoric diester. — Hydrochloric acid (0.1M, 2 mL) that contained 5% (v/v) of D_2O was equilibrated at 60° in a 10-mm, n.m.r. tube. A sample of HIB ($\bar{L}$ = 44; 30 mg) was added, and ^{31}P-n.m.r. spectra were then recorded as a function of time over a 1-h reaction period. The initial rate of disappearance of the phosphoric diester signal was used to estimate the pseudo-first-order rate-constant for hydrolysis of phosphoric diester: $-k' \geqslant \sim 0.01$ min^{-1} ($\tau_{1/2} \leqslant \sim 60$ min.)

Preparation and characterization of samples of hib_n. — Two samples of HIB isolated from *H. influenzae* strain 1482 (ref. 4) were provided by Dr. Rachel Schneerson (Office of Biologics). These samples were shown by ^{31}P-n.m.r. analysis[1] to have $\bar{L}$ = 44 and 84, and are herein referred to as HIB_{44} and HIB_{84}, respectively. A portion (518 mg) of HIB_{44} was added to a 125-mL Erlenmeyer flask that contained water (45 mL) and a magnetic stirring-bar. Hydrochloric acid (M; 5 mL) was added with rapid stirring at 25°, and the flask was then continuously swirled in a 60° water-bath for 15 min. The stirred solution was cooled in an ice–water bath, and made neutral with M NaOH (~5 mL). The neutral hydrolyzate was concentrated to 10 mL (Amicon MU 05 apparatus, 500 molecular-weight cutoff), and the retained material was then

passed through a column of Sephadex G-100. Fractions 75–92 were pooled, dialyzed, and lyophilized, to afford the hib_n product (sample 1: 375 mg, 72% recovery). The hydrolysis procedure was repeated with a second portion (640 mg) of HIB_{44}; the heating period was 7 min, and the neutral hydrolyzate was lyophilized prior to size-exclusion chromatography. Fractions 64–94 were pooled to afford the hib_n product (sample 2: 560 mg, 88% recovery). In a third hydrolysis reaction, a solution of HIB_{84} (500 mg) in water (45 mL) was allowed to equilibrate thermally in the 60° bath before the addition of M HCl (5 mL; also pre-equilibrated at 60°). The depolymerization was terminated after 3 min by the addition of M NaOH (5 mL), and the reaction-mixture was cooled, and the pH adjusted to 7, before lyophilization and size-exclusion chromatography. ^{31}P-N.m.r. spectra were recorded for fractions 50, 60, 70, and 80. Fractions 60–80 were pooled to afford the hib_n product (sample 3; 344 mg, 69% recovery).

The following characterization of sample 1 of hib_n is representative of the procedures that were used. A portion (852 μg) of sample 1 (repeating-unit formula weight = 365) was found to have 0.167 ±0.008 μmol.equiv. of D-ribofuranose end-groups per 2.33 repeating-units, which was normalized to a value of 7.2 ±0.4 μmol. equiv. of D-ribofuranose end-groups per 100 repeating-units. Four integrations of a ^{13}C-n.m.r. spectrum recorded for hib_n sample 1 gave a value of 9.3 ±0.3 D-ribofuranose end-groups per 100 repeating-units (*cf.*, Results and Discussion), which was averaged with the corresponding, chemically derived quantity to give a final value of 8.2 ±1.0 D-ribofuranose end-groups per 100 repeating-units. ^{31}P-N.m.r. integrations obtained with a solution of hib_n sample 1 at pH 7 gave 16.7 monophosphate (4.09 p.p.m.) end-groups per 100 phosphoric diester linkages (1.03 and 1.79 p.p.m.). The sample was treated with DEAC (*vide infra*), and ^{31}P-n.m.r. integrations for the product gave 16.8 D-ribofuranose 2,3-cyclophosphate (20.90 p.p.m.) end-groups per 100 phosphoric diester linkages and 1.0 D-ribitol 4,5-cyclophosphate (18.92) end-group per 100 phosphoric diester linkages, for an average value (before, and after, reaction with DEAC) of 17.2 ±0.6 phosphorus-containing end-groups per 100 repeating-units. The total value of 25.4 ±1.6 D-ribofuranose and phosphorus-containing end-groups per 100 repeating-units indicated that $\bar{L} = 3.9 \pm 0.2$; hence, oligosaccharide sample 1 was designated hib_4. These results are summarized in Table I, which also lists the corresponding analytical data for oligosaccharide samples 2 and 3, respectively designated hib_7 and hib_{12}.

Reaction of hib_n samples with DEAC. — A solution of the oligosaccharide (10–20 mg) in 0.1M imidazole buffer [pH 7, 20% (v/v) of D_2O, 1.5 mL] was treated with an aliquot (50 μL) of a freshly prepared solution of DEAC in the same buffer (140 mg/mL). A second aliquot (50 μL) of a freshly prepared solution of DEAC in the imidazole buffer (280 mg/mL) was added after 12 h at 25°. ^{31}P-N.m.r. spectra were recorded after an additional 12 h at 25°.

Reaction of hib_4 with hydroxylamine hydrochloride. — A solution of hib_4 (13 mg; 2.2 μmol.equiv. of D-ribofuranose end-groups) in water (1 mL) was added to a neutralized solution of $H_2NOH \cdot HCl$ (4.6 mg, 66 μmol) in water (0.2 mL),

and M NaOH was used to maintain the pH of the solution at ~7. The mixture was lyophilized after 12 h at 25°, and a solution of the resultant solid in D_2O (0.3 mL) was used to record the ^{13}C-n.m.r. spectrum.

Reaction of hib_4 with AAD. — A neutralized solution of AAD (3 mg, 17.2 μmol) in water (0.1 mL) was added to hib_4 (20 mg, 3.3 μmol.equiv. of D-ribofuranose end-groups), and, after 24 h of stirring at 25°, the solution was diluted with D_2O (0.2 mL) prior to recording of the ^{13}C-n.m.r. spectrum.

Preparation and analysis of BSA-AH. — A modified version of a published[4] procedure was employed as follows. Recrystallized[34] AAD (2 g, 11.5 mmol) was added to a magnetically stirred solution of purified[35] BSA (2 g) in 0.1M NaCl (80 mL) at 25°, and the pH of the solution was adjusted to 4.9 with 0.1M HCl before addition of DEAC (0.6 g, 3.14 mmol) in one portion. The 0.1M HCl titrant was used to maintain pH 4.8–5.0 for 2 h, and the mixture was then dialyzed against 0.2M NaCl, divided into three 50-mL portions, and each portion passed through a column of Sephadex G-100. In each case, fractions 30–60 were pooled, dialyzed, and lyophilized, to afford BSA-AH. The combined material, which contained 4% of moisture, was found by amino acid analysis[36] to contain 931 μg of protein/mg of sample. The TNBS assay indicated 0.135 ±0.006 μmol.equiv. of $RNHNH_2$/mg of sample, which was equal to 9.7 ±0.4 mol.equiv. of $RNHNH_2$ mol of BSA (molecular weight ~67,000). This estimate of the adipyl hydrazide content was combined with the value measured by D-ribose incorporation (*vide infra*) to give an average value of 9.0 ±0.8 mol.equiv. of $RNHNH_2$/mol of BSA. The derivatized protein was thus designated BSA-AH_9.

Conjugation of D-ribose with BSA-AH_9. — A solution of BSA-AH_9 (10 mg) and D-ribose (5 mg) in 0.2M NaCl (0.1 mL) was stirred for 20 h at 25°, and the solution was then passed through a column of Sephadex G-100. Fractions 30–60 were pooled, dialyzed, and then lyophilized; the unreacted D-ribose was contained in fractions 90–105. Automated sugar[37] and amino acid[36] analyses of the lyophilized product gave values of 98 nmol of D-ribose per mg of sample and 799 μg of protein per mg of sample, respectively, and thus indicated 8.2 mol.equiv. of $RNHNH_2$/mol of BSA, assuming 100% efficiency for the conjugation of the $RNHNH_2$ groups with D-ribose. The D-ribose content in this conjugate was calculated after correcting for the relatively small amount of D-ribose (3.8 nmol) that was incorporated by BSA (1 mg, 14.9 nmol) in a corresponding control-reaction using BSA (10 mg) and D-ribose (5 mg).

Preparation and analysis of ^{13}C-enriched BSA-AH. — A solution of hexanedioic acid-*1,6*-$^{13}C_2$ (90 atom-% isotopic purity, Merck & Co., Inc.; 490 mg, 3.31 mmol) in absolute CH_3OH (5 mL) that contained 4 drops of conc H_2SO_4 was boiled under reflux for 140 min in a round-bottomed flask that was equipped with a $CaCl_2$ drying tube. The reaction mixture was cooled to 25°, and added to a separatory funnel that contained water (25 mL), and the dimethyl ester of hexanedioic acid-*1,6*-$^{13}C_2$ was extracted with ether (5 mL, 5 times). The extracts were combined, washed with water (5 mL, twice), dried (anhydrous $MgSO_4$), and evaporated in a rotary evaporator (without heating) to a colorless oil (520 mg, 2.95 mmol, 89%) which was boiled under reflux for 2.5 h with a mixture of absolute ethanol (1.6 mL) and H_2NNH_2 ·

H_2O (0.55 mL). The mixture was cooled to 25°, suction-filtered, and the solid quickly washed with ice-cold, absolute ethanol (1 mL) to give, after drying *in vacuo*, AAD-$^{13}C_2$ (420 mg, 2.39 mmol, 72%): m.p. 179–181° (corr.), lit.[34] m.p. 182° and[38] 171°.

Reported[4] procedures were used to couple AAD-$^{13}C_2$ with BSA, and to analyze the BSA-AAD-$^{13}C_2$ product, which was found to contain ~33 mol.equiv. of $RNHNH_2$/mol of BSA. A solution of this material (27.6 mg) in D_2O (0.5 mL) at pH 7.4 was analyzed by ^{13}C-n.m.r. spectroscopy (25.0 MHz) using a standard set of data-acquisition parameters that included a $\pi/4$ pulse, suppression of the nuclear Overhauser effect (nOe), a 5-s pulse repetition-time, and 500 pulses. The same solution (sample A) was also examined by ^{13}C-n.m.r. spectroscopy at a higher field-strength (67.9 MHz), which was provided by a previously described[39], home-built instrument. The standard acquisition-parameters at 25.00 MHz were also used to obtained the ^{13}C-n.m.r. spectrum of a surrogate solution (sample B) that contained BSA (27.6 mg) and AAD-$^{13}C_2$ (0.58 mg) in D_2O (0.5 mL); a second spectrum for sample B was recorded by using a 10-s pulse repetition-time. All of the time-domain spectra were exponentially multiplied so as to obtain 1 Hz of additional line-broadening after Fourier transformation, and the resultant, frequency-domain spectra were displayed at the same noise-level for measurement of integrated signal-intensities by the "cut-and weigh" method. The relative signal-intensities (I_{rel}) measured with sample B were equal, within the estimated limits of experimental error (±10%): I_{rel} = 1.000 for the 5-s repetition-time, and I_{rel} = 0.91 for the 10-s repetition-time. Compared with the average value of these measurements (I_{rel} = 0.96), the signal intensity for sample A (I_{rel} = 4.93) indicated 0.61 nmol of ^{13}C-labeled adipyl hydrazide/mg of BSA-AH-$^{13}C_2$, which was equivalent to 41 ±5 mol.equiv. of $RNHNH_2$ per mol of BSA.

Conjugation of BSA-AH$_9$ with hib$_n$ samples. – Table II lists the pertinent synthetic and analytical details for the various conjugation reactions. The following is a representative procedure. BSA-AH$_9$ (10 mg) and hib$_4$ (10 mg) were dissolved in 0.2M NaCl (0.1 mL), and the viscous solution was magnetically stirred for 20 h at 25°. The reaction mixture was passed through the size-exclusion column, and fractions 30–40 were pooled, dialyzed, and then lyophilized. The D-ribose content of the resultant product was measured by the orcinol assay, and the protein content was estimated from the A_{280} value *vs.* the A_{280} value given by BSA-AH.

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