Synthesis of a spacer-containing nonasaccharide fragment of *Streptococcus pneumoniae* 19F capsular polysaccharide

Magnus Nilsson and Thomas Norberg

Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, S-750 07 Uppsala, Sweden

The nonasaccharide 1, representing three repeating units of the capsular polysaccharide from *Streptococcus pneumoniae* 19F, has been synthesized. The synthetic strategy is to first synthesize, by an AB + C route, trisaccharide derivative 14, having *O*-benzyl groups as persistent, and 1-*O*-allyl and 4″-*O*-acetyl groups as temporary, protecting groups. Then, specific replacement of the 1-*O*-allyl group with an α-H-phosphonate monoester gives trisaccharide 17, which is coupled to a spacer. The obtained derivative 18 is then extended from the 4″ end in a stepwise fashion to give compound 20, using trisaccharide 17 and solution-phase H-phosphonate chemistry. Finally, removal of the persistent O-protecting groups gives the nonasaccharide 1.

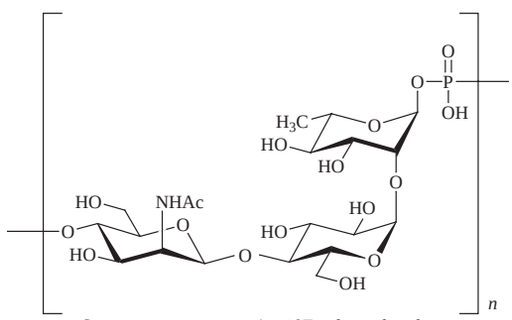
Introduction

Bacterial capsular polysaccharides are important bacterial surface components that have been studied for nearly a century by immunologists and chemists. Chemical structures are now known for a great number of bacterial species,[1] and their immunological properties have been well characterized. Immunologically, bacterial capsular polysaccharides give rise to highly specific immune responses. Because of this, they have been used in vaccines, replacing less efficient whole-cell vaccines. Such polysaccharide vaccines, especially the modern varieties consisting of polysaccharides conjugated to immunogenic proteins (conjugate vaccines[2]), have proved to be very efficient in preventing human disease. For example, conjugate vaccines against *Haemophilus influenzae* type b (HiB) are today used widely as paediatric vaccines. Their use has dramatically reduced the incidence of childhood meningitis, a life-threatening disease caused by the HiB organism. Spurred by the success of HiB conjugate vaccines, intense efforts are now being made in both industry and academia to further develop the conjugate vaccine concept.

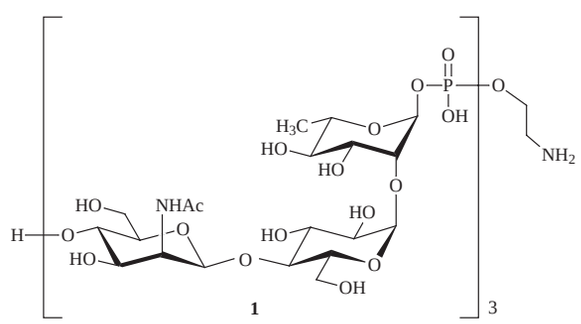
In most previous work, the source of the bacterial capsular polysaccharides has been bacterial cell cultures. After culturing and harvesting of the bacterial cells, the polysaccharides are isolated from the cells by centrifugation, precipitation and chromatography, and then typically subjected to some kind of chemical modification that makes possible conjugation to a protein. These processes, culture, isolation and chemical modification, are not always trivial, and the whole process can be hampered by difficulties in each of the steps.

An alternative source of bacterial polysaccharide structures is chemical synthesis. It is today possible, using multistep organic synthesis, to synthesize fragments of bacterial polysaccharides several repeating units in length. Although these synthetic structures are of lower relative molecular mass than the natural polysaccharides, they are often long enough to function very well as immunogenic components of conjugate vaccines.[3] The advantage of a synthetic product over an isolated one is its more defined structure and definite lack of bacterial contaminants, and, in some cases, its economy of production. Only very small amounts of synthetic material are required in a vaccine, typically less than 10 micrograms per dose. This means that a synthetic production facility can operate on a quite modest scale, even though the synthetic process will involve numerous chemical steps.

Several research groups have initiated synthetic programmes



Streptococcus pneumoniae 19F polysaccharide



aimed at preparation of bacterial polysaccharide fragments. Our own efforts in this regard include synthesis of penta- and deca-meric fragments[4,5] and a pentameric analogue[6] of *H. influenzae* type B capsular polysaccharide. We now report synthesis of the nonasaccharide fragment **1** of the capsular polysaccharide from *Streptococcus pneumoniae* 19F (native structure, see above). Since this organism is an important pathogen (it is a common cause of respiratory infections in children), it is not surprising that synthetic efforts have been reported before. The repeating-unit trisaccharide has been synthesized by several groups.[7–10] However, the crucial joining together of several repeating units by phosphodiester linkages to form larger structures, presumably required for a reliable immune response, has, to our knowledge, not been reported.

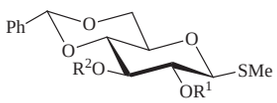
Results and discussion

General strategy

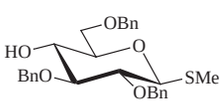
Our general strategy for the synthesis of compound **1** was the following: first, trisaccharide derivative **14** was synthesized, having *O*-benzyl groups as persistent, and 1-*O*-allyl and 4″-*O*-

acetyl groups as temporary protecting groups. Then, specific replacement of the 1-*O*-allyl group with an α-H-phosphonate monoester gave a trisaccharide **17** which could be coupled to a spacer (useful later in the conjugation to proteins). The obtained derivative **18** was then extended from the 4″ end in a stepwise fashion to give nonasaccharide **20**, using substrate **17** and solution-phase H-phosphonate chemistry. Finally, removal of the persistent O-protecting groups gave the nonasaccharide **1**.

(1) Synthesis of trisaccharide 14. As noted in the Introduction section, several syntheses have been described of the *S. pneumoniae* 19F trisaccharide repeating unit, using either A + BC[7] or AB + C[8–10] strategies. To make synthesis of larger structures possible, a protected trisaccharide repeating unit should contain selectively removable temporary protecting groups in the 1- and 4″-position. Our approach was to synthesize such a trisaccharide **14**, using thioglycoside blocks in an AB + C strategy. Many of the intermediates were crystalline, and the chromatographic purifications necessary were not of the most difficult type, which allowed for the production of gram amounts of compound **14**. The following steps were carried out: Donor **4**, suitably protected for later manipulation at the 2- and 4-position, was prepared from substrate **2**[11] by selective benzylation at the 3-position as described[12] (minor modifications), giving compound **3** in 55% yield. Benzoylation of compound **3** then gave compound **4** in 88% crystalline yield.

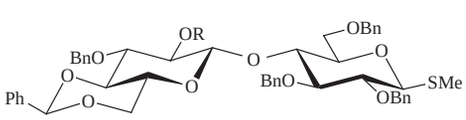


2 $R^1 = R^2 = H$
3 $R^1 = H$, $R^2 = Bn$
4 $R^1 = Bz$, $R^2 = Bn$

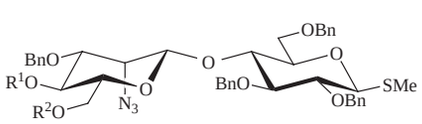


5

Conversion of fully protected monosaccharide **4** into the bromo sugar followed by silver triflate (AgOTf)-promoted coupling with compound **5**[13] gave disaccharide **6** in 66% yield. Debenzoylation of compound **6** using methanolic sodium methoxide in 1,4-dioxane gave mono-ol **7** in 96% yield, with a free 2′-OH group. Reaction of compound **7** with triflic anhydride in dichloromethane–pyridine and subsequent reaction of the product **8** with sodium azide in DMF gave azide **9** in 75% yield (from mono-ol **7**). The *manno* configuration in product **9** was evident from the ^{1}H NMR coupling pattern.



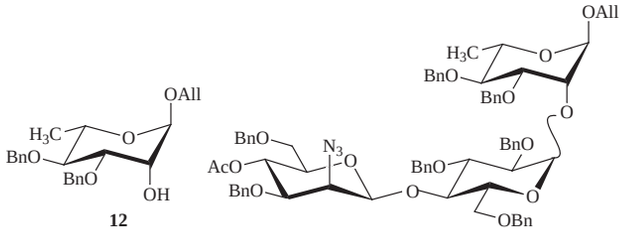
6 R = Bz
7 R = H
8 R = Tf



9 $R^1 = R^2$ = benzylidene
10 $R^1 = H$, $R^2 = Bn$
11 $R^1 = Ac$, $R^2 = Bn$

Regioselective opening of the benzylidene acetal[14] in compound **9** gave alcohol **10** in 81% yield, which was 4′-O-acetylated to give donor **11** in 94% yield. Methyl triflate-promoted coupling of fully protected compound **11** with acceptor **12**[15] in dichloromethane gave a 2:1 α/β-mixture of trisaccharides (**13** α/β) in 87% overall yield. Efforts to improve the α:β ratio in this coupling included both change of solvent (diethyl ether or diethyl ether–dichloromethane mixtures) and change of coupling method (silver triflate- or halide-ion-promoted coupling with the bromide derived from compound **11**). No significant improvement was observed.

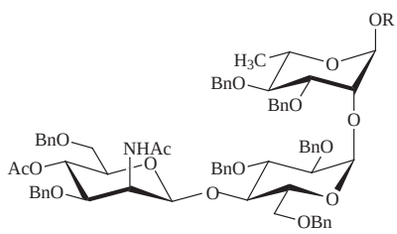
The obtained α/β mixture was difficult to separate by chromatography, but after conversion of the azide group into an acetamido group, the corresponding mixture of trisaccharides was easily separated.



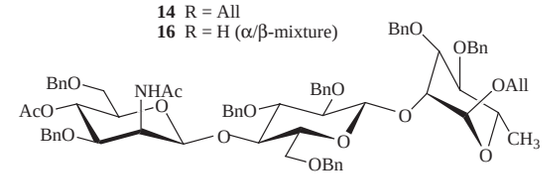
12

13 α/β

The conversion **13**α/β ⟶ **14/15** was accomplished by, first, reaction of azide **13**α/β with triphenylphosphine, then hydrolysis of the formed imine, and finally acetylation of the amine by using pyridine–acetic anhydride to give the desired compounds **14** (α-form) and **15** (β-form) in 58 and 28% yield, respectively (from **13**α/β). Deallylation of trisaccharide **14** using, first, Wilkinson's catalyst and then 80% acetic acid gave compound **16** in 80% yield.

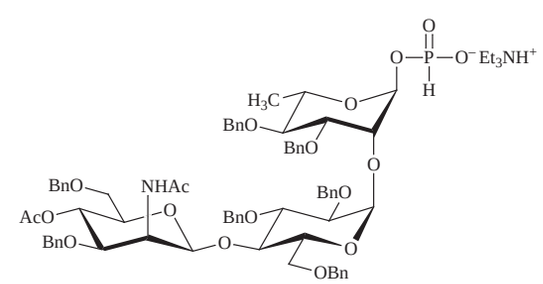


14 R = All
16 R = H (α/β-mixture)



15

Treatment of compound **16** with phosphorous acid and an excess of 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane in pyridine gave compound **17** in 61% yield. The α configuration of product **17** was indicated by $J_{C\text{-}1,H\text{-}1}$ (180 Hz) in the NMR spectrum. Very little β product was detected.



17

(2) Nonasaccharide synthesis. The attachment of a phosphate-linked spacer group at the reducing end of compound **17** and subsequent stepwise oligomerization to give a protected phosphodiester-linked nonasaccharide **20** was carried out in solution (with chromatographic purification after each coupling cycle) using a common H-phosphonate protocol (coupling with pivaloyl chloride in pyridine, then oxidation with iodine in water–pyridine). Thus, compound **17** was first coupled with Cbz-ethanolamine[16] and the product was then deacetylated using methanolic sodium methoxide to give the spacer-containing trisaccharide acceptor **18** in 91% yield.

Using compound **17**, the H-phosphonate coupling/deprotection cycle was performed twice on phosphate **18**, giving, first, the hexasaccharide **19** and then the nonasaccharide **20** in 87 and 83% yield, respectively. The deprotection steps

Table 1 $^1H/^{13}C$ Chemical shift (*J*/Hz in parentheses)[a] of compound **3**

	1	2	3	4	5	6a	6b
Glc	4.37 (9.4) 86.6	3.60 (8.6) 72.5	3.65–3.73 81.7*	3.65–3.73 81.4*	3.50 70.9	3.77 (10.2) 68.7	4.36 (4.8)

[a] Further $^1H/^{13}C$ chemical shifts are 2.21/12.1 (s, 3 H, S*CH*$_3$), 5.57/101.4 (s, 1 H, Ph*CH*O).

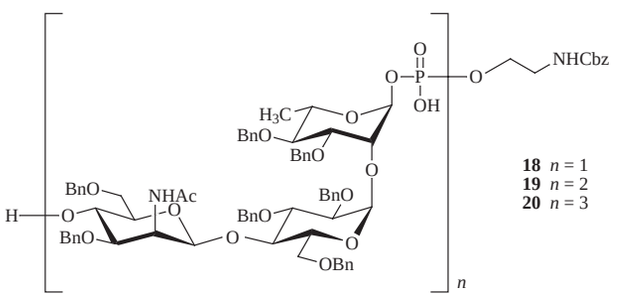
Table 2 $^1H/^{13}C$ Chemical shift (*J*/Hz in parentheses)[a] of compound **4**

	1	2	3	4	5	6a	6b
Glc	4.52 (9.9) 83.9	5.36 (8.9) 71.2	3.91 (9.4) 79.3	3.83 (9.1) 81.8	3.58 70.8	3.82 (10.5) 68.7	4.41 (5.1)

[a] Further $^1H/^{13}C$ chemical shifts are 2.19/11.4 (s, 3 H, S*CH*$_3$), 5.61/101.3 (s, 1 H, Ph*CH*O) and 165.2 (*C*=O).

were monitored by MALDI-TOF MS† and FAB-MS,‡ since TLC monitoring was found to be difficult.

Hydrogenolysis of compound **20** (Pd/C in ethanol–acetic acid–water; atmospheric pressure) gave the deprotected nonasaccharide **1** in 73% yield.



18 *n* = 1
19 *n* = 2
20 *n* = 3

The ^1H NMR spectrum of compound **1** was well resolved and showed doubling of the signals of the three anomeric protons in the ratio 2:1, which could be explained by assuming that the terminating trisaccharide, C (the terminating trisaccharide refers to the one with unsubstituted OH-4 of ManNAc), differs slightly in chemical shift from the other two units, A and B, or that the signals from ManNAc-C, Rha-A and Glc-A give slightly different chemical shifts from the rest of the residues. Chemical shifts of compound **1** are presented according to the first assumption. Nuclear Overhauser Enhancement Spectroscopy (NOESY) experiments showed NOE correlation between the H-1s of rhamnose and the H-1s of glucose, indicating α anomeric configurations of the rhamnose units. Also, a coupled 2D heteronuclear multiple quantum-filtered coherence (HMQC) spectrum showed rhamnose $J_{H\text{-}1,C\text{-}1}$ couplings indicating α anomeric configurations (174 Hz). Finally, the structure of compound **1** was verified by the agreement between the measured (HRMS, 1833.5186 Da) and calculated (1833.5142 Da) relative molecular mass of the M − H ion.

Experimental

General methods

Concentrations were performed at reduced pressure (<40 °C bath temperature, unless otherwise stated). Weighed yields are calculated from residues dried *in vacuo* (0.5 mmHg) overnight. Optical rotations were measured at 23 °C (*c* 1.0, $CHCl_3$), using a Perkin-Elmer 341 polarimeter; $[\alpha]_D$-values are given in 10^{-1} deg cm^2 g^{-1}. NMR spectra were recorded for solutions in $CDCl_3$ (internal Me_4Si, δ 0.00) or D_2O (internal acetone, δ_H 2.225, δ_C 31.0) at 300 K unless otherwise stated with Bruker DRX 400 or DRX 600 spectrometers. Only selected NMR data are reported. Assignments were corroborated by appropriate 2-D experiments. Pairwise assignments marked with an asterisk could be reversed. Coupling constants *J* are given within brackets, and are measured in Hz. FAB-MS spectra were recorded with a JEOL JMS-SX/SX-102A instrument. Ions were produced by a beam of Xe atoms (6 keV), using a matrix of glycerol or *m*-nitrobenzyl alcohol. For HRMS, poly(ethylene glycol) (PEG) was used as an internal standard. For MALDI-TOF MS, an LDI-1700XP instrument was used (negative-ion mode), using trihydroxyacetophenone as a matrix; optionally the sample was mixed with aq. 0.1 M ammonium hydrogen citrate, TLC was performed on Silica Gel F_{254} (Merck, Darmstadt, Germany) with detection by UV light and by staining with 5% sulfuric acid in ethanol or 0.5% ninhydrin in butan-1-ol. Column chromatography was performed on Matrex silica gel 60 Å (35–70 μm, Amicon). Molecular sieves (powdered 3 Å and 4 Å) were dried at 280 °C/0.5 mmHg overnight. Dichloromethane was distilled from P_2O_5 when necessary. Pyridine was of *pro analysi* quality and, when used for H-phosphonate coupling, was first distilled from ninhydrin and then fractionally distilled from anhydrous barium oxide.

Methyl 3-*O*-benzyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside 3. A mixture of methyl 4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside **2**[11] (2.00 g, 6.7 mmol) and sodium hydride (80% in mineral oil; 0.50 g, 16.8 mmol) in DMF (16 ml) was stirred at room temperature (rt) for 2 h. Copper(II) chloride (0.90 g, 6.7 mmol) was added and the mixture was stirred for 15 min, then benzyl bromide (1.2 ml, 10.0 mmol) was added and the mixture was stirred for 18 h. Additional benzyl bromide (1 ml, 8.4 mmol) was then added and stirring was continued for another 24 h. Methanol (1.5 ml) was added to destroy excess of reagent. The mixture was diluted with ethyl acetate, washed successively with 5% aq. ammonium hydroxide and brine, dried (Na_2SO_4), filtered and concentrated. Column chromatography (dichloromethane–acetone 95:5) of the residue gave the title compound (1.43 g, 55%) as a solid, which could be recrystallized from ethyl acetate–petroleum spirit (65–70 °C) to give needles, mp 173–174 °C; $[\alpha]_D$ +49; $^1H/^{13}C$ NMR data ($CDCl_3$) are shown in Table 1 [HRMS: Calc. for $C_{21}H_{25}O_5S$: 389.1423. Found: 389.1427 (M + H$^+$)].

Methyl 2-*O*-benzoyl-3-*O*-benzyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside 4. To a cooled (0 °C) solution of compound **3** (2.82 g, 7.25 mmol) in pyridine (40 ml) was added benzoyl chloride (1.3 ml, 11 mmol) and the solution was stirred for 3 h. Water (1.5 ml) was added and the solution was then diluted with dichloromethane, washed successively with 2 M sulfuric acid and 1 M aq. sodium hydrogen carbonate, dried (Na_2SO_4), filtered and concentrated. The residue was recrystallized from ethanol to give the title compound (3.16 g, 88%) as needles, mp 134–136 °C; $[\alpha]_D$ +26. $^1H/^{13}C$ NMR data ($CDCl_3$) are shown in Table 2 [HRMS: Calc. for $C_{28}H_{29}O_6S$: 493.1685. Found: 493.1713 (M + H$^+$)].

Methyl 4-*O*-(2-*O*-benzoyl-3-*O*-benzyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-2,3,6-tri-*O*-benzyl-1-thio-β-D-glucopyranoside 6. To a cooled (0 °C) mixture of compound **4** (1.13 g, 2.3 mmol) and molecular sieves (4 Å) in dichloromethane (25 ml) was

† Matrix-assisted laser-desorption time-of-flight mass spectrometry.
‡ Fast-atom bombardment mass spectrometry.

Table 3 $^1H/^{13}C$ Chemical shift (*J*/Hz in parentheses)[a] of compound **6**

	1	2	3	4	5	6a	6b
Glc	4.22 (9.7)	3.40 (9.2)	3.56 (9.2)	3.99 (9.7)	3.15	3.47	3.61 (2.6, 11.3)
	85.2	80.4	84.5	76.5	78.6	67.7	
Glc′	4.73 (8.7)	5.22 (8.7)	3.68 (9.2)	3.73 (9.2)	3.25	3.47	4.21 (5.1, 10.5)
	100.7	73.9	77.9	81.9	66.1	68.6	

[a] Further $^1H/^{13}C$ chemical shifts are 2.14/12.7 (s, 3 H, S*CH*$_3$), 5.51/101.2 (s, 1 H, Ph*CH*O) and 164.8 (*C*=O).

Table 4 $^1H/^{13}C$ Chemical shift (*J*/Hz in parentheses)[a] of compound **9**

	1	2	3	4	5	6a	6b
Glc	4.35 (9.7)	3.46 (nd)	3.67 (9.2)	3.99 (9.2)	3.47	3.75	3.75
	85.5	80.6	84.9	77.6	78.6*	68.8	
Man	4.67 (1.0)	3.83 (3.6)	3.48 (9.7)	3.90 (9.2)	3.02	3.51	3.99 (5.1, 10.7)
	100.3	63.6	76.7*	78.5	67.3	68.4	

[a] Further $^1H/^{13}C$ chemical shifts are 2.29/12.8 (s, 3 H, S*CH*$_3$) and 5.49/101.6 (s, 1 H, Ph*CH*O).

Table 5 $^1H/^{13}C$ Chemical shift (*J*/Hz in parentheses)[a] of compound **11**

	1	2	3	4	5	6a	6b
Glc	4.34 (9.4)	3.38 (9.1)	3.68 (9.1)	3.96 (9.7)	3.48	3.75	3.79 (11.7, 3.2)
	85.5	80.8	84.8	76.8	78.4	69.0	
Man	4.65 (nd)	3.85 (3.5)	3.34 (9.7)	5.09 (9.7)	3.27	3.31	3.99 (4.1, 10.8)
	99.4	61.7	78.2	68.8	74.4	70.2	

[a] Further $^1H/^{13}C$ chemical shifts are 1.86/20.9 (s, 3 H, OCO*CH*$_3$) and 2.20/12.3 (s, 3 H, S*CH*$_3$).

added bromine (0.12 ml, 2.3 mmol). The mixture was stirred for 18 min, then cyclohexene (0.53 ml, 5.2 mmol) was added and the mixture was stirred at 0 °C for 10 min. After cooling of the mixture to −40 °C, monosaccharide **5**[13] (1.00 g, 2.1 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (0.427 g, 2.1 mmol) were added and stirring was continued for another 30 min. A solution of silver triflate (0.617 g, 2.4 mmol) in toluene (25 ml) was added dropwise to the mixture during 30 min at −40 to −50 °C, and, after complete addition, the mixture was stirred for another 40 min. Pyridine (1.3 ml) was added, the mixture was filtered through Celite, and the filtrate was diluted with dichloromethane, washed with 10% aq. sodium thiosulfate, dried (Na_2SO_4), filtered and concentrated. Column chromatography of the residue (stepwise gradient elution, toluene–ethyl acetate 9:1–1:1) gave compound **6** as a solid (1.27 g, 66%), which could be recrystallized from ethyl acetate–ethanol as needles, mp 187–191 °C; $[\alpha]_D$ +23; $^1H/^{13}C$ NMR data ($CDCl_3$) are shown in Table 3 [HRMS: Calc. for $C_{55}H_{56}NaO_{11}S$: 947.3441. Found: 947.3457 (M + Na^+)].

Methyl 4-*O*-(2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-mannopyranosyl)-2,3,6-tri-*O*-benzyl-1-thio-β-D-glucopyranoside 9. To a stirred solution of benzoate **6** (2.41 g, 2.60 mmol) in 1,4-dioxane (20 ml) was added 0.5 M methanolic sodium methoxide (20 ml). The solution was heated to 40 °C and stirred for 2 h 45 min. The solution was diluted with 1,4-dioxane (25 ml), neutralized with Dowex 50 (H^+) resin, filtered and concentrated. Column chromatography of the residue (toluene–ethyl acetate 7:1) gave the free mono-ol **7** as a solid (2.05 g, 96%).

A solution of the latter compound (2.00 g, 2.44 mmol) in dichloromethane–pyridine (2:1; 30 ml) was stirred and cooled (0 °C) while triflic anhydride (1 ml, 5.95 mmol) was added. The solution was kept at 0 °C, and additional triflic anhydride was added after 2 h 45 min (1 ml, 5.95 mmol), and after 10 h (0.5 ml, 2.98 mmol). After 11 h the reaction mixture was diluted with dichloromethane, washed successively with 2 M sulfuric acid, 1 M aq. sodium hydrogen carbonate and water, dried (Na_2SO_4), filtered and concentrated.

A mixture of the crude residue containing triflate **8** (yellow foam) and sodium azide (0.80 g, 12.3 mmol) in DMF (40 ml) was stirred at 70 °C for 4 h 30 min, then diluted with ethyl acetate, washed with water, dried (Na_2SO_4), filtered and concentrated. Column chromatography of the residue with ethyl acetate in petroleum spirit (stepwise gradient elution, 25–33%) gave the title compound **9** as a syrup (1.54 g, 75%), $[\alpha]_D$ −18; $^1H/^{13}C$ NMR data ($CDCl_3$) are shown in Table 4 [HRMS: Calc. for $C_{48}H_{52}N_3O_9S$: 846.3424. Found: 846.3476 (M + H^+)].

Methyl 4-*O*-(4-*O*-acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-mannopyranosyl)-2,3,6-tri-*O*-benzyl-1-thio-β-D-glucopyranoside 11. To a cooled (ice) mixture of compound **9** (1.44 g, 1.70 mmol), sodium cyanoborohydride (0.60 g, 8.50 mmol) and molecular sieves (3 Å) in THF (20 ml) was added dropwise fluoroboric acid (54% in diethyl ether; 1.2 ml, 8.5 mmol). Additional fluoroboric acid was added after 30 min (0.3 ml, 2.2 mmol), and again after 90 min. After 110 min, the reaction mixture was filtered, diluted with ethyl acetate, washed with 1 M aq. sodium hydrogen carbonate, dried (Na_2SO_4), filtered and concentrated. Column chromatography of the residue with ethyl acetate in petroleum spirit (1:2) gave mono-ol **10** as a syrup (1.18 g, 81%), which then was treated with pyridine–acetic anhydride (2:1; 15 ml) for 14 h. Concentration and co-concentration with toluene of the reaction mixture gave a residue, which was purified by column chromatography with petroleum spirit–ethyl acetate (2:1) as eluent. The appropriate fractions were collected and concentrated to give compound **11** as an amorphous compound (1.16 g, 94%), $[\alpha]_D$ +20 (*c* 0.2); $^1H/^{13}C$ NMR data ($CDCl_3$) are shown in Table 5 [HRMS: Calc. for $C_{50}H_{55}N_3NaO_{10}S$: 912.3506. Found: 912.3535 (M + Na^+)].

Allyl *O*-[2-acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-β-D-mannopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl]-(1→2)-3,4-di-*O*-benzyl-α-L-rhamnopyranoside 14 and allyl *O*-[2-acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-β-D-mannopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-benzyl-β-D-glucopyranosyl]-(1→2)-3,4-di-*O*-benzyl-α-L-rhamnopyranoside 15. A mixture of disaccharide **11** (1.17 g, 1.32 mmol), rhamnoside **12**[15] (0.66 g, 1.71 mmol) and molecular sieves (4 Å) in dichloromethane (30 ml) was stirred at rt for 30 min. Then methyl triflate (0.43 ml, 3.95 mmol) was added and stirring was continued at rt for 24 h. Triethylamine (1.65 ml, 11.8 mmol) was added to destroy excess of methyl triflate, and the mixture was filtered

Table 6 $^1H/^{13}C$ Chemical shift (*J*/Hz in parentheses)[a] of compound **14**

	1	2	3	4	5	6a	6b
Rha	4.82 (1.5)	4.02 (3.2)	3.88 (9.4)	3.58 (9.4)	3.73 (6.1)	1.36	
	96.6	75.1	79.1	80.3	68.3	18.0	
Glc	4.90 (3.5)	3.52 (9.1)	3.92 (9.1)	3.97 (9.7)	4.08	3.22	3.36 (2.3, 10.8)
	97.0	79.7	80.6	75.9	69.7	68.0	
Man	4.52 (nd)	4.58 (nd)	3.17 (9.7)	4.99 (nd)	3.17	3.33 (4.1)	3.44 (3.5, 10.8)
	99.4	49.5	77.2	68.0	73.8	69.0	

[a] Further $^1H/^{13}C$ chemical shifts are 1.73/23.2 (NHCOCH_3), 1.93/20.8 (OCOCH_3), 3.90 and 4.14/67.7 (CH_2CH=CH_2), 5.20/117.2 (CH_2CH=CH_2), 5.72/170.9 (N*H*Ac) and 5.86/134.0 (CH_2CH=CH_2).

Table 7 $^1H/^{13}C$ Chemical shift (*J*/Hz in parentheses)[a] of compound **17**

	1	2	3	4	5	6a	6b
Rha	5.67 (1.5)	4.07 (2.5)	3.92 (9.2)	3.59 (9.2)	3.94 (6.2)	1.33	
	94.3 (180)	75.2	79.1	79.6	70.2	17.9	
Glc	4.89 (3.8)	3.49 (9.4)	3.87 (9.1)	3.95 (nd)	4.06	3.18	3.32 (11.7)
	97.2 (171)	79.4	80.6	75.8	69.9	68.0	
Man	4.50 (nd)	4.57 (4.4)	3.15 (9.5)	4.98 (9.7)	3.16	3.33 (4.1)	3.42 (3.2, 10.5)
	99.4 (163)	49.4	77.2	68.0	73.9	69.0	

[a] Further $^1H/^{13}C$ chemical shifts are 1.35/8.6 [(CH_3CH_2)$_3$N], 1.71/22.8 (NHCOOCH_3), 1.93/20.5 (OCOCH_3), 3.06/45.8 [(CH_3CH_2)$_3$N], 94.3 (C-1, $J_{C,P}$ 5.2) and 6.85 (d, $J_{H,P}$ 684, HPO_3^-).

through Celite, diluted with dichloromethane, washed with water, dried (Na_2SO_4), filtered and concentrated. Column chromatography of the residue (stepwise gradient elution toluene–ethyl acetate 9:1–1:1) gave the anomeric mixture of trisaccharides (**13α/β**) as a syrup (1.40 g, 87%, α:β-ratio 68:32) which was not separated.

This mixture (1.39 g, 1.13 mmol) and triphenylphosphine (0.43 g, 1.64 mmol) in dichloromethane (25 ml) was stirred at 37 °C for 23 h. Then the formed imine was hydrolysed by addition of water (25 ml), and stirring was continued for 10 h at 37 °C. The organic layer and a dichloromethane wash were combined, dried (Na_2SO_4), filtered and concentrated. The residue was stirred overnight at rt with pyridine–acetic anhydride (2:1; 15 ml), concentrated, and co-concentrated twice from toluene. Column chromatography of the residue (stepwise gradient elution, petroleum spirit–ethyl acetate 3:2–1:2) gave first compound **14** as a foam (0.81 g, 58% from **13α/β**), $[\alpha]_D$ +18 and then compound **15** as a syrup (0.388 g, 28% from **13α/β**), $[\alpha]_D$ −11; $^1H/^{13}C$ NMR data ($CDCl_3$) of **14** are in Table 6 [HRMS: Calc. for $C_{74}H_{83}NNaO_{16}$: 1264.5609. Found: 1264.5774 (M + Na^+)]. $^1H/^{13}C$ NMR data ($CDCl_3$) of **15**: δ 4.65/104.9 (1 H, d, $J_{1',2'}$ 8.3, H-1′), 4.73/99.6 (m, H-1″) and 4.96/98.8 (d, $J_{1,2}$ 1.5, H-1) [HRMS: Calc. for $C_{74}H_{83}NNaO_{16}$: 1264.5609. Found: 1264.5731 (M + Na^+)].

Triethylammonium *O*-(2-acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-β-D-mannopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl)-(1→2)-3,4-di-*O*-benzyl-α-L-rhamnopyranosyl hydrogen phosphonate 17. Trisaccharide **14** (0.80 g, 0.64 mmol) and tris(triphenylphosphine)rhodium(I) chloride (0.06 g, 64 μmol) in ethanol (12 ml), toluene (4.8 ml) and water (1.5 ml) was refluxed for 4 h. The reaction mixture was diluted with water and extracted with diethyl ether. The combined etheral layers were washed with saturated aq. potassium chloride, dried (Na_2SO_4), filtered and concentrated. The residue was stirred with 80% aq. acetic acid (40 ml) overnight at 80 °C, then diluted with ethyl acetate, washed with 1 M aq. sodium hydrogen carbonate, dried (Na_2SO_4), filtered and concentrated. Column chromatography of the residue (stepwise gradient elution, toluene–ethyl acetate 3:1–3:2) gave compound **16** as an amorphous product (0.61 g, 80%).

Compound **16** (0.614 g, 0.51 mmol) as a solution in pyridine (1.9 ml) was mixed with a 2 M solution of phosphorous acid in pyridine (2.6 ml), then 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane (0.47 g, 2.55 mmol) was added and the mixture was stirred for 54 h at rt. Additional 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane (0.25 g, 1.35 mmol) was added, the reaction mixture was heated to 40 °C, and stirring was continued for 17 h. Then 1 M aq. triethylammonium hydrogen carbonate (2 ml) was added to destroy excess of reagent, and the mixture was diluted with dichloromethane, washed with 0.5 M aq. triethylammonium hydrogen carbonate, dried (Na_2SO_4), filtered and concentrated. The residue was purified by column chromatography (ethyl acetate–acetic acid–methanol–water 40:3:3:2), then was dissolved in dichloromethane and washed with 0.25 M aq. triethylammonium hydrogen carbonate. The organic layer was separated, concentrated and co-concentrated from dichloromethane (5 × 10 ml), which gave, after drying *in vacuo*, the title compound **17** as a foam (0.427 g, 61%), $[\alpha]_D$ +21 (*c* 0.2). $^1H/^{13}C$ NMR data ($CDCl_3$) are shown in Table 7 [HRMS: Calc. for $C_{71}H_{79}NO_{18}P$: 1264.5034. Found: 1264.4949 (M − H^+)].

Preparation of deprotected nonasaccharide 1. To a stirred solution of compound **17** (49.7 mg, 36.3 μmol) and Cbz-ethanolamine[16] (14.0 mg, 71.7 μmol) in pyridine (0.7 ml) under N_2 at rt was added pivaloyl chloride (13 μl, 109 μmol). After 20 min the formed H-phosphonate diester was oxidized during 15 min by addition of water (50 μl) and iodine (28 mg, 110 μmol). Then the reaction mixture was diluted with dichloromethane, washed successively with 10% aq. sodium thiosulfate, 2 M aq. sulfuric acid and 1 M aq. triethylammonium hydrogen carbonate and the dichloromethane layer was evaporated under a stream of N_2. The residue was dissolved in 0.2 M methanolic sodium methoxide (3.0 ml) and stirred overnight at rt. An excess of acetic acid was added and the mixture was concentrated using a Rotavapor (bath temp. 30 °C). Column chromatography of the residue (stepwise gradient elution, ethyl acetate–methanol–acetic acid–water 100:3:3:2 and 40:3:3:2), followed by concentration of appropriate fractions, gave a residue, which was dissolved in dichloromethane and washed with 1 M aq. triethylammonium hydrogen carbonate. The dichloromethane layer was evaporated with a stream of N_2 and the residue was dried *in vacuo* to give compound **18** as a foam (50.3 mg, 91%) [HRMS: Calc. for $C_{79}H_{88}N_2O_{20}P$: 1415.5667. Found: 1415.5669 (M − H^+)].

To a stirred suspension of compounds **17** (43.1 mg, 31.4 μmol) and **18** (47.9 mg, 31.5 μmol) in pyridine (0.8 ml) under N_2 at rt was added pivaloyl chloride (8 μl, 65.3 μmol). Additional pivaloyl chloride (5 μl, 40.8 μmol) was added after 20 min, and

Table 8 $^1H/^{13}C$ Chemical shift (J/Hz in parentheses)[a] of compound **1**

	1	2	3	4	5	6a	6b
Rha AB	5.50	4.03	3.94	3.51	3.89	1.31	
	$J_{H,P}$ 7.0					$J_{6,5}$ 6.2	
	94.5	77.5	69.7	72.4	71.2*	17.4	
	$J_{H,C}$ 174						
Rha C	5.47	4.00	(ol)	3.53	(ol)	(ol)	
	$J_{H,P}$ 7.5						
	94.4	77.6	(ol)	(ol)	(ol)	(ol)	
	$J_{H,C}$ 174						
Glc AB	5.02	3.58	3.89	3.70	4.08	3.72–3.80	3.72–3.80
	$J_{1,2}$ 3.7						
	98.4	71.8	70.9*	79.4	71.0*	60.5	
	$J_{H,C}$ 172						
Glc C	5.03	3.59	(ol)	(ol)	(ol)	(ol)	(ol)
	$J_{1,2}$ 3.7						
	98.4	(ol)	(ol)	(ol)	(ol)	(ol)	
	$J_{H,C}$ 172						
Man AB	4.91	4.58	4.00	4.07	3.56	3.85	3.95
		$J_{2,3}$ 4.0					
	100.0	53.9	72.0*	72.9*	76.4	61.2	
	$J_{H,C}$ 165						
Man C	4.89	4.55	3.82	3.52	3.45	3.82	3.93
		$J_{2,3}$ 4.0					
	100.0	54.1	72.6	67.2	77.3	(ol)	
	$J_{H,C}$ 165						

[a] Further $^1H/^{13}C$ chemical shifts are 2.07/22.8 ($CCOCH_3$), 2.08/22.8 (AB, $COCH_3$), 3.28/40.8 ($OCH_2CH_2NH_2$), 4.12/62.8 ($OCH_2CH_2NH_2$) and 176.2 (C=O).

after 50 min water (50 μl) and iodine (24 mg, 94.5 μmol) were added, and stirring was continued for 20 min. Work-up, deacetylation of the obtained material, and isolation of compound **19** were performed as described for the previous step, giving product **19** as a foam (77.5 mg, 87%) (FABMS: Calc. for $C_{148}H_{164}N_3O_{37}P_2$: 2637.05. Found: *m/z*, 2637.044).

To a stirred suspension of compounds **17** (36.5 mg, 26.7 μmol) and **19** (75.8 mg, 26.7 μmol) in pyridine (0.9 ml) under N_2 at rt was added pivaloyl chloride (10 μl, 81.7 μmol). Additional pivaloyl chloride (3 × 5 μl, 40.8 μmol) was added after 20, 40 and 60 min. Water (50 μl) and iodine (20 mg, 80 μmol) were added after 90 min and then, after 115 min, work-up, deacetylation of the obtained material, and isolation of product **20** were performed as described for the previous step, giving compound **20** as a foam (92.6 mg, 83%).

A mixture of compound **20** (44.3 mg, 10.6 μmol) and Pd/C (7.3 mg; 10%) in ethanol–acetic acid–water (2:1:1; 3 ml) was hydrogenolyzed at atmospheric pressure for 8 h, then additional Pd/C (12.2 mg; 10%) was added and the mixture was hydrogenolyzed for another 17 h. TLC (Pr^iOH–water–pyridine 4:2:1) and MALDI-TOF MS of the reaction mixture indicated that the reaction was complete. The mixture was filtered, concentrated (bath temperature 30 °C) and the residue was purified by gel filtration on a Bio-Gel P2 column, using water–butan-1-ol (95:5) as eluent. Lyophilization of the appropriate fractions gave the final product **1** (14.2 mg, 73%) as a powder. $^1H/^{13}C$ NMR data ($CDCl_3$) are shown in Table 8 (ol = overlapping signals) [HRMS: Calc. for $C_{64}H_{108}N_4O_{52}P_3$: 1833.5142. Found: 1833.5186 ($M - H^+$)].

References

1 H. J. Jennings, *Adv. Carbohydr. Chem. Biochem.*, 1983, **41**, 155.
2 J. M. Cruse and R. E. Lewis, Jr., *Conjugate Vaccines*, Karger, Basel, 1989.
3 C. Peters, D. Evenberg, P. Hoogerhout, H. Käyhty, L. Saarinen, C. van Boeckel, G. van den Marel, J. van Boom and J. Poolman, *Infect. Immun.*, 1992, **60**, 1826.
4 S. Nilsson, M. Bengtsson and T. Norberg, *J. Carbohydr. Chem.*, 1992, **11**, 265.
5 A. Y. Chernyak, T. Norberg and S. Nilsson, unpublished results, 1992.
6 M. Nilsson and T. Norberg, *J. Carbohydr. Chem.*, 1998, **17**, 305.
7 T. Sugawara and K. Igarashi, *Carbohydr. Res.*, 1988, **172**, 195.
8 H. Paulsen, B. Helpap and J. P. Lorentzen, *Carbohydr. Res.*, 1988, **179**, 173.
9 L. Panza, F. Ronchetti, G. Russo and L. Toma, *J. Chem. Soc., Perkin Trans. 1*, 1987, 2745.
10 E. Kaji, F. W. Lichtenthaler, Y. Osa, K. Takahashi and S. Zen, *Bull. Chem. Soc. Jpn.*, 1995, **68**, 2401.
11 P. Fügedi, W. Birberg, P. J. Garegg and Å. Pilotti, *Carbohydr. Res.*, 1987, **164**, 297.
12 A.-C. Helland, *PhD Thesis*, Stockholm University, Sweden, 1991.
13 M. Bols and H. C. Hansen, *Chem. Lett.*, 1994, 1049.
14 J.-M. Mallet, G. Meyer, F. Yvelin, A. Jutand, C. Amatore and P. Sinaÿ, *Carbohydr. Res.*, 1993, **244**, 237.
15 P. Westerduin, P. E. de Haan, M. J. Dees and J. H. van Boom, *Carbohydr. Res.*, 1988, **180**, 195.
16 R. King, F. Cooper and C. Bishop, *Carbohydr. Res.*, 1977, **55**, 83.

Paper 7/09293H
Received 24*th December* 1997
Accepted 2*nd March* 1998