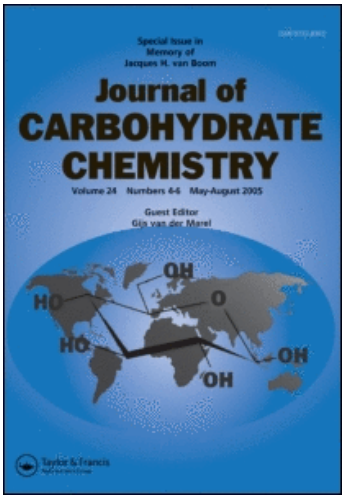

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Synthesis of Fragments of the Capsular Polysaccharide of Haemophilus Influnzae Type B Part II. Preparation and Structural Analysis of Fragments Comprising two and Three Repeating Units

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SYNTHESIS OF FRAGMENTS OF THE CAPSULAR POLYSACCHARIDE OF *HAEMOPHILUS INFLUENZAE* TYPE B

Part II.[1,2] PREPARATION AND STRUCTURAL ANALYSIS OF FRAGMENTS COMPRISING TWO AND THREE REPEATING UNITS.

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ABSTRACT

The synthesis of spacer-containing fragments of the capsular polysaccharide of *H. influenzae*, comprising two and three repeating units (**9a** and **9b**, respectively), is presented. Ribosylribitol building blocks **1** and **2** were coupled with *bis*[1-benzotriazolyl]-2-chlorophenyl phosphate (**3**) to give the phosphotriester derivative **5a** (72%), which was selectively deprotected at O-5 of the terminal ribitol residue (cleavage of the *trans*-1-propenyl ether) to afford compound **6a** (82%). Repeated coupling of building block **2** with **6a** (chain elongation), followed by propenyl-cleavage, gave analogue **6b**. Both derivatives **6a** and **6b** were coupled with spacer **7** (chain termination). The fully protected fragments **8a** (84%) and **8b** (49%, starting from **6a**) were thus obtained. Complete deprotection of **8a** and **8b** was achieved in three steps. After purification and cation-exchange, **9a** and **9b** were obtained in 41 and 68% yields, respectively. The structural integrity of the largest fragment (**9b**) was confirmed by FAB MS and various NMR spectroscopic techniques.

INTRODUCTION

The bacterium *Haemophilus influenzae* type b (Hib) produces a type-specific capsular polysaccharide antigen, polyribosylribitol phosphate (pRRP) in which phosphodiesters link O-3 of the ribose to O-5 of the ribitol residues.[3,4] Conjugates of this antigen with proteins are being tested as vaccines to prevent Hib-diseases, such as meningitis, in infants.[5] Until recently, polysaccharide or rather large oligomers from natural sources have been used to prepare such conjugates. However, application of well-defined synthetic Hib antigens or haptens seems advantageous.[6]

First syntheses of the repeating unit of pRRP were reported by Garegg *et al.*[7,8] We developed a synthetic route which is suitable for preparation of larger fragments.[1] In the previous part[2] we described the synthesis of two properly protected ribosylribitol building blocks (1 and 2) which are key-intermediates in our synthetic approach. In this paper we wish to present full experimental details of the application of 1 and 2 in the synthesis of the spacer-containing pRRP-fragments **9a** and **9b**. Furthermore, proof of the structural integrity of these compounds, particularly of the "three repeating unit fragment" in **9b**, will be presented.

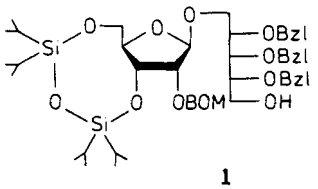
RESULTS AND DISCUSSION

Synthesis of Fragments 9a and 9b.

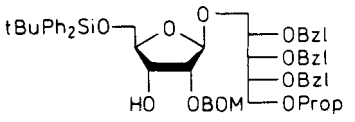
A sequential strategy was followed for the synthesis of pRRP-fragments using ribosylribitol building block 1 as a terminal unit and building block 2 as an "elongation unit".

Compound 2 was phosphorylated with a slight excess of *bis*[1-benzotriazolyl]-2-chlorophenyl phosphate[9] (3) in pyridine/dioxane to give the reactive intermediate 4, which was not isolated but immediately added to a solution of ribosylribitol 1 and a catalytic amount of *N*-methylimidazole in pyridine. The product, the fully protected phosphotriester derivative 5a, was obtained in 72% yield after short column chromatography over silica gel. Selective cleavage of the *trans*-1-propenyl group by reaction with mercury oxide/ mercury chloride in acetone/water[10] furnished derivative 6a (82%).

In this stage of the synthesis two options were available: (a) chain termination and (b) further chain extension. Chain termination was effected



1



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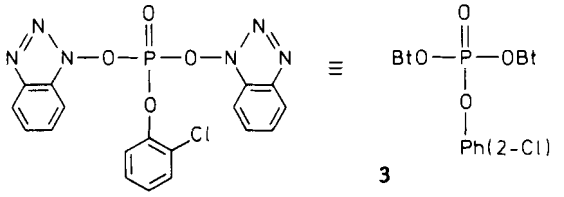


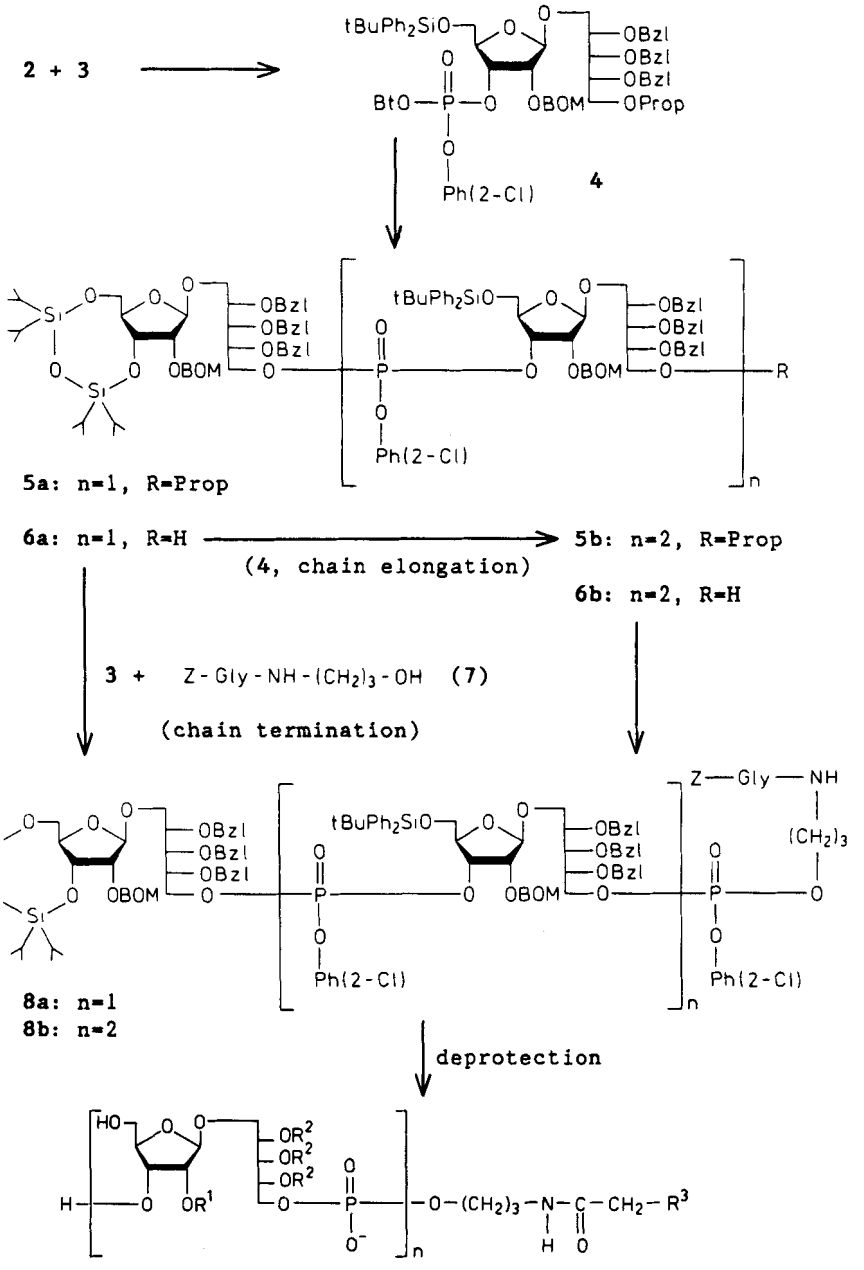
3

BOM=benzyloxymethyl
Bt=1-benzotriazolyl
Bzl=benzyl
Ph(2-Cl)=2-chlorophenyl
Prop=trans-1-propenyl

by coupling of **6a** and *N*-benzyloxycarbonylglycine-3-hydroxy-1-propylamide (7) with reagent **3**, which afforded the fully protected derivative **8a** (84%). In this way a second phosphotriester and a protected amino function were introduced simultaneously. The presence of an amino function in the final product facilitates the conjugation with a protein (a prerequisite for the preparation of suitable immunogens[6]).

Total deprotection of compound **8a** was achieved by a three-step process. The 2-chlorophenyl phosphate protecting groups were removed by reaction with excess of *syn*-pyridine-2-carboxaldoxime[11] (30 eq) and N^1,N^1,N^3,N^3-tetramethylguanidine (20 eq) in dry tetrahydrofuran at room temperature. Under these conditions a dinucleoside phosphotriester is normally deprotected within 30-60 min. However, in this case the rate of the reaction was extremely slow, as monitored by ^{31}P NMR spectroscopy. Complete conversion of **8a** into a *bis*[phosphodiester] derivative required 48 h. The crude product thus obtained was treated with tetra-*n*-butylammonium fluoride[12] in dry dioxane to cleave the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPS) and the *tert*-butyldiphenylsilyl group (16 h, room temperature). After gel filtration over Sephadex LH-20, the partially deprotected product (**9**, n=2, R^1=BOM, R^2=Bzl, R^3=NHZ) was obtained as its *bis*[tetra-*n*-butylammonium] salt.

In the final step, the benzyl, benzyloxymethyl and benzyloxycarbonyl groups were removed by hydrogenolysis. This was initially performed by catalytic transfer hydrogenation, using ammonium formate (0.5 M in methanol) as the hydrogen donor[13] (1 h, reflux). Unfortunately, this process was accompanied by severe methylation (up to 50%) of the liberated spacer



Gly=glycyl, Z=benzyloxycarbonyl

amino group, as detected by ^{1}H NMR and FAB MS. Apparently, a reductive methylation of the amino group with formaldehyde had occurred. The benzyloxymethyl groups seemed to be the source of formaldehyde, since catalytic transfer hydrogenation using ammonium formate as the hydrogen donor was successfully applied to cleave *N*-benzyloxycarbonyl[14] and *N*-benzyl groups[15] (in methanol, at room temperature and under reflux, respectively). In later experiments glycinamide was added to the reaction mixture as a formaldehyde scavenger. Under the conditions of the transfer hydrogenation this had little effect. Much better results were obtained by using a standard catalytic hydrogenation (at atmospheric pressure) of 9 (n=2, R^1=BOM, R^2=Bzl, R^3=NHZ, *bis*[tetra-*n*-butylammonium] salt) in *tert*-butanol/water in the presence of excess glycinamide. As shown by ^{1}H NMR spectroscopy, the crude product contained large amounts of *N*-methyl- and *N,N*-dimethylglycinamide. However, the final product **9a** (two repeating units + spacer), obtained in 41% yield after gel filtration over Sephadex G-25 and ion-exchange by filtration over SP-Sephadex C-25 (Na^+-form), contained less than 1% of the *N*-methyl-analogue (9, n=2, R^1=R^2=H, R^3=$NH_2CH_3^+[Na^+]$).

Having established satisfactory conditions for the synthesis of **9a**, attention was turned to further chain extension of intermediate **6a**. Thus, building block **2** was again phosphorylated with reagent **3** to give compound **4**, which was coupled with **6a** to give compound **5b**. *Via* intermediate **6b** the fully protected product **8b** was obtained in 49% over-all yield. Complete deprotection of **8b**, followed by gel filtration and ion-exchange gave **9b** (three repeating units + spacer, 68%).

Confirmation of the Structure of Compound 9b.

The structural integrity of the "three repeating unit fragment" **9b** was firmly established by FAB MS and NMR spectroscopy. Table 1 shows FAB mass spectral data. The mass spectra confirmed the molecular weight of 1170 expected for $C_{35}H_{69}O_{35}N_2P_3$ (*e.g.* **9b**, n=3, R^1=R^2=H R^3=$NH_3^+[H^+]_2$). The FAB(-) spectrum suggested the neutral loss of a ribosylribitol ($[M-C_{10}H_{19}O_8]^-$) and the presence of a ribosylribitol phosphate dimeric fragment ($[C_{20}H_{39}O_{23}P_2]^-$).

The ^{1}H NMR spectrum with signal assignments is shown in FIG. 1. For convenience the ribosylribitol phosphate repeating units are numbered (1)

TABLE 1.

Mass Spectral Data of Compound **9b**[a].

FAB(+) glycerol		
m/z	relative intensity	ion structure
1193	m	$[M+Na]^+$
1171	s	$[M+H]^+$

FAB(-) glycerol		
m/z	relative intensity	ion structure
1193	m	$[M+Na]^-$
1170	s	$[M]^{-}\cdot$
903	m	$[M-C_{10}H_{19}O_8]^-$
709	s	$[C_{20}H_{39}O_{23}P_2]^-$

[a] M=1170 (C_{35} $H_{69}N_2O_{35}P_3$, *e.g.* [**9b**-$2Na^+$+$2H^+$])

TABLE 2.

^{13}C NMR Chemical Shifts[a] of Compound **9b**.

Unit	Position				
	C-1	C-2	C-3	C-4	C-5
spacer	166.9	40.5	36.4	29.2[b]	63.6[c]
ribitol(1)	68.5	71.4	70.2	71.0[c]	66.4[c]
ribose(1)	106.4	73.8[d]	74.3[c]	82.0[c]	62.3
ribitol(2)	68.6	71.4	70.1	71.0[c]	66.7[c]
ribose(2)	106.7	73.8[d]	74.3[c]	82.0[c]	62.3
ribitol(3)	68.6	71.4	70.1	71.0[c]	66.7[c]
ribose(3)	106.9	74.3	70.6	82.7	62.6

[a] In D_2O at 297 K, referred to external acetone (30.5 ppm)
[b] Split by $J_{CP} \approx 7 \pm 1.3$ Hz
[c] Split by $J_{CP} \approx 5 \pm 1.3$ Hz
[d] Split by $J_{CP} \approx 3 \pm 1.3$ Hz

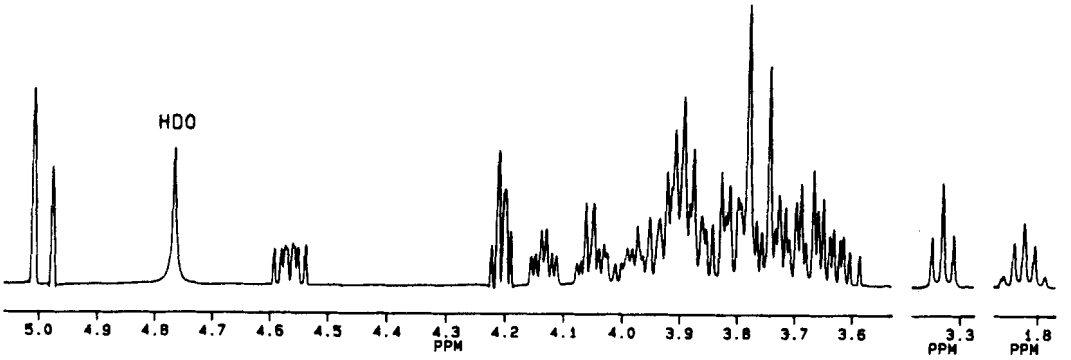
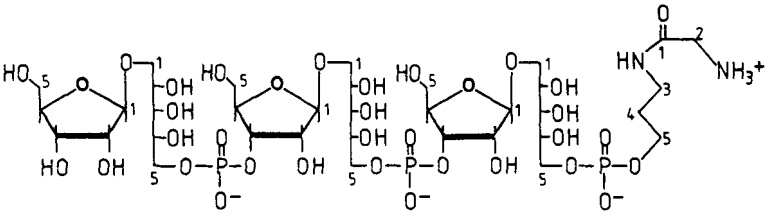


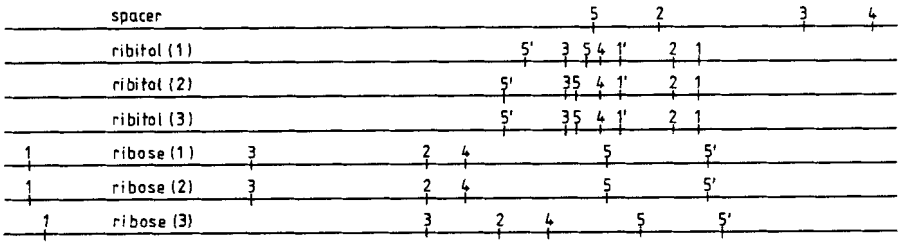
FIG. 1. 360 MHz ^{1}H NMR spectrum and proton assignment of compound 9b (in D_2O at 297 K, referred to internal HDO at 4.765 ppm).

to (3), unit (1) being attached to the spacer. The ^{13}C NMR data are compiled in Table 2.

The assignment of the protons and carbons in FIG. 1 and Table 2 is based on the chemical shifts and their correlations, as determined with ^{1}H-^{1}H (FIG. 2) and ^{13}C-^{1}H (FIG. 3) correlation spectroscopy, and on the carbon multiplicities (CH_2/CH/C). FIG. 4 shows a ^{1}H-coupled ^{31}P spectrum and, finally, FIG. 5 shows a ^{31}P-^{1}H correlation spectrum. The ^{13}C and ^{31}P NMR spectra confirm the presence of 35 carbon and 3 phosphorus atoms.



ribose (3) ribose (2) ribose (1) spacer
ribitol (3) ribitol (2) ribitol (1)
phosphate (3) phosphate (2) phosphate (1)



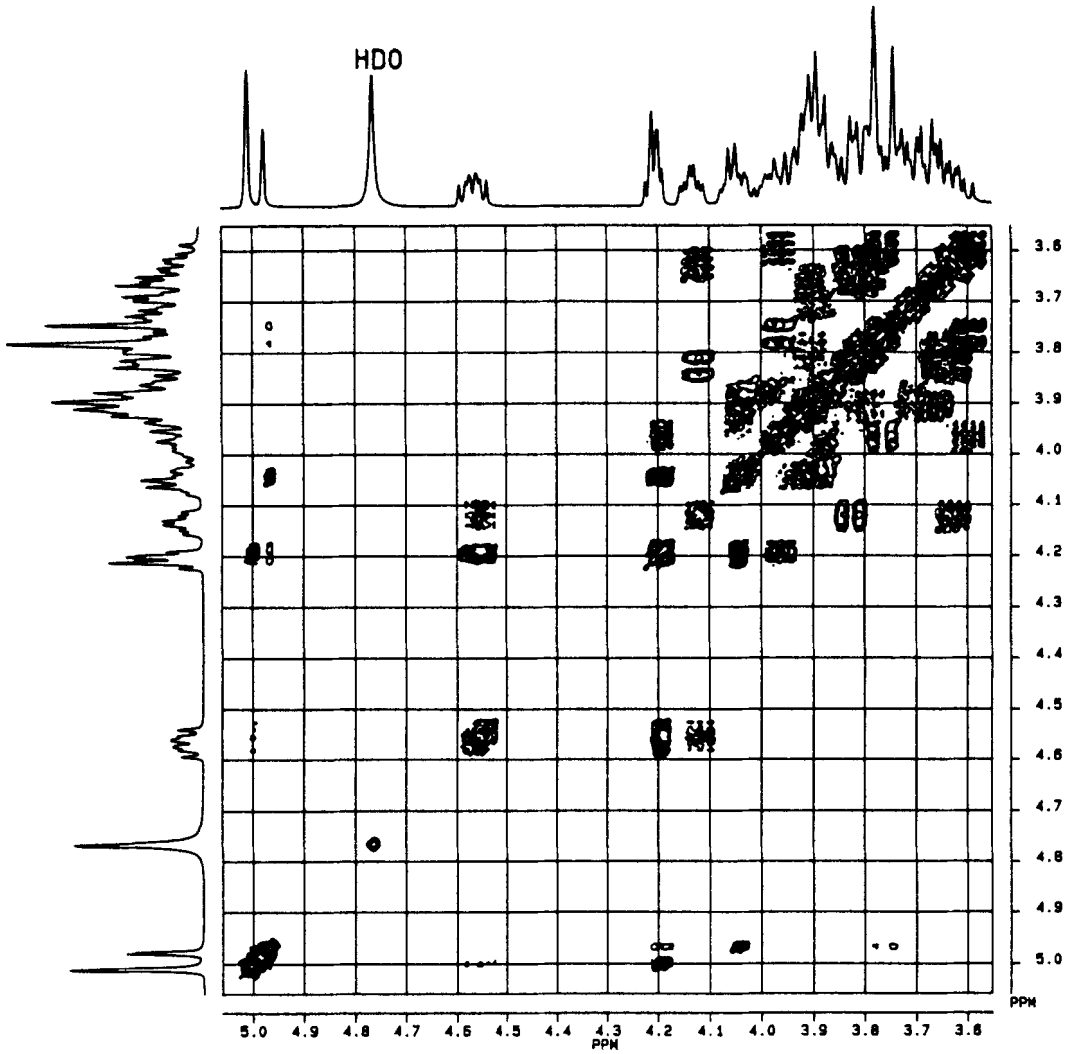


FIG. 2. Expanded region (3.55-5.05 ppm) of the ^{1}H-^{1}H correlation (COSY) spectrum of compound **9b**. The normal 1D spectrum is plotted along the axes.

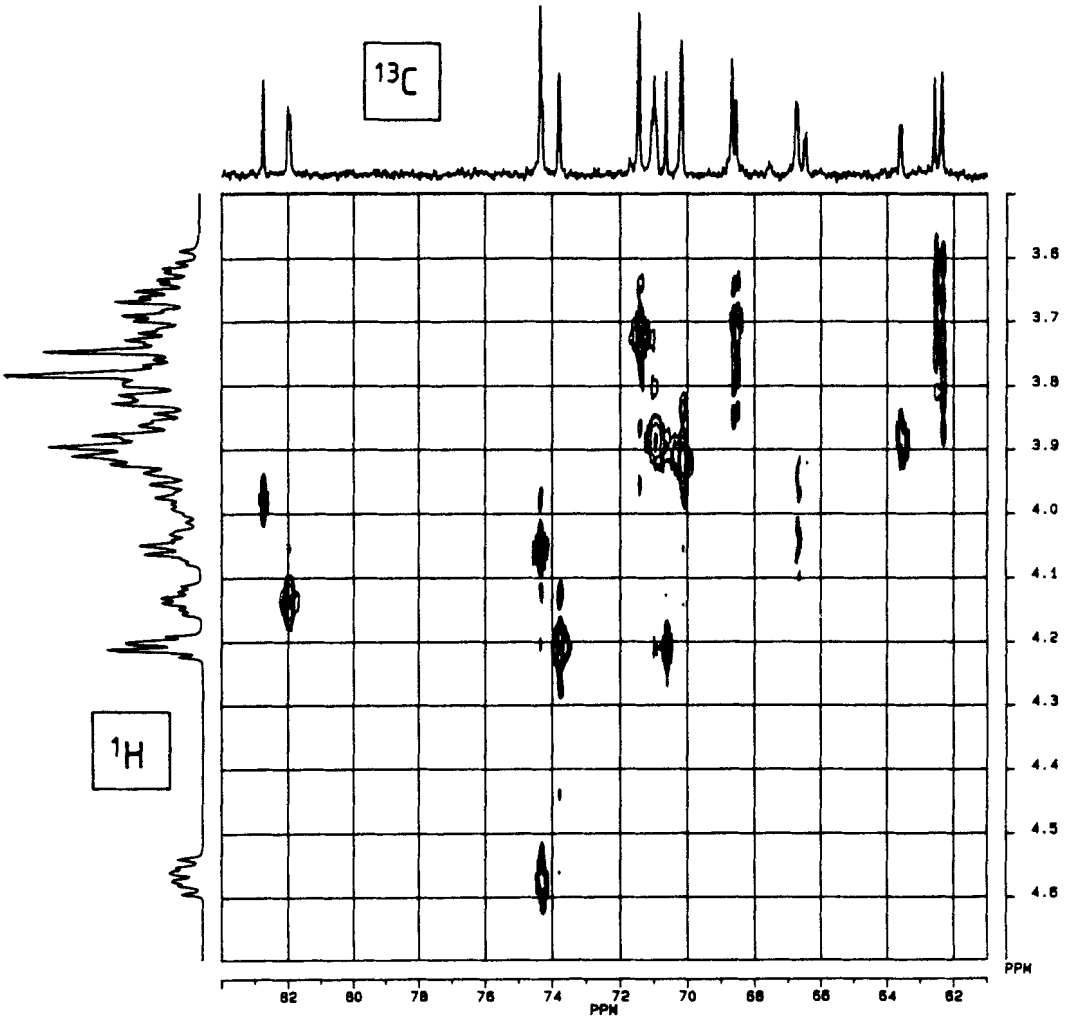


FIG. 3. Expanded region (^{1}H: 3.5-4.7 ppm; ^{13}C: 61-84 ppm) of the ^{13}C-^{1}H correlation spectrum of compound **9b**. The respective 1D spectra are plotted along the axes.

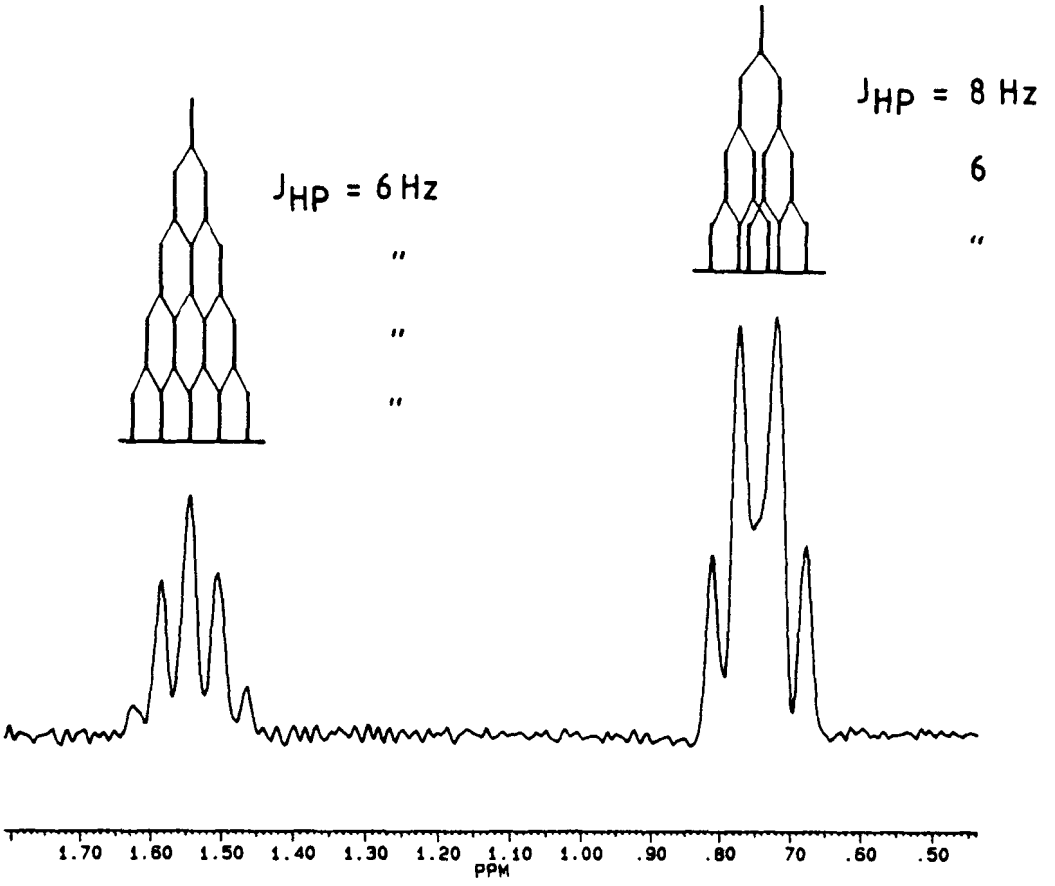


FIG. 4. 145 MHz ^{31}P NMR spectrum of compound **9b**. Phosphate(1): δ 1.55 ppm; phosphate(2) and -(3): δ 0.78 ppm. The ^{31}P-^{1}H couplings are indicated by the branching diagram.

The chemical shifts of C-1 of the ribitols are about 3 ppm higher than for unsubstituted ribitol (65.5 ppm[16]), whereas C-2 and C-3 are 4-5 ppm lower; this can only be rationalized if the riboses are linked to C-1 of the ribitols.

The location of phosphate(1) between C-5 of the spacer and C-5 of ribitol(1) is confirmed by the detection of ^{31}P-couplings to H-5, H-5', C-4 and C-5 of the spacer and to C-4 and C-5 (but not C-1, C-2 and C-3) of ribitol(1). Overlap prevents the observation of the splitting patterns of H-5 and H-5' of ribitol(1), but the ^{31}P-^{1}H correlation spectrum (FIG. 5) proves the connection between phosphate(1) and both H-5/H-5' of ribi-

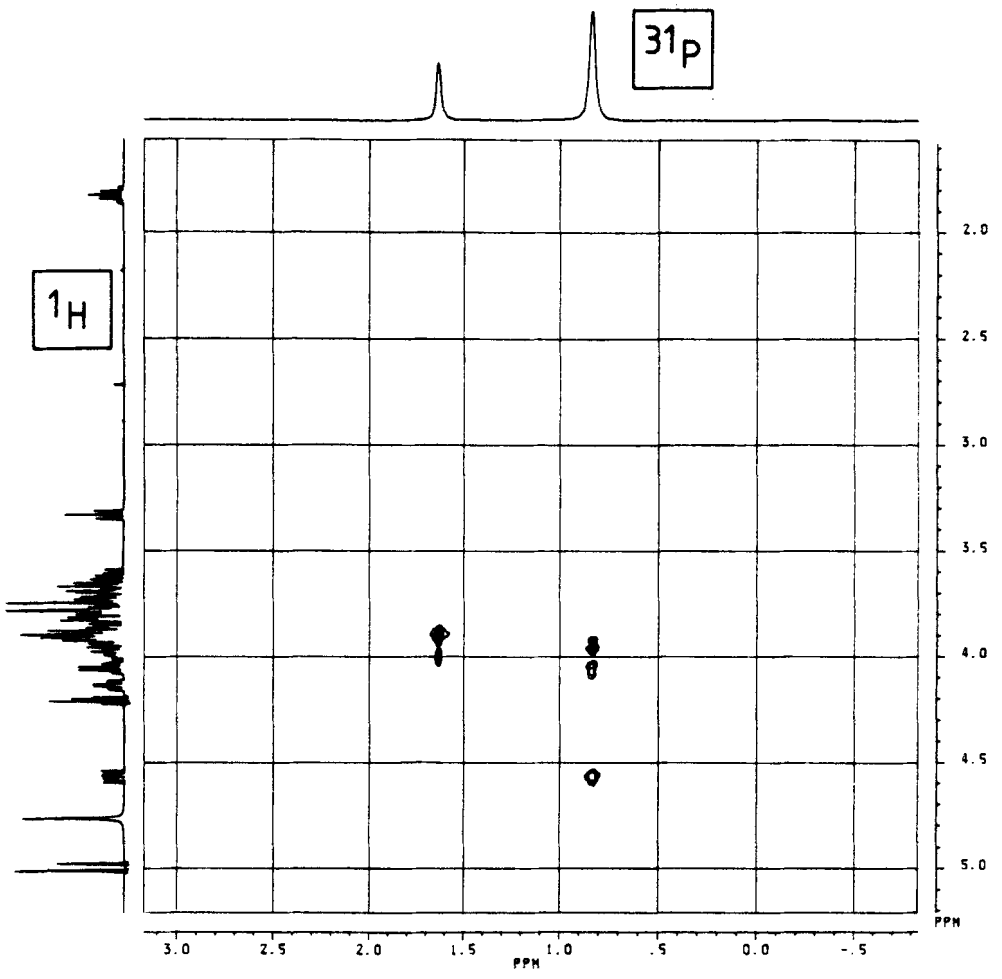


FIG. 5. ^{31}P-^{1}H chemical-shift-correlation spectrum of compound **9b**. For reference purposes a $^{31}P\{^{1}H\}$ spectrum and a ^{1}H spectrum are plotted along the horizontal and vertical axes, respectively.

tol(1) and H-5/H-5' of the spacer. The 6 Hz quintet pattern of the ^{31}P of phosphate(1) reconfirms its location between two CH_2-groups (FIG. 4).

In the same way the location of phosphate(2) (and -(3)) between C-3 of ribose(1) (and -(2)) and C-5 of ribitol(2) (and -(3)) is confirmed by ^{31}P-couplings to H-3, C-2, C-3 and C-4 of both riboses and to C-4 and C-5 of both ribitols. Again the splitting patterns of H-5/H-5' of ribitol(2) and -(3) could not be observed because of severe overlap, but the correlation of these protons, and of H-3 of ribose(1) and -(2) with the ^{31}P of phosphate(2) and -(3) is nicely shown in the ^{31}P-^{1}H correlation spectrum.

Finally, the double-triplet pattern (6, 6 and 8 Hz) of the ^{31}P of phosphate(2) and -(3) (FIG. 4) is in agreement with its location between a CH- and a CH_2-group.

In conclusion a synthetic route suitable for the preparation of pRRP-fragments, following a sequential strategy, is reported. So far, spacer-containing fragments comprising two and three repeating units (**9a** and **9b**) were prepared. The structural integrity of the largest fragment (**9b**) was confirmed by FAB MS and NMR spectroscopy. Furthermore, it has already been proved that conjugates of **9a**/**9b** with tetanus toxoid induce a pRRP-specific antibody response in mice,[6] rabbits[6] and monkeys.

The assemblage of larger pRRP-fragments, either by the approach presented in this paper or by a solid-phase method, will be the subject of further investigations.

EXPERIMENTAL

Materials and Methods. General materials and methods were as described in Part I.[2] Pyridine which was used for phosphorylation experiments was distilled three times, from calcium hydride, *p*-toluenesulfonyl chloride and sodium hydroxide, respectively, and stored over molecular sieves 4 Å. A standard solution of *bis*[1-benzotriazolyl]-2-chlorophenyl phosphate (3) in dioxane was prepared according to ref 17.

A Jeol FX-200 spectrometer was routinely used for recording ^{1}H, $^{13}C\{^{1}H\}$ (with APT, attached proton test) and $^{31}P\{^{1}H\}$ NMR spectra at 200, 50.1 and 80.7 MHz, respectively. If not stated otherwise, the compounds were dissolved in $CDCl_3$ and $^{1}H/^{13}C$ and ^{31}P chemical shifts are given in ppm, relative to internal tetramethylsilane and external 85% H_3PO_4, respectively. A Bruker AM 360 spectrometer was used for recording ^{1}H, $^{13}C\{^{1}H\}$ (with DEPT multiplicity analysis) and ^{31}P spectra, and ^{1}H-^{1}H, ^{13}C-^{1}H and ^{31}P-^{1}H chemical-shift-correlated 2D spectra of compound **9b** in D_2O. The operating frequencies were 360 MHz for ^{1}H, 90 MHz for ^{13}C and 145 MHz for ^{31}P.

The FAB mass spectrum of compound **9a** was recorded in the negative ion mode on a VG ZAB 3F mass spectrometer using Krypton atoms for ionization. The FAB spectra of compound **9b** were recorded both in the positive and the negative ion mode on a VG ZAB 2HF mass spectrometer using Xenon atoms for ionization. The matrix used for dissolving the compounds was glycerol.

Fully Protected Phosphotriester 5a. Compounds **2** (254 mg, 0.27 mmol) and **1** (229 mg, 0.25 mmol) were separately concentrated from dry pyridine (2.5 mL) three times (the last time to a volume of about 1 mL). *N*-methylimidazole (35 µL) was added to the solution of **1** in pyridine.

First step: 1.5 mL of a 0.2 M solution of **3**[17] was added to **2**. The solution was stirred at room temperature for 30 min. TLC (hexane/ethyl acetate, 7/3) indicated complete conversion of **2** (R_f 0.41) into baseline material, that is, into the intermediate active phosphotriester **4**.

Second step: The solution of **4**, as obtained in the first step, was immediately added to the mixture of **1** and *N*-methylimidazole under anhydrous conditions. After stirring at room temperature for 2 h, TLC showed complete disappearance of **1** (R_f 0.34) and formation of **5a** (R_f 0.51 and 0.49, diastereoisomers). The solution was diluted with diethyl ether (50 mL) and washed with 1 M triethylammonium bicarbonate (TEAB) (2 x 25 mL), 1M KH_2PO_4 (2 x 25 mL) and 1 M TEAB (25 mL). The organic layer was dried and concentrated to give a syrup, which was purified by chromatography on a 4 x 2 cm column of Kieselgel 60, 230-400 mesh (Merck). Elution: hexane/ethyl acetate 10/0 to 8/2. The appropriate fractions were pooled and concentrated to give **5a** (368 mg, 72%) as a syrup. ^{31}P NMR: δ -6.42 and -6.77 (diastereoisomers).

Selectively Deprotected Derivative 6a (Propenyl-cleavage from 5a). Compound **5a** (368 mg, 0.18 mmol) was treated with $HgO/HgCl_2$ as described for the preparation of **1**.[2] The product was purified by chromatography on a 4 x 2 cm column of Kieselgel 60. Elution: hexane/ethyl acetate 9/1 to 7/3. Pure **6a** (296 mg, 82%) was obtained as a syrup. TLC (hexane/ethyl acetate 7/3): R_f 0.25. ^{31}P NMR: δ -6.27 and -6.57 (diastereoisomers).

***N*-Benzyloxycarbonylglycine-3-hydroxy-1-propylamide (7).** *N*-Benzyloxycarbonylglycine pentachlorophenyl ester[18,19] (3.0 g, 6.6 mmol) was dissolved in dioxane (20 mL) and 3-amino-1-propanol (1.25 mL, 16.4 mmol) in dioxane (10 mL) was added. The reaction mixture was stirred at room temperature for 1 h, and concentrated. The residue was taken-up in 1M $KHSO_4$ (50 mL) and extracted with dichloromethane (5 x 50 mL). The combined organic layers were dried and concentrated. The product was crystallized from ethyl acetate/hexane. Yield: 1.7 g (97%). Mp 94-96 °C. 200 MHz ^1H NMR: δ 7.4-7.25 (5 arom. H), 6.74 (br t, -NH- amide), 5.70 (br t, -NH-urethane), 5.11 (s, Ph-CH_2-O-), 3.79 (d, -NH-CH_2-CO-, J 8 Hz), 3.60 (br t, -CH_2-CH_2-OH), 3.39 (q, -NH-CH_2-CH_2-, J 6.5 Hz), 2.9 (br, -OH), 1.69

(qui, $-CH_2-CH_2-CH_2-$). ^{13}C NMR ($CDCl_3$/ CD_3OD 4/1): δ 170.9, 157.6, 136.5, 128.7, 128.4, 128.2, 67.4, 59.7, 44.3, 36.8, 31.9.

Anal. Calcd for $C_{13}H_{18}N_2O_4$: C, 58.63; H, 6.81; N, 10.52. Found: C, 58.74; H, 6.71; N, 10.49.

In later preparations the ester of benzyloxycarbonylglycine and *N*-hydroxysuccinimide[20] was used as the starting compound. In this case the salt, formed by the liberated *N*-hydroxysuccinimide and excess of 3-amino-1-propanol, precipitates from the reaction mixture. Compound 7 was obtained in 70-80% yield after a single crystallization.

Fully Protected Derivative 8a. This derivative was prepared in a manner similar to that described for **5a**. Compound 7 (59 mg, 0.22 mmol) was phosphorylated with 3 (1.1 mL 0.2 M solution in dioxane). The solution of the intermediate active phosphotriester thus obtained was added to a mixture of **6a** (296 mg, 0.15 mmol) and *N*-methylimidazole (20 μL). After stirring at room temperature for 2 h, TLC (hexane/ethyl acetate 7/3) showed approximately 50% conversion of **6a** into baseline material, that is into **8a**. Extra 7 (80 mg, 0.30 mmol) was phosphorylated with 0.2 M 3 in dioxane (1.5 mL) and, 30 min later, added to the reaction mixture. After 2 h, the conversion of **6a** was complete, as judged from TLC. The product was purified by chromatography on a 4 x 2 cm column of Kieselgel 60. Elution: chloroform/acetone 100/0 to 95/5. Pure **8a** (305 mg, 84%) was obtained as a syrup. TLC ($CHCl_3$/acetone 95/5): R_f 0.29. ^{31}P NMR: δ -5.38 (phosphotriester between ribitol and spacer), -6.32 and -6.59 (phosphotriester between ribitol and ribose).

Complete Deprotection of 8a. a. Removal of the 2-chlorophenyl groups.[11] Compound **8a** (285 mg, 0.12 mmol) was concentrated from dry dioxane (5 mL) twice and redissolved in dry tetrahydrofuran (4.8 mL). *Syn*-pyridine-2-carboxaldoxime (0.44 g, 3.6 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (0.30 mL, 2.4 mmol) were added successively and the reaction mixture was left at room temperature for 48 h. TLC (chloroform/acetone 9/1) showed complete conversion of **8a** (R_f 0.36) into baseline material. ^{31}P NMR (0.4 mL of the reaction mixture + 0.1 mL THF-D_8) gave signals of phosphodiesters at 0.30 and -0.35 ppm. Dowex 50WX4 in the triethylammonium-form (1.8 g) was added and the reaction mixture was stirred at room temperature for 90 min. The ion-exchange resin was removed by filtration and washed with tetrahydrofuran. The combined filtrate and washings were concentrated. The oil thus obtained was used in the next step without purification.

b. Removal of the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl and the tert-butyldiphenylsilyl group.[12] The oil, as obtained in the previous step, was dissolved in dry dioxane (4.0 mL) and 1 M tetra-*n*-butylammonium fluoride in dioxane (2.0 mL) was added. After stirring for 16 h at room temperature, TLC (chloroform/methanol/ triethylamine 90/10/0.1) showed complete conversion of the starting compound (R_f 0.33) into a more polar product (R_f 0.18). ^{31}P NMR (0.4 mL of the reaction mixture + 0.1 mL THF-D_8) gave two resonances at 0.95 and 0.50 ppm. The reaction mixture was concentrated. The residue was dissolved in methanol and applied to a column of Sephadex LH-20 (45 x 3 cm). Elution was effected with methanol and fractions of 4 mL were collected. Aliquots of each fraction were spotted on TLC-plates. Spots were visualized by spraying with methanolic H_2SO_4, followed by heating. Sugar-positive fractions (nr 36-45) were pooled and concentrated to give a syrup (**9**, n=2, R^1=BOM, R^2=Bzl, R^3=NHZ, *bis*[tetra-*n*-butylammonium] salt). ^{31}P NMR (CD_3OD): δ 1.95 and 1.56.

c. Removal of the benzyloxycarbonyl, benzyloxymethyl and benzyl groups. The syrup, obtained in step b, was coevaporated with *tert*-butanol/water 95/5, redissolved in *tert*-butanol/water 1/2 (9.6 mL) and 1 M acetic acid (120 µL), 265 mg (2.4 mmol) glycinamide hydrochloride and 400 mg 10% Pd/C were added. The reaction mixture was stirred under a gentle stream of hydrogen (atmospheric pressure) for 24 h. The catalyst was removed by filtration and washed with water. The combined filtrate and washings were lyophilized. The material was redissolved in water (10 mL) and again hydrogenated for 24 h in the presence of 400 mg of fresh Pd/C. After work-up (as described above), the product was purified twice by gel filtration over a column (140 x 2 cm) of Sephadex G-25 (fine). Elution: 0.01 M triethylammonium bicarbonate pH 7, 25 mL/h. Ninhydrine and sugar positive fractions were pooled, lyophilized and again lyophilized from water (3x) to give **9** (n=2, R^1=R^2=H, R^3=NH_2, mixed tetra-*n*-butylammonium/triethylammonium salt). This material was passed over a 4 x 2 cm column of SP-Sephadex C-25, Na^+-form (dry weight 1.8 g) in water. After lyophilization, **9a** was obtained as a white powder (42 mg, 41%). ^1H NMR (D_2O, reference HDO, set at 4.77 ppm): 5.02 (s, H-1 Ribf), 4.98 (s, H-1 Ribf), 4.6-4.5 (m, 8 lines, 1H), 3.76 (s, CH_2 Gly), 3.33 (t, -NH-CH_2-CH_2-, spacer), 1.83 (t, -CH_2-CH_2-CH_2-, spacer). The remaining protons give a highly complex pattern at 4.3-3.5 ppm. Less than 1% of $NHCH_3$-analogue (δ 2.74) and 0.4% of $N(CH_3)_2$-analogue (δ 2.89) was detected. ^{13}C

NMR (external reference tetramethylammonium chloride δ 56.2): δ 167.8 (s), 107.7 (s, C-1 Ribf), 107.5 (s, C-1 Ribf), 83.5 (s, CH), 82.8 (d, CH, J_{CP} 5.8 Hz), 75.2 (s, CH), 75.1 (d, CH, J_{CP} 3.4 Hz), 74.6 (br s, CH, J_{CP} unresolved), 72.3 (s, 2 x CH), 71.8 (d, 2 x CH, J_{CP} 8.8 Hz), 71.4 (s, CH), 71.0 (s, 2 x CH), 6.95 (s, CH_2), 69.3 (s, CH_2), 67.5 (d, CH_2, J_{CP} 4.4 Hz), 67.3 (d, CH_2, J_{CP} 4.4 Hz), 64.4 (d, CH_2, J_{CP} 5.9 Hz), 63.4 (s, CH_2), 63.2 (s, CH_2), 41.3 (s, CH_2 Gly), 37.2 (s, CH_2), 30.0 (d, CH_2, J_{CP} 7.3 Hz). ^{31}P NMR: δ 1.54 and 0.74. The FAB(-) MS showed m/z 823 ($[C_{25}H_{49}N_2O_{24}P_2]^-$, *e.g.* **9a**-$Na^+$) as the most abundant signal in the high mass region.

Compounds 5b, 6b and 8b. These derivatives were prepared in a similar manner as the corresponding compounds with n=1. On a 3.5 mmol scale **8a** was obtained in 49% over-all yield, starting from **6a** and **2**. TLC: **5b** (hexane/ethyl acetate 7/3) R_f 0.53, **6b** (hexane/ ethyl acetate 7/3) R_f 0.24, **8b** ($CHCl_3$/acetone 95/5) R_f 0.30. ^{31}P NMR: **5b**, δ -6.33 (two overlapping signals), -6.62, -6.69; **6b**, δ -6.32, -6.35, -6.60, -6.69; **8b**, δ -5.35 (phosphotriester between ribitol and spacer), -6.33, -6.39, -6.63, -6.68.

Compound 9b (Three Repeating Units + Spacer). Compound **8b** (4.5 g, 1.3 mmol) was treated with 7.0 g (58 mmol) *syn*-2-pyridinecarboxaldoxime and 4.8 mL N^1,N^1,N^3,N^3-tetramethylguanidine in dry tetrahydrofuran (22 mL), followed by treatment with 0.5 M tetra-*n*-butylammonium fluoride in dioxane (65 mL), as described for deprotection of **8a** (steps a and b). The product was purified in two portions by gel filtration over Sephadex LH-20 (140 x 4 cm). Elution: methanol, 3.5 mL/min. Sugar positive fractions (elution volume 440-510 mL) were pooled and concentrated. The combined product of two runs was subjected to repeated gel filtration under the same conditions to give 3.8 g (1.26 mmol) of syrupy **9** (n=3, R^1=BOM, R^2=Bzl, R^3=NHZ, *tris*[tetra-*n*-butylammonium] salt).

The benzyl-type protecting groups were removed, as described for deprotection of **8a** (step c), by two successive hydrogenations (in the presence of 30 eq glycinamide hydrochloride, 1.3 mmol acetic acid and 4.25 g 10% Pd/C) in *tert*-butanol/water 1/2 (105 mL) and water (60 mL), respectively. The product, obtained after lyophilization, was divided in four equal parts. Each sample was dissolved in 1 M triethylammonium bicarbonate (TEAB) pH 7 (2.5 mL), briefly degassed, and applied to a 140 x 2 cm column of Sephadex G-25 (fine). Elution was effected with 0.01 M

TEAB pH 7. The appropriate fractions (elution volume 165-240 mL) were lyophilized. The material obtained from two runs was combined, dissolved in a small volume of 0.01 M TEAB pH 7 and subjected to repeated gel filtration under the same conditions. A total amount of 1.20 g of product was obtained. This material was passed over a 18 x 2 cm column of SP Sephadex C-25 (Na^+-form) in water and lyophilized to give 1.05 g (68%) of **9b**. FAB mass spectral data, Table 1; 360 MHz ^1H NMR spectrum, FIG. 1; ^{13}C NMR spectral data, Table 2; 145 MHz ^{31}P NMR spectrum, FIG. 4.

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REFERENCES

1. Preliminary communication: P. Hoogerhout, D. Evenberg, C. A. A. van Boeckel, J. T. Poolman, E. C. Beuvery, G. A. van der Marel, and J. H. van Boom, *Tetrahedron Lett.*, **28**, 1553 (1987).

2. Part I: J. P. G. Hermans, L. Poot, M. Kloosterman, G. A. van der Marel, C. A. A. van Boeckel, D. Evenberg, J. T. Poolman, P. Hoogerhout, and J. H. van Boom, *Recl. Trav. Chim. Pays-Bas*, **106**, 498-504 (1987).

3. R. M. Crisel, R. S. Baker, and D. E. Dorman, *J. Biol. Chem.*, **250**, 4926 (1975).

4. P. Branefors-Helander, C. Erbing, L. Kenne, and B. Lindberg, *Acta Chem. Scand. Ser. B*, **30**, 276 (1976).

5. D. M. Granoff, and R. S. Munson, Jr., *J. Infect. Dis.*, **153**, 448 (1986).

6. P. Hoogerhout, D. Evenberg, C. A. A. van Boeckel, J. T. Poolman, E. C. Beuvery, and J. H. van Boom, manuscript in preparation.

7. P. J. Garegg, and B. Samuelsson, *Carbohydr. Res.*, **86**, 293 (1980).

8. P. J. Garegg, R. Johansson, I. Lindh, and B. Samuelsson, *Carbohydr. Res.*, **150**, 285 (1985).

9. G. A. van der Marel, C. A. A. van Boeckel, G. Wille, and J. H. van Boom, *Tetrahedron Lett.*, 3887 (1981).

10. R. Gigg, and C. D. Warren, *J. Chem. Soc. (C)*, 1903 (1968).

11. C. B. Reese, and L. Zard, *Nucl. Acids Res.*, **9**, 639 (1981).

12. E. J. Corey, and A. Venkateswarlu, *J. Am. Chem. Soc.*, 94, 6190 (1972).

13. T. Bieg, and W. Szeja, *Synthesis*, 76 (1985).

14. M. K. Anwer, and A. F. Spatola, *Synthesis*, 929, (1980).

15. S. Ram, and L. D. Spicer, *Tetrahedron Lett.*, **28**, 515 (1987).

16. W. Bremser, L. Ernst, W. Fachinger, R. Gerhards, A. Hardt, and P. M. E. Lewis, *Carbon-13 NMR Spectral Data*, VCH, Weinheim, spectrum CNMR4906.

17. J. H. van Boom, and C. T. J. Wreesman in: *Oligonucleotide synthesis: a practical approach*; M.J. Gait, Ed.; I.R.L. Press, Oxford, U.K. (1984), pp 153-183.

18. G. Kupryszewski, and M. Formela, *Roczniki Chem.*, **35**, 1533 (1961); *Chem. Abstr.*, **57**, 7373 (1962).

19. M. Itoh, *Chem. Pharm. Bull.*, **18**, 784 (1970).

20. G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).