Towards a Synthetic Glycoconjugate Vaccine Against *Neisseria meningitidis* A

Ali Berkin, Bruce Coxon, and Vince Pozsgay*[a]

Abstract: Albumin conjugates of synthetic fragments of the capsular polysaccharide of the Gram-negative bacterium *Neisseria meningitidis* serogroup A were prepared. The fragments include monosaccharides **1** [α-D-Man*p*NAc-(1→O)-$(CH_2)_2NH_2$] and **2** [6-*O*-P(O)-$(O^-)_2$-α-D-Man*p*NAc-(1→O)-$(CH_2)_2NH_2$], disaccharide **3** {α-D-Man*p*NAc-[1→O-P(O)(O^-)→6]-α-D-Man*p*NAc-(1→O)-$(CH_2)_2NH_2$}, and trisaccharide **4** {α-D-Man*p*NAc-[1→O-P(O)(O^-)→6]-α-D-Man*p*NAc-[1→O-P(O)(O^-)→6]-α-D-Man*p*NAc-(1→O)-$(CH_2)_2NH_2$}. Two monosaccharide blocks were employed as key intermediates. The reducing-end mannose unit featured the NHAc group at C-2, and contained the aminoethyl spacer as the aglycon for the final bioconjugation. The interresidual phosphodiester linkages were fashioned from an anomerically positioned *H*-phosphonate group in a 2-azido-mannose building block. The spacer-linked saccharides **1**–**4** were N-acylated with hepta-4,6-dienoic acid and the resulting conjugated diene-equipped saccharides were subjected to Diels–Alder-type addition with maleimidobutyryl-group functionalized human serum albumin to form covalent conjugates containing up to 26 saccharide haptens per albumin molecule. Complete ^1H, ^{13}C, and ^{31}P NMR assignments for **1**–**4** are given. Antigenicity of the neoglycoconjugates containing **1**–**4** was demonstrated by a double immunodiffusion assay which indicated that a fragment as small as a monosaccharide is recognized by a polyclonal meningococcus group A antiserum and that the O-acetyl group(s) present in the natural capsular material is not essential for antigenicity.

Keywords: Diels–Alder reaction · glycoconjugates · immunology · *Neisseria meningitidis* · vaccines

Introduction

Neisseria meningitidis serogroup A, a Gram-negative bacterium, causes endemic and epidemic meningitis in Sub-Saharan Africa (the meningitis belt) and sporadically throughout the world.[1–3] Meningitis, inflammation of the brain's outer layers termed meninges, is a potentially deadly disease that when left untreated had a mortality rate of 60–80%. Antibiotic therapy decreased the mortality rate to about 10%. Several lines of evidence indicate that protection against meningococcal infections including serogroup A is directly related to the presence of humoral IgG antibodies against the bacterium's capsular polysaccharide (CPS): a critical level of bactericidal antibodies confers immunity to meningococcal meningitis. There is an inverse relation between the presence of bactericidal antibodies and the age-related incidence of meningococcal meningitis.[4] Most newborns have bactericidal antibodies to groups A, B, and C meningococci which decline to non-detectable levels at about three months of age. Thereafter, there is a gradual increase so that approximately 70% of young adults have protective levels of anti group A meningococcal antibodies. The stimulus for these group A meningococcal polysaccharide antibodies includes cross-reacting, non-pathogenic bacteria. During the antibody-free period, when placentally-acquired antibodies have declined, and adult levels have not yet been reached, the incidence of meningitis is the highest. In adults, protective IgG antibodies against the CPS of *N. meningitidis* serogroup A can be induced by vaccination with the purified capsular material (currently used in combination with the CPS's of serogroups C, Y, and W135), and the efficacy of this vaccine in controlling epidemics of *N. meningitidis* in individuals of all ages has been documented.[5] In contrast to other medically-important polysaccharides, group A meningococcal polysaccharide induces a booster response before the age of two years.[6] However, multiple injections over a short period of time are required to induce protective levels of meningococcal polysaccharide antibodies. As a consequence of the developmental regulation of the immune system, the CPS vaccine is poorly immunogenic in infants. Improvement of the group A meningococcal polysaccharide by covalently binding

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it to tetanus toxoid has produced conjugates with improved immunogenicity in all ages.[7, 8]

The CPS of *N. meningitidis* A consists of α-(1→6)-linked 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate residues **A** (Figure 1). Bundle et al.[9] reported the presence of

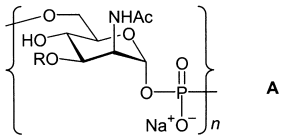

Figure 1. Structure of the repeating unit of the capsular polysaccharide of *Neisseria meningitidis* A. Varying degrees of 3-O- or 4-O-acetylation have been observed.

O-acetyl groups at position O-3 in approximately 70% of the ManNAc residues, as determined by ^{1}H and ^{13}C NMR spectroscopy. A more recent study[10] using a different sample found approximately 95% O-acetylation at positions O-3 or O-4 of the ManNAc residues.

A synthetic oligosaccharide–protein conjugate[11] for *Shigella dysenteriae* type 1 has been shown to be more immunogenic than a similar product prepared with the natural polysaccharide.[12] A similar finding was reported by Verez-Bencomo (personal communication) who found that a protein conjugate of the synthetic pentamer of the CPS of *Haemophilus influenzae* type b elicited a higher anticapsular immune response in humans than did the commercial polysaccharide–protein conjugate vaccine. Other examples for the preparation and immunological evaluation of protein conjugates of structurally well-defined, synthetic bacterial oligosaccharides may be found in Reference [13]. Following these precedents, our strategy is to investigate whether synthetic fragments of the CPS of *N. meningitidis* A could serve as components of a conjugate vaccine.

As our first approach to the long-term goal of conjugate vaccine development, we report the synthesis of fragments **1**–**4** (Figure 2) that correspond to the CPS of *N. meningitidis* A. We also report the covalent attachment of these fragments to human serum albumin using a novel bioconjugation method recently proposed by us which relies on the Diels–Alder cycloaddition reaction for linking (oligo)saccharides to proteins.[14] Further, we demonstrate an evaluation of the antigenicity of the synthetic fragments by reactions with a meningococcal A antiserum.

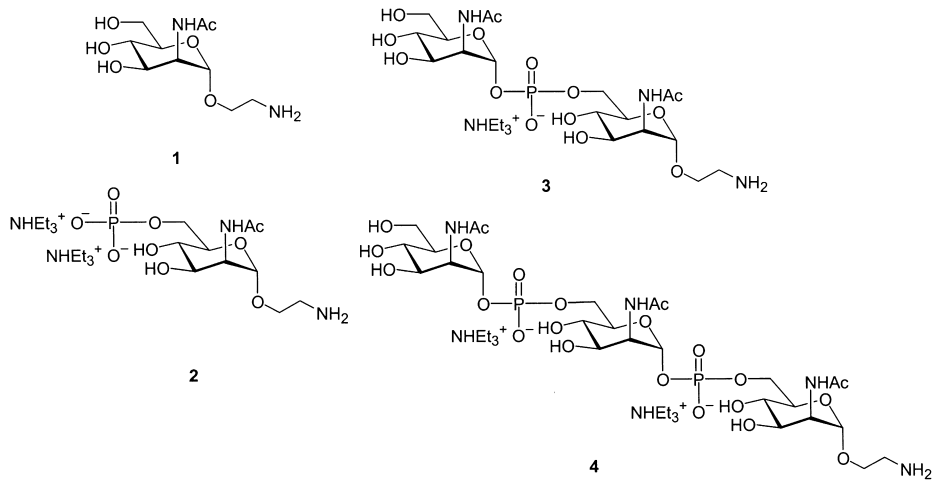

Figure 2. Chemical structures of synthetic target compounds as analogues of the capsular polysaccharide of *Neisseria meningitidis* A.

Results and Discussion

Chemical synthesis: An approach to structures related to **A** has been studied by Shibaev et al.[15] who reported the synthesis of glycosyl phosphosugars containing *N*-acetyl-D-mannosamine residues using *H*-phosphonate chemistry for the introduction of the phosphodiester linkages. However, the anomeric purity of their product was not convincingly demonstrated. More recently, Oscarson reported the assembly of a pentamer analogue corresponding to the repeating unit **A** of the CPS of *N. meningitidis* A in a fully protected form containing azido groups in place of the acetamido groups, which was not deprotected.[16] Our approach to synthetic fragments of the CPS of *N. meningitidis* A is shown in Scheme 1 and relies on *H*-phosphonate chemistry for the formation of the interglycosidic phosphodiester moiety.[17, 18] Briefly, two monosaccharide building blocks were prepared. The 2-acetamido-2-deoxy-α-D-mannopyranoside (**14**) bears a protected aminoethyl spacer group that allows for conjugation to a carrier protein, and has its HO-6 hydroxyl group unprotected for attachment of the subsequent unit. The precursor to the interglycosidic phosphate moiety is the 2-azido-2-deoxy-α-D-mannopyranoside intermediate **10** in which the *H*-phosphonate residue is stereoselectively preinstalled in the α-anomeric position. In this unit, HO-6 can be regioselectively deprotected for chain elongation. The use of the corresponding analogue with an acetamido group in place of the azido group proved to be abortive.

The *H*-phosphonate derivative of 2-azido-2-deoxy-α-D-mannopyranose (**10**) was synthesized according to Scheme 2. Methyl 2-azido-2-deoxy-α-D-mannopyranoside (**5**) was obtained from methyl α-D-glucopyranoside in five steps as described,[19, 20] and was converted to the fully protected derivative **7** by successive tritylation (→**6**) and benzylation (→**7**) in 92% overall yield, using standard protocols. Acetolysis (Ac_2O/H_2SO_4) afforded an anomeric mixture of **8*α*** and its β-anomer **8*β*** in a 3:2 ratio (TLC) from which the α-anomer could be isolated in pure form by column chromatography, followed by fractional crystallization in 56% overall yield. The regioselective removal of the anomeric acetate group of **8*α*** was achieved according to van Boom's method[18] with a 2M solution of dimethylamine in THF to afford 6-*O*-acetyl-2-

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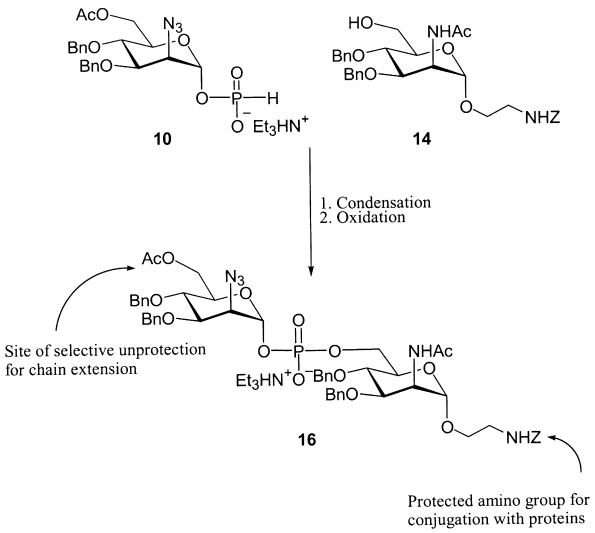
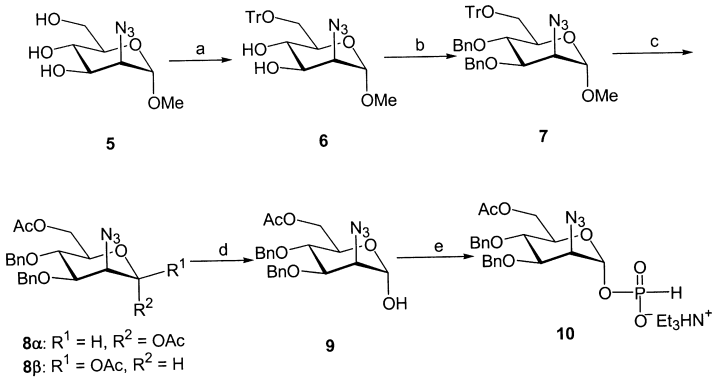


Scheme 1. Synthetic strategy for the synthesis of oligomeric analogues of the capsular polysaccharide of *Neisseria meningitidis* A.

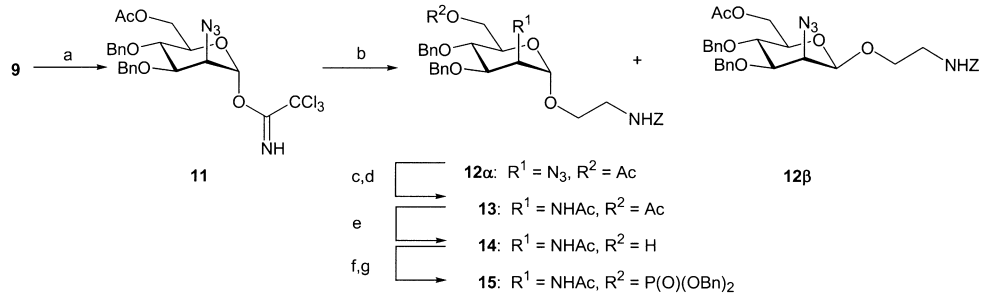


Scheme 2. Synthesis of *H*-phosphonate **10**: a) 1.2 equiv TrCl, pyridine, 4-dimethylaminopyridine (cat.), 40 °C, 24 h, 99 %; b) 2.4 equiv BnBr, NaH, DMF, 0 → 23 °C, 1 h, 93 %; c) Ac_2O, H_2SO_4 (cat.), 23 °C, 15 min, 56 %; d) 2.5 equiv Me_2NH, THF, 0 → 23 °C, 2 h, 100 %; e) 7.0 equiv $(PhO)_2P(O)H$, C_5H_5N, 23 °C, 1 h, 84 %.

azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranose (**9**) in a quantitative yield following column chromatographic purification. The ^1H NMR spectrum of the material obtained in this way lacked the anomeric signal corresponding to the β-anomer, both before and after the chromatographic purification. Compound **9** was converted into anomerically pure *H*-phosphonate derivative **10** following the method of Jankowska et al.[21] Briefly, **9** was treated with diphenylphosphite in pyridine, followed by the addition of an aqueous solution of triethylamine to afford a mixture of the triethylammonium salt of 6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α- (**10**) and β-D-mannopyranosyl hydrogenphosphonate in an approximate ratio of 93:3 (^1H NMR spectrum). The pure α-mannoside **10**, free of the β-anomer, was obtained by C-18 reverse-phase column chromatography in 84 % yield.

The synthesis of the spacer-bearing, reducing-terminal mannopyranoside unit (**14**) is shown in Scheme 3. Thus, treatment of compound **9** with CCl_3CN in the presence of DBU afforded the corresponding anomeric trichloroacetimidate (**11**, 79 %) which was used to glycosylate benzyl *N*-(2-hydroxyethyl)carbamate in the presence of a catalytic amount of trimethylsilyl trifluoromethanesulfonate as a promoter, to afford a mixture of 2-(benzyloxycarbonylamino)ethyl 6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α- (**12*α***) and β-D-mannopyranoside (**12*β***) in a 3:2 ratio as judged by TLC. Chromatographic resolution of the mixture afforded the α-mannoside **12*α*** in 54 % yield. The α anomeric configuration in **12*α*** was ascertained by a measurement of the one-bond ^1H-1 – ^{13}C-1 coupling constant of $J = 169$ Hz in the ^{13}C NMR spectrum. Subsequent conversion of the N_3 group to the NHAc group using a combination of $NiCl_2$ and $NaBH_4$, followed by treatment with Ac_2O, afforded the protected acetamido-mannoside derivative **13** (90 %); subsequent deacetylation (NaOMe/MeOH) provided the monosaccharide acceptor **14** (93 %). Treatment of the alcohol **14** with dibenzyl *N*,*N*-diisopropylphosphoramidate followed by in situ oxidation of the intermediate with 3-chloroperoxybenzoic acid, afforded the 6-*O*-phosphate derivative **15** as the pure α-anomer in 99 % yield following chromatographic purification.

Having both key building blocks in hand, the stage was set for the synthesis of oligomers corresponding to the CPS **A**, as shown in Scheme 4. The *H*-phosphonate **10** and the alcohol **14** were combined and condensed in the presence of pivaloyl chloride in pyridine according to a standard protocol.[18] The resulting intermediate *H*-phosphonate diester (not identified) was oxidized in situ by iodine in a mixture of pyridine and water to afford the anomerically pure phosphodiester-linked disaccharide **16** in 95 % yield following column chromatography. Next, the azido group in **16** was converted into the acetamido group by nickel boride reduction ($NiCl_2/NaBH_4$) followed by in situ N-acetylation with Ac_2O to afford the disaccharide **17**. For further chain elongation, the fully



Scheme 3. Synthesis of mannosides **14** and **15**: a) 2.2 equiv CCl_3CN, 0.3 equiv 1,8-diazabicyclo[5.4.0]unde-7-ene, CH_2Cl_2, 0 °C, 30 min, 79 %; b) 2.5 equiv benzyl *N*-(2-hydroxyethyl)carbamate, TMSOTf (cat.), CH_2Cl_2, 0 °C, 30 min, 54 %; c) 4.8 equiv $NiCl_2 \cdot 6H_2O$, 8.0 equiv $NaBH_4$, MeOH, 0 → 10 °C, 30 min; d) Ac_2O, MeOH, 0 °C, 30 min, 90 % for two steps; e) NaOMe, MeOH, 23 °C, 30 min, 93 %; f) 2.5 equiv $(BnO)_2PN(iPr)_2$, 4.0 equiv tetrazole, CH_3CN, 23 °C, 2.0 h; g) *m*CPBA −40 °C → 23 °C, 1 h, 99 % for two steps.

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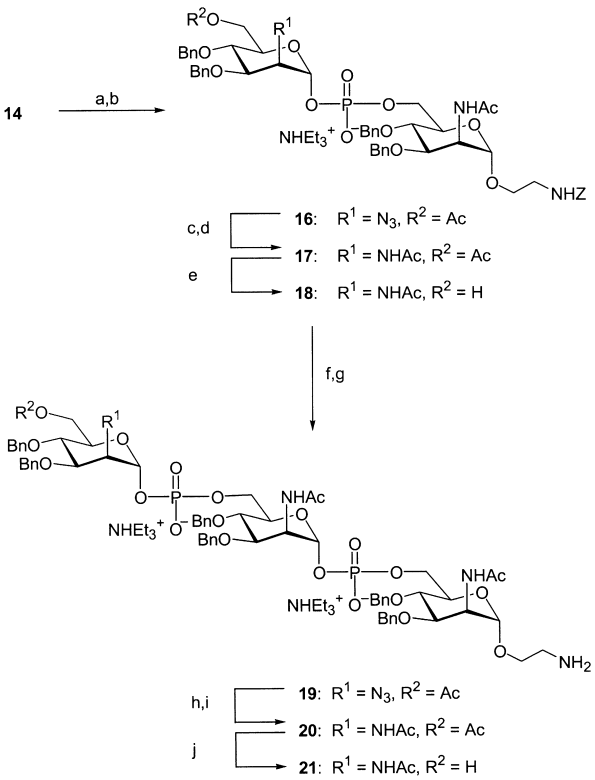

Scheme 4. Synthesis of dimer and trimer analogues of the capsular polysaccharide of *Neisseria meningitidis* A: a) 0.8 equiv of **10**, 2.2 equiv PivCl, C_5H_5N, 23 °C, 30 min; b) 2.0 equiv I_2, $C_5H_5N \cdot H_2O$ (95:1), 0 °C, 30 min, 95% for two steps; c) 4.8 equiv $NiCl_2 \cdot 6H_2O$, 8.0 equiv $NaBH_4$, MeOH, 0 → 10 °C, 30 min; d) Ac_2O, MeOH, 0 °C, 30 min, 99% for two steps; e) NaOMe, MeOH, 23 °C, 30 min, 95%; f) 1.0 equiv **10**, 1.5 equiv PivCl, C_5H_5N, 23 °C, 5 min; g) 2.0 equiv I_2, C_5H_5N/H_2O (95:1), 0 °C, 30 min, 74% for two steps; h) 4.8 equiv $NiCl_2 \cdot 6H_2O$, 8.0 equiv $NaBH_4$, MeOH, 0 → 10 °C, 30 min; i) Ac_2O, MeOH, 0 °C, 30 min, 80% for two steps; j) NaOMe, MeOH, 23 °C, 30 min, 99%.

protected dimer **17** was deacetylated (NaOMe/MeOH) to provide the acceptor alcohol **18** (95%).

The synthesis of the trimer fragment of the CPS of *N. meningitidis* A followed a procedure similar to that just described for the dimer **16**. However, the reaction times for the coupling and oxidation steps needed careful tuning to suppress cleavage of the phosphodiester bond(s). Under an optimized set of conditions, pivaloyl chloride-mediated coupling of **10** and **18**, followed by in situ oxidation with iodine, afforded the fully protected trimer **19** in 74% yield. Nickel boride reduction followed by in situ N-acetylation as described for the preparation of the dimer **17** afforded the trimeric fragment **20** in 80% yield. Subsequent de-O-acetylation (NaOMe/MeOH) produced the alcohol **21** (99%). Attempted chain elongation of **21** with the *H*-phosphonate **10** under conditions similar to those described for the preparation of **16** and **19** failed to produce the desired tetrameric fragment. This situation could not be improved by using a variety of conditions including an excess of the *H*-phosphonate and the coupling reagent. The cause of this is not understood at this time, but may be related to the instability of the phosphodiester linkage in the *N*-acetylmannosamine environment to the oxidative conditions employed. This reasoning is based on the chromatographic observation that both reaction partners, namely compounds **10** and **21** disappeared during the initial condensation with pivaloyl chloride. Degradation was noted during the course of the second stage, namely during the oxidation. A similar observation was noted recently by Oscarson et al. for the synthesis of phosphodiester-linked oligomers of the repeating units of the CPS's of *Haemophilus influenza* types c and f.[22]

Catalytic hydrogenolysis of each of the intermediates **14**, **15**, **18**, and **21** in the presence of 10% Pd/C and 1M triethylammonium acetate (pH 7.0) simultaneously removed the O-benzyl and N-benzyloxycarbonyl protecting groups to afford the target saccharides **1**–**4** which were isolated as amorphous solids. The identity and purity of the target saccharides were demonstrated by one- and two-dimensional ^1H, ^{13}C, and ^{31}P NMR spectroscopic analyses, including COSY, TOCSY, and HSQC techniques that allowed determination of the chemical shifts and coupling constants for **1**–**4** shown in Tables 1 and 2.

Synthesis of neoglycoconjugates: Saccharides **1**–**4** were covalently attached to human serum albumin (HSA) using a recently developed method that makes use of the high efficiency of Diels–Alder cycloaddition reactions in water.[14] In this protocol, the aminoethyl glycosides **1**–**4** were derivatized with the *N*-hydroxysuccinimide ester of hepta-4,6-dienoic acid (**22**) in methanol to afford the conjugated diene-equipped constructs **23**–**26** (Figure 3) which were purified by solid-phase extraction using a C-18 reverse-phase column as the adsorbent, and methanol/water mixtures as the eluents. The matching dienophil groups were attached to the protein **27** using the commercial reagent sulfosuccinimidyl 4-maleimidobutyrate **28** (probably at the ε-amino groups of the 58 available lysine residues) to yield the maleimide-derivatized protein **29** (Scheme 5). Depending on the excess of the reagent, up to 38 maleimidobutyryl groups could be introduced per HSA molecule. Uncatalyzed reaction of the derivatized HSA **29** with the diene-equipped saccharide **23** in unbuffered, aqueous solutions afforded neoglycoconjugates that contained up to an average of 26 saccharide moieties per HSA molecule, as determined by MALDI-TOF mass spectrometry (see Table 3). Similarly, saccharide-diene constructs **24**–**26** were attached to **29**. The uncoupled saccharide-dienes could be recovered in their original, bioconjugatable form by a simple diafiltration step and their identity and purity were verified by NMR and MS data.

NMR spectroscopy: The structures and pyranose ring forms of compounds **1**–**4** were confirmed by detailed NMR studies at 500 MHz, which allowed most of the vicinal $^1H-^1H$ coupling constants to be measured. ^1H NMR assignments (see Table 1) were determined from one-dimensional subspectra of individual sugar residues obtained as F_2 slices of two-dimensional TOCSY spectra acquired with highly rectangular data sets. Further confirmation of these assignments was obtained from 2D COSY data. ^{13}C NMR assignments (see Table 2) were determined principally by *z*-gradient selected, HSQC experiments, based on the aforementioned ^1H NMR assignments. Additional confirmation of carbon type was obtained from ^1H coupled and decoupled 1D DEPT-135

Table 1. ^{1}H and ^{31}P NMR chemical shifts (ppm) and ^{1}H – ^{1}H and ^{1}H – ^{31}P NMR coupling constants (Hz ± 0.1 Hz) for **1** – **4**.

^{1}H	Compound residue[b]	**1**	**2**[a]	**3**[a]		**4**[a]		
		I	I	I	II	I	II	III
H-1		4.825d	4.835dd	4.832d	5.403dd	4.831d	5.395dd	5.410d
H-2		4.378dd	4.401dd	4.395dd	4.424dd	4.396dd	4.427dd	4.416dd
H-3		4.044dd	4.067dd	4.052dd	4.108dd	4.055dd	4.122dd	4.117dd
H-4		3.623t	3.776t	3.688t	3.661t	3.690t	3.744t	3.663t
H-5		3.673m	3.733m	3.781m	3.878m	3.783m	3.978m	3.876m
H-6		3.873m	4.083m	4.172m	3.864s	4.194m	4.196s	3.869s
H-6′		3.830m	4.016m	4.130m	3.864s	4.133m	4.152s	3.869s
$^{\alpha}CH_2$		3.820m	3.985m	3.974m	–	3.993m	–	–
		3.589m	3.717m	3.703m	–	3.718m	–	–
$^{\beta}CH_2$		3.003m	3.280m	3.282m	–	3.302m	–	–
				3.270m	–	3.272m	–	–
NAc		2.051m	2.058m	2.055	2.063	2.058	2.066	2.064
^{31}P chemical shifts		–	5.75[c]	– 0.04q[d]	–	0.01q[e,f]	– 0.10q[e,g]	–
$J_{1,2}$		1.6	1.6	1.4	1.6	1.5	1.7	1.8
$J_{2,3}$		4.9	4.4	4.7	4.6	4.9	4.7	4.7
$J_{3,4}$		9.7	9.6	10.0	10.2	10.1	10.3	10.2
$J_{4,5}$		9.6	9.5	10.0	9.6	9.9	9.9	9.6
$J_{5,6}$		2.2	3.6	2.7	[h]	1.8	3.6	[h]
$J_{5,6'}$		4.4	≈ 0.5	4.8	[h]	5.0	1.8	[h]
$J_{6,6'}$		12.3	11.9	11.5	[h]	11.5	11.7	[h]
$J_{1,P}$		–	7.5	–	7.5	–	5.6	5.4
$J_{6,P}$		–	6.1	5.4	–	5.7	–	–
$J_{6',P}$		–	4.6	6.7	–	7.0	–	–

[a] Compounds **2** – **4** were analyzed as sodium salts. [b] Residues are numbered I, II, III, starting from the potential reducing end. [c] Broad singlet. [d] Spacings 6.6, 6.5, and 6.5 Hz. [e] Assignments interchangeable. [f] Spacings 6.3, 6.4, and 7.4 Hz. [g] Spacings 7.5, 6.6, and 7.0 Hz. [h] Couplings not measurable owing to degeneracy of H-5, H-6, and H-6′.

Table 2. ^{13}C NMR chemical shifts (ppm) and ^{13}C – ^{1}H and ^{13}C – ^{31}P NMR coupling constants (Hz) for saccharides **1** – **4**.[a]

^{13}C	Compound Residue	**1**[b]	**2**[c]	**3**[d]		**4**[e]		
		I	I	I	II	I	II	III
C-1		99.65	99.83	99.69	95.98	99.73	95.95	95.94
C-2		53.28	53.10	53.13	53.87	53.14	53.94	53.94
C-3		69.77	69.28	69.55	69.40	69.55	69.22	69.40
C-4		67.42	66.99	67.11	67.11	67.18	66.77	67.15
C-5		73.21	72.86	72.29	74.18	72.40	73.14	74.19
C-6		61.14	63.62	65.41	61.00	65.50	65.25	60.99
$^{\alpha}CH_2$		67.63	64.33	64.27	–	64.31	–	–
$^{\beta}CH_2$		40.33	39.76	39.75	–	39.78	–	–
NAc		22.66	22.64	22.67	22.69	22.73[f]	22.72[f]	22.70[f]
C=O		175.57	175.63	175.69[g]	175.49[g]	175.64[h]	175.53[h]	175.47[h]

[a] Compounds **2** – **4** were analyzed as sodium salts. [b] $J_{C\text{-}1,H\text{-}1} = 174.9$ Hz. [c] $J_{C\text{-}1,H\text{-}1} = 174.9$, $J_{C\text{-}5,P} = 6.7$, $J_{C\text{-}6,P} = 4.3$ Hz. [d] Residue I, $J_{C\text{-}1,H\text{-}1} = 173.0$, $J_{C\text{-}5,P} = 7.8$, $J_{C\text{-}6,P} = 5.3$ Hz; residue II, $J_{C\text{-}1,H\text{-}1} = 174.8$, $J_{C\text{-}1,P} = 5.6$, $J_{C\text{-}2,P} = 8.8$ Hz. [e] Residue I, $J_{C\text{-}1,H\text{-}1} = 173.8$, $J_{C\text{-}5,P} = 7.5$, $J_{C\text{-}6,P} = 5.2$ Hz; residue II, $J_{C\text{-}1,H\text{-}1} = 176.7$, $J_{C\text{-}1,P} = 5.4$, $J_{C\text{-}2,P} = 9.0$, $J_{C\text{-}5,P} = 8.0$, $J_{C\text{-}6,P} = 5.3$ Hz; residue III, $J_{C\text{-}1,H\text{-}1} = 176.7$, $J_{C\text{-}1,P} = 5.6$, $J_{C\text{-}2,P} = 9.0$ Hz. [f] Assignments interchangeable. [g] Assignments interchangeable. [h] Assignments interchangeable.

Table 3. Composition of the saccharide-HSA conjugates.

Saccharide	Average ratio[a]
ManNAc **1**	26
ManNAc-P **2**	19
dimer **3**	15
dimer **3**	15
trimer **4**	8
trimer **4**	5

[a] Saccharide to HSA [mol mol^{-1}].

^{13}C NMR spectra. The α anomeric configurations of all of the sugar residues were indicated by the large $^1J_{C\text{-}1,H\text{-}1}$ values (172.0 – 174.9 Hz) measured from ^{1}H coupled ^{13}C NMR spectra. The phosphate groups in compounds **2** – **4** were characterized by ^{1}H coupled and decoupled ^{31}P NMR spectra. ^{31}P NMR chemical shifts and spacings corresponding to coupling patterns are shown in Table 1.

Double immunodiffusion assay: The antigenicity of conjugates derived from **23** – **26** was evaluated by use of a meningococcus group A antiserum (H-49)[23] by double immunodiffusion compared with the native purified CPS of *N. meningitidis* A, which served as a positive reference. Bearing in mind that the assay is not designed to lead to quantitative conclusions, Figure 4 shows that each conjugate derived from **23** – **26**, and the native CPS precipitated with H-49 antiserum. The dienophile-derivatized HSA did not precipitate with H-49 (not shown). These results suggest that a fragment as small as a monosaccharide is antigenic, even without the phosphate group. Additionally, the presence of the O-acetyl group(s) may not be essential for antigenicity.

In conclusion, we have synthesized mono- and phosphodiester-linked oligomeric fragments of the CPS of *N. meningitidis* serogroup A, for which complete NMR assignments are presented. We have covalently attached these saccharides to human serum albumin using a novel bioconjugation method based on the Diels – Alder cycloaddition reaction. Serologic evaluation of the conjugates demonstrated that a polyclonal anti-*Neisseria meningitidis* A antiserum can recognize a monosaccharide fragment of its CPS, and that the O-acetyl group of the native CPS may not be essential for antigenicity. Evaluation of the immunogenicity of the new glycoconjugates and synthesis of partially O-acetylated fragments of the CPS are planned.

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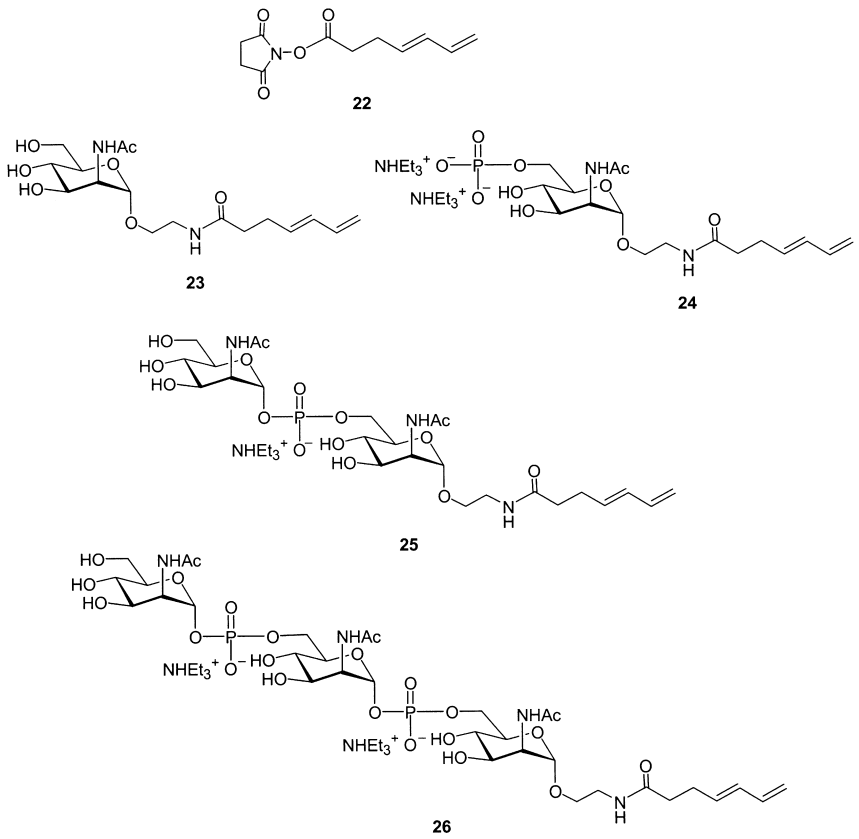
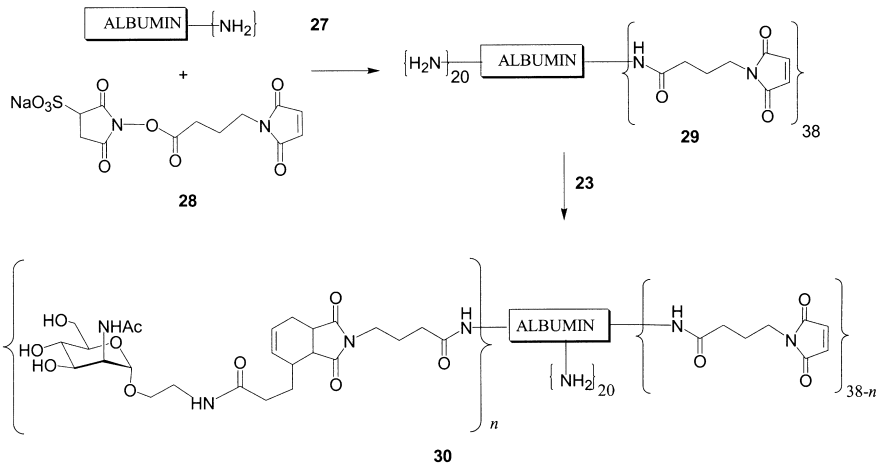


Figure 3. The structure of the diene donor **22** and the diene-functionalized saccharides **23**–**26**.



Scheme 5. General reaction Scheme for the conjugation of synthetic fragments of the CPS of *N. meningitidis* A to human serum albumin using the Diels–Alder bioconjugation method.

Experimental Section

General: All chemicals were commercial grade and were used without purification. Anhydrous solvents were obtained from Sigma-Aldrich. Human serum albumin (defatted) was purchased from Sigma-Aldrich, and was purified by ultrafiltration through a YM10 Diaflow membrane in an Amicon ultrafiltration cell, using five changes of water, followed by freeze-drying. Column chromatography was performed on silica gel 60 (0.040–0.063). Thin-layer chromatography (TLC) was performed using glass plates precoated with silica gel (250 μm, 60 Å). Reverse-phase column chromatography was performed on preparative C18, 125 Å (Waters). Melting points were determined in a Meltemp capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 341 polarimeter for solutions in a 1 dm cell at 22 °C in $CHCl_3$ unless stated otherwise. The mass spectra were recorded at the Laboratory of Bioorganic Chemistry, NIDDK, or in the Laboratory of Cellular and Molecular Biophysics, NICHD, NIH, Bethesda, MD (USA). The fast atom bombardment (FAB) mass spectra were obtained using 6 keV Xe atoms to ionize samples from a dithiothreitol/dithioerythritol, 3-nitrobenzyl alcohol, or glycerol matrix. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA (USA).

Methyl 2-azido-2-deoxy-6-*O*-triphenylmethyl-α-D-mannopyranoside (6): Freshly prepared triphenylmethyl chloride (4.58 g, 16.4 mmol) and 4-dimethylaminopyridine (cat.) were added to a stirred solution of methyl 2-azido-2-deoxy-α-D-mannopyranoside (**5**; 3.0 g, 13.7 mmol) in anhydrous pyridine (20 mL). After 24 h at 40 °C, the mixture was diluted with CH_2Cl_2 (100 mL), treated with $NaHCO_3$ (2 g), and concentrated. The residue was coevaporated with toluene. The resulting solid was treated with CH_2Cl_2. Filtration followed by concentration of the filtrate under reduced pressure afforded a residue which was subjected to column chromatography (EtOAc/hexanes 1:3+0.1% Et_3N) to afford **6** (6.4 g, 99%) as a white solid. R_f = 0.21 (EtOAc/hexanes 1:3); m.p. 98–99 °C; $[\alpha]_D = +27$ ($c = 0.5$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.46–7.22$ (m, 15H), 4.69 (br s, 1H), 3.98 (m, 1H), 3.88 (m, 1H), 3.74 (t, $J = 9.4$ Hz, 1H), 3.59 (m, 1H), 3.42 (m, 2H), 3.36 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 143.5, 128.6, 127.9, 127.3, 99.1, 87.4, 71.3, 70.5, 70.0, 64.7, 62.8, 55.0; MS (FAB): calcd for $C_{26}H_{27}N_3Na_1O_5$ $[M+Na]^+$: 484.2; found: 484.3; elemental analysis calcd (%) for $C_{26}H_{27}N_3O_5$ (461.5): C 67.66, H 5.90; found C 67.47, H 5.97.

Methyl 2-azido-3,4-di-*O*-benzyl-2-deoxy-6-*O*-triphenylmethyl-α-D-mannopyranoside (7): 60% NaH (2.1 g, 52 mmol) was added in portions to an ice-cold, stirred solution of **6** (6.0 g, 13.0 mmol) in DMF (6 mL). After 20 min, the reaction mixture was treated dropwise with benzyl bromide

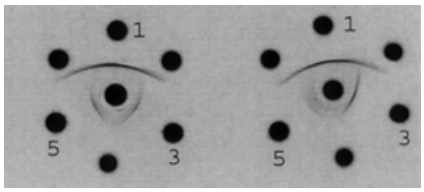


Figure 4. Double immunodiffusion assay of synthetic conjugates against meningococcus group A antiserum (horse 49). Left: 1) Native *meningococcal* A CPS. 3) Monomer-HSA conjugate. 5) Monomer-6-P-HSA conjugate. Center well-Horse 49 antiserum. Right: 1) Native *meningococcal* A CPS. 3) Dimer-HSA conjugate. 5) Trimer-HSA conjugate. Center well-Horse 49 antiserum.

(3.70 mL, 31.2 mmol). After 1 h, MeOH (2 mL) was added and the mixture was diluted with CH_2Cl_2 (200 mL), washed with H_2O (3 × 200 mL), dried (Na_2SO_4), and concentrated. The residue was co-evaporated with toluene. Column chromatography of the residue (EtOAc/hexanes 1:9) afforded **7** (7.7 g, 93%) as an oil which solidified upon trituration. $R_f = 0.72$ (EtOAc/hexanes 1:3); m.p. 104–105 °C; $[\alpha]_D = +36$ ($c = 0.5$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.50-6.85$ (m, 25H), 4.78 (d, $J = 1.8$ Hz, 1H), 4.70 (m, 3H), 4.25 (d, $J = 10.6$ Hz, 1H), 4.01 (dd, $J = 3.5$, 8.8 Hz, 1H), 3.94 (dd, $J = 1.8$, 4.1 Hz, 1H), 3.86 (t, $J = 9.0$ Hz, 1H), 3.76 (m, 1H), 3.48 (dd, $J = 1.75$, 10.0 Hz, 1H), 3.23 (dd, $J = 4.7$, 9.4 Hz, 1H), 3.40 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 143.9$, 128.8, 128.5, 128.2, 128.1, 127.8, 127.7, 127.6, 126.9, 98.9, 79.8, 75.2, 74.7, 72.6, 71.5, 62.7, 61.3, 54.7; HRMS (FAB): calcd for $C_{40}H_{39}Li_1N_3O_5$ $[M+Li]^+$: 648.3050; found: 648.3060; elemental analysis calcd (%) for $C_{40}H_{39}N_3O_5$ (641.8): C 74.86, H 6.13; found C 75.11, H 6.19.

1,6-Di-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranose (8α): A solution of **7** (7.0 g, 10.9 mmol) in acetic anhydride (60 mL) was treated with conc. sulfuric acid (30 drops) at rt. After 15 min, the solution was diluted with CH_2Cl_2 (800 mL), washed sequentially with H_2O (150 mL), aqueous $NaHCO_3$ (satd, 150 mL), H_2O (150 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Column chromatography (EtOAc/hexanes 1:9 → 1:5) afforded **8α** (2.85 g, 56%) as a clear oil which crystallized on standing. $R_f = 0.30$ (EtOAc/hexanes 1:3); m.p. 60–62 °C; $[\alpha]_D = +57$ ($c = 1.7$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.38-7.31$ (m, 10H), 6.03 (d, $J = 1.2$ Hz, 1H), 4.90 (d, $J = 10.5$ Hz, 1H), 4.75 (s, 2H), 4.59 (d, $J = 10.6$ Hz, 1H), 4.27 (d, $J = 1.8$ Hz, 2H), 4.04 (m, 1H), 3.92–3.84 (m, 3H), 2.07 (s, 3H), 2.06 (s, 3H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 170.7$, 168.3, 137.5, 137.2, 128.7–128.0, 91.9, 79.0, 75.4, 73.3, 72.7, 72.1, 62.6, 60.0, 20.8; HRMS (FAB): calcd for $C_{24}H_{28}N_3O_7$ $[M+H]^+$: 470.1927; found: 470.1924; elemental analysis calcd (%) for $C_{24}H_{27}N_3O_7$ (469.5): C 61.40, H 5.80; found C 61.44, H 5.81.

6-*O*-Acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranose (9): A stirred solution of **8** (2.00 g, 4.26 mmol) in THF (2.0 mL) was treated with a solution of dimethylamine in THF (2 M, 5.3 mL, 10.7 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature. After 2 h, the mixture was concentrated under reduced pressure. A solution of the residue in $CHCl_3$ (100 mL) was washed with H_2O (10 × 25 mL), dried (Na_2SO_4), and concentrated. Column chromatography of the residue (EtOAc/hexanes 1:2 to 2:1+1% Et_3N) afforded **9** (1.82 g, 100%) as a clear oil. $R_f = 0.12$ (EtOAc/hexanes 1:3); $[\alpha]_D = +42$ ($c = 1.6$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.39-7.26$ (m, 10H), 5.21 (d, $J = 1.5$ Hz, 1H), 4.90 (d, $J = 11.0$ Hz, 1H), 4.74 (dd, $J = 11.3$, 15.6 Hz, 2H), 4.59 (d, $J = 11.0$ Hz, 1H), 4.34 (dd, $J = 1.8$, 11.7 Hz, 1H), 4.24 (dd, $J = 4.4$, 11.7 Hz, 1H), 4.15 (dd, $J = 3.7$, 9.2 Hz, 1H), 4.02 (m, 1H), 3.94 (m, 1H), 3.81 (dd, $J = 9.5$, 1H), 2.06 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.0$, 137.8, 137.5, 128.7–127.9, 92.6, 79.2, 75.3, 74.0, 72.6, 70.0, 63.2, 61.2, 20.9; HRMS (FAB): calcd for $C_{22}H_{26}N_3O_6$ $[M+H]^+$: 428.1822; found: 428.1827; elemental analysis calcd (%) for $C_{22}H_{25}N_3O_6$ (427.5): C 61.82, H 5.90; found C 61.59, H 5.79.

6-*O*-Acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl hydrogenphosphonate, triethylammonium salt (10): Diphenylphosphite (9.60 mL, 50.1 mmol) was added to a stirred solution of **9** (3.06 g, 7.16 mmol) in anhydrous pyridine (30 mL). After 1 h, the reaction mixture was cooled to 0 °C and was treated with a solution of triethylamine in H_2O (1:1, 30 mL). After 30 min, the mixture was concentrated under reduced pressure. The residue was co-evaporated with toluene. Reverse-phase (C-18) column chromatography (MeOH/H_2O 3:7 → 1:1) afforded **10** (3.56 g, 84%) as a clear oil. $R_f = 0.39$ (MeOH/CH_2Cl_2 1:8+1% Et_3N); $[\alpha]_D = +36$ ($c = 1.1$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 12.4$ (br s, 1H), 7.98–7.26 (m, 10H), 6.92 (d, 1H), 5.59 (dd, $J = 1.5$, 8.5 Hz, 1H), 4.90 (d, $J = 10.5$ Hz, 1H), 4.71 (dd, $J = 11.7$ Hz, 2H), 4.59 (d, $J = 11.1$ Hz, 2H), 4.32–4.21 (m, 3H), 4.10–4.05 (m, 2H), 3.85 (dd, $J = 9.4$ Hz, 1H), 3.04 (m, 6H), 2.03 (s, 3H), 1.32 (t, $J = 7.3$ Hz, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.9$, 138.0, 137.7, 128.5–127.4, 93.5 (d, $J \approx 4$ Hz), 79.4, 75.0, 73.8, 72.4, 70.7, 62.9, 61.8 (d, $J \approx 6$ Hz), 58.3, 55.4, 52.1, 45.5, 20.8, 8.5; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = 0.25$; HRMS (FAB): calcd for $C_{22}H_{25}N_3O_8P_1$ $[M-Et_3NH]^-$: 490.1379; found: 490.1361; elemental analysis calcd (%) for $C_{28}H_{43}N_4O_9P_1$ $[M+H_2O]$ (610.6): C 61.40, H 5.80; found C 61.44, H 5.81.

6-*O*-Acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl trichloroacetimidate (11): Trichloroacetonitrile (14.1 mL, 140 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (2.9 mL, 19.2 mmol) were added to an ice-cold solution of **9** (27.4 g, 64.0 mmol) in CH_2Cl_2 (50 mL). After 30 min, triethylamine was added (1 mL) and the reaction mixture was concentrated under reduced pressure. Column chromatography of the residue (EtOAc/hexanes 1:4+0.1% Et_3N) afforded **11** (28.6 g, 79%) as a clear oil. $R_f = 0.71$ (EtOAc/hexanes 1:2); $[\alpha]_D = +66$ ($c = 1.4$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.64$ (s, 1H), 7.39–7.31 (m, 10H), 6.18 (d, $J = 1.8$ Hz, 1H), 4.92 (d, $J = 10.6$ Hz, 1H), 4.80 (d, $J = 11.7$ Hz, 1H), 4.74 (d, $J = 11.7$ Hz, 1H), 4.62 (d, $J = 10.6$ Hz, 1H), 4.32 (d, $J = 2.0$, 12.3 Hz, 1H), 4.24 (d, $J = 4.0$, 12.3 Hz, 1H), 4.11 (m, 1H), 3.94 (m, 2H), 3.92 (dd, $J = 9.9$ Hz, 1H), 2.04 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.7$, 159.9, 137.5, 137.2, 128.6–128.1, 95.8, 78.9, 75.5, 73.2, 73.0, 72.6, 62.5, 59.9, 20.8; HRMS (FAB): calcd for $C_{24}H_{25}Cl_3Li_1N_4O_6$ $[M+Li]^+$: 577.1000; found: 577.1030; elemental analysis calcd (%) for $C_{24}H_{25}Cl_3N_4O_6$ (571.8): C 50.41, H 4.41; found C 50.52, H 4.50.

2-(Benzyloxycarbonylamino)ethyl 6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside (12): Trimethylsilyl trifluoromethanesulfonate (10 μL) was added at 0 °C to a mixture of **11** (6.94 g, 12.1 mmol) and benzyl *N*-(2-hydroxyethyl)carbamate (8.29 g, 42.5 mmol) in CH_2Cl_2 (40 mL). After 30 min, Et_3N (1.0 mL) was added and the reaction mixture diluted with $CHCl_3$ (300 mL), washed with H_2O (3 × 50 mL), dried (Na_2SO_4), concentrated, and toluene added to and evaporated from the residue under reduced pressure. Column chromatography (EtOAc/hexanes 2:3) afforded **12** (3.94 g, 54%) as a yellowish, white oil which crystallized on standing. $R_f = 0.33$ (EtOAc/hexanes 1:2); m.p. 66–68 °C; $[\alpha]_D = +17$ ($c = 1.3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.41-7.26$ (m, 15H), 5.10 (s, 2H), 5.08 (m, 1H), 4.87 (d, $J = 10.6$ Hz, 1H), 4.78 (d, $J = 1.8$ Hz, 1H), 4.75 (d, $J = 11.4$ Hz, 1H), 4.69 (d, $J = 11.4$ Hz, 1H), 4.57 (d, $J = 10.9$ Hz, 1H), 4.25 (m, 2H), 4.04 (m, 1H), 3.88 (m, 1H), 3.81–3.73 (m, 2H), 3.73–3.35 (m, 4H), 2.04 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.8$, 156.3, 137.6, 137.5, 128.5–127.9, 98.3, 79.5, 75.3, 73.9, 72.6, 70.1, 67.3, 66.9, 63.1, 60.9, 40.6, 20.8; HRMS (FAB): calcd for $C_{32}H_{37}N_4O_8$ $[M+H]^+$: 605.2611; found: 605.2623; elemental analysis calcd (%) for $C_{32}H_{36}N_4O_8$ (604.7): C 63.56, H 6.00; found C 63.33, H 5.92.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-6-*O*-acetyl-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside (13): Slowly sodium borohydride (4.40 g, 116 mmol) was added to an ice-cooled solution of **12** (8.80 g, 14.6 mmol) and nickel chloride hexahydrate (16.6 g, 69.9 mmol) in MeOH (150 mL) while maintaining the temperature below 10 °C. After 1 h, acetic anhydride (13.8 mL, 146 mmol) was added dropwise to the reaction mixture, which was stirred for an additional 30 min at 5 °C. The solvent was removed under reduced pressure, and the residue was dissolved in $CHCl_3$ (600 mL); the solution was washed with H_2O (400 mL). The aqueous layer was extracted with $CHCl_3$ (3 × 75 mL), and the organic extracts were combined, dried (Na_2SO_4), and concentrated to a brown oil. Column chromatography (EtOAc) afforded **13** (8.15 g, 90%) as a clear oil. $R_f = 0.45$ (EtOAc); $[\alpha]_D = +17$ ($c = 1.4$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.42-7.25$ (m, 15H), 5.87 (d, $J = 8.2$ Hz, 1H), 5.20 (m, 1H), 5.10 (s, 2H), 4.88 (d, $J = 10.5$ Hz, 1H), 4.85 (d, $J = 1.2$ Hz, 1H), 4.71 (d, $J = 11.1$ Hz, 1H), 4.65 (m, 1H), 4.48 (d, $J = 10.8$ Hz, 1H), 4.32 (dd, $J = 5.5$, 12.0 Hz, 1H), 4.24 (dd, $J = 1.8$, 12.0 Hz, 1H), 4.06 (dd, $J = 4.7$, 9.4 Hz, 1H), 3.83 (m, 1H), 3.70 (m, 1H), 3.56–3.36 (m, 3H), 2.04 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.6$, 170.4, 137.7, 137.5, 133.1–127.9, 99.2, 75.1, 74.0, 71.2, 69.1, 67.4, 66.8, 63.4, 49.2, 40.7, 23.4, 20.8; HRMS (FAB): calcd for $C_{34}H_{41}N_2O_9$ $[M+H]^+$: 621.2812; found: 621.2802; elemental analysis calcd (%) for $C_{34}H_{40}N_2O_9$ (620.7): C 65.79, H 6.50; found C 65.74, H 6.64.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside (14): Compound **13** (8.15 g, 13.1 mmol) in MeOH (60 mL) was treated with a solution of 25% NaOMe/MeOH (2.5 mL) for 30 min at rt. The solution was neutralized with Amberlite IR-120 (H^+) resin and concentrated under reduced pressure in the presence of Et_3N. Column chromatography (EtOAc/MeOH 1:0 → 95:5) afforded **14** (7.03 g, 93%) as a clear oil. $R_f = 0.37$ (EtOAc/MeOH 95:5); $[\alpha]_D = +21$ ($c = 0.6$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.36-7.26$ (m, 15H), 5.78 (d, $J = 8.2$ Hz, 1H), 5.10 (s, 2H), 5.09 (m, 1H), 4.92 (d, $J = 11.1$ Hz, 1H), 4.86 (d, $J = 1.2$ Hz, 1H), 4.69 (d, $J = 11.1$ Hz, 1H), 4.62 (d, $J = 11.1$ Hz, 1H), 4.50 (d, $J = 11.1$ Hz, 1H), 4.05 (m, 1H), 3.81–3.31 (m, 9H), 2.03 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.9$, 138.0, 137.8, 128.5–127.9, 99.2, 77.3, 75.2, 73.6, 71.2, 67.1, 66.8, 61.7, 49.5, 40.7, 23.4; HRMS (FAB): calcd for $C_{32}H_{38}N_2O_8$ $[M+H]^+$: 579.2706; found: 579.2707; elemental analysis calcd (%) for $C_{32}H_{38}N_2O_8$ (578.7): C 66.42, H 6.62; found: C 66.20, H 6.66.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-6-*O*-dibenzylphosphate-α-D-mannopyranoside (15): Tetrazole (97 mg, 1.38 mmol) was added at rt to a solution of **14** (200 mg, 0.346 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidate (298 μL, 0.864 mmol) in

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CH_3CN (2.5 mL). After 30 min, the mixture was cooled to $-40\,^{\circ}C$ and 3-chloroperoxybenzoic acid (262 mg, 0.864 mmol) was added and stirring was continued for an additional 5 min. The mixture was stirred for 30 min at 0 °C, and then stirred for 30 min at rt. The mixture was diluted with $CHCl_3$ (20 mL), washed with aq 5% $NaHCO_3$ (3 × 10 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Column chromatography afforded **15** (0.290 g, 100%) as a clear oil. $R_f = 0.19$ (EtOAc/hexanes 1:1); $[\alpha]_D = +39$ ($c = 0.6$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.41-7.24$ (m, 25H), 5.10 (s, 2H), 5.06–4.89 (m, 4H), 4.92 (d, $J = 11.1$ Hz, 1H), 4.82 (d, $J = 10.5$ Hz, 1H), 4.74 (ddd, $J = 1.2$, 3.2, 9.9 Hz, 1H), 4.65 (d, $J = 1.2$ Hz, 1H), 4.54 (d, $J = 11.1$ Hz, 1H), 4.47 (d, $J = 10.5$ Hz, 1H), 4.28 (m, 1H), 4.25 (m, 1H), 4.00 (dd, $J = 4.1$, 9.4 Hz, 1H), 3.84 (t, $J = 9.6$ Hz, 1H), 3.60 (m, 2H), 3.37 (m, 2H), 3.26 (m, 1H), 2.06 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.9$, 156.3, 138.2–135.8, 128.6–127.7, 99.9, 75.3, 73.1, 71.1, 70.6, 69.8 (d, $J \approx 5$ Hz), 69.5 (d, $J = 5.5$ Hz), 68.1 (d, $J = 6.6$ Hz), 67.1, 66.8, 53.4, 48.3, 40.6, 23.2; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = -2.0$; HRMS (FAB): calcd for $C_{46}H_{52}N_2O_{11}P_1$ $[M+H]^+$: 839.3309; found: 839.3331; elemental analysis calcd (%) for $C_{46}H_{51}N_2O_{11}P_1$ (838.9): C 65.86, H 6.13; found: C 65.67, H 6.23.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside 6-(6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate), triethylammonium salt (16): Freshly distilled trimethylacetyl chloride (39 μL, 0.318 mmol) was added at rt to a mixture of **10** (70 mg, 0.118 mmol) and **14** (85 mg, 0.147 mmol) in anhydrous pyridine (1 mL). After 30 min, the reaction mixture was cooled to 5 °C and a solution of I_2 (60 mg, 0.235 mmol) in pyridine/H_2O (95:1, 2 mL) was added. Stirring was continued for an additional 30 min. The reaction mixture was diluted with $CHCl_3$ (60 mL) and washed sequentially with ice-cooled 1M Na_2SO_4 (2 × 10 mL), 1M triethylammonium bicarbonate buffer (pH 8.5, 2 × 10 mL), dried (Na_2SO_4), and concentrated under reduced pressure at 20 °C. Column chromatography (MeOH/CH_2Cl_2 0:1 → 1:9) afforded **16** (120 mg, 95.3%) as an amorphous solid. $R_f = 0.60$ (MeOH/CH_2Cl_2 1:7+1% Et_3N); $[\alpha]_D = +22$ ($c = 0.4$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.63$ (br d, $J = 8.4$ Hz, 1H), 7.37–7.22 (m, 25H), 5.56 (dd, $J = 1.8$, 6.5 Hz, 1H), 5.15 (m, 1H), 5.09 (s, 2H), 4.92 (d, $J = 11.1$ Hz, 1H), 4.87 (d, $J = 11.1$ Hz, 1H), 4.81–4.61 (m, 6H), 4.58 (d, $J = 11.1$ Hz, 1H), 4.47 (d, $J = 11.1$ Hz, 1H), 4.32 (dd, $J = 1.8$, 11.7 Hz, 1H), 4.24–3.84 (m, 9H), 3.70–3.60 (m, 2H), 3.38–3.24 (m, 3H), 2.79 (q, $J = 7.2$ Hz, 6H), 2.03, 1.99 (2s, 6H), 1.17 (t, $J = 7.2$ Hz, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.9$, 156.3, 138.6, 138.2, 138.1, 137.7, 136.5, 128.5–127.6, 99.8, 94.5 (d, $J = 4.6$ Hz), 79.4, 77.2, 75.2, 75.0, 73.9, 73.7, 72.3, 71.6 (d, $J = 4.0$ Hz), 71.0, 70.4, 66.9, 66.7, 65.5 (d, $J = 6.3$ Hz), 62.6, 61.8, 61.5, 48.4, 45.7, 40.7, 23.1, 20.9, 8.5; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = -3.0$; HRMS (FAB): calcd for $C_{54}H_{61}N_5O_{16}P_1$ $[M - Et_3NH]^-$: 1066.3851; found: 1066.3879.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside 6-(2-acetamido-6-*O*-acetyl-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate), triethylammonium salt (17): Sodium borohydride (18 mg, 0.483 mmol) was added slowly to an ice-cooled solution of **16** (65 mg, 0.060 mmol) and nickel chloride hexahydrate (69 mg, 0.289 mmol) in MeOH (2.5 mL) while maintaining the temperature below 10 °C. After 1 h, acetic anhydride (0.5 mL, 5.30 mmol) was added dropwise to the reaction mixture which was stirred at 5 °C. After 30 min, the reaction mixture was processed as described for the preparation of **13**. Column chromatography (MeOH/CH_2Cl_2 1:7+0.1% Et_3N) afforded **17** (65 mg, 99%) as an amorphous solid. $R_f = 0.33$ (MeOH/CH_2Cl_2 1:9); $[\alpha]_D = +4$ ($c = 0.8$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.71$ (d, $J = 9.6$ Hz, 1H), 7.36–7.21 (m, 25H), 5.79 (d, $J = 8.2$ Hz, 1H), 5.60 (dd, $J = 1.2$, 7.0 Hz, 1H), 5.28 (m, 1H), 5.09 (s, 2H), 4.90 (d, $J = 10.5$ Hz, 1H), 4.88 (d, $J = 11.1$ Hz, 1H), 4.78 (d, $J = 11.1$ Hz, 1H), 4.73–4.65 (m, 5H), 4.53 (d, $J = 11.1$ Hz, 1H), 4.47 (d, $J = 11.1$ Hz, 1H), 4.46 (d, $J = 10.5$ Hz, 1H), 4.33–4.15 (m, 6H), 4.05–3.89 (m, 3H), 3.69–3.53 (m, 4H), 3.46–3.20 (m, 4H), 2.60 (q, $J = 7.2$ Hz, 6H), 2.06, 2.02, 1.97 (3s, 9H), 1.07 (t, $J = 7.2$ Hz, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.8$, 170.4, 170.0, 156.2, 138.5, 138.2, 138.0, 137.6, 136.4, 128.4–127.7, 99.7, 95.1 (d, $J = 5.7$ Hz), 77.2, 75.3, 74.8, 73.8, 73.7, 71.6 (d, $J = 4.3$ Hz), 71.3, 71.0, 69.4, 66.8, 66.7, 65.2 (d, $J = 6.2$ Hz), 63.2, 50.1, 50.0, 48.5, 46.1, 40.7, 25.4, 23.6, 23.2, 20.9, 11.2; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = -2.9$; MS (MALDI-TOF): calcd for $C_{56}H_{66}N_3O_{17}P_1$ $[M - Et_3N]^-$: 1083.4; found: 1083.4; HRMS (FAB+, CsI): calcd for $C_{56}H_{65}Cs_2N_3O_{17}P_1$ $[M - 1Et_3NH + 2Cs]^+$: 1348.2160; found: 1348.2130.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside 6-(2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate), triethylammonium salt (18): Compound **17** (9.67 g, 8.16 mmol) in MeOH (50 mL) was treated with a solution of 25% NaOMe/MeOH (1.7 mL) at room temperature. After 30 min, the reaction mixture was processed as described for the preparation of **14**. Column chromatography (MeOH/CH_2Cl_2 1:5+1% Et_3N) afforded **18** (8.85 g, 95%) as an amorphous solid. $R_f = 0.58$ (MeOH/CH_2Cl_2 1:9+1% Et_3N); $[\alpha]_D = +9$ ($c = 1.2$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.67$ (d, $J = 10.0$ Hz, 1H), 7.34–7.20 (m, 25H), 6.74 (d, $J = 8.8$ Hz, 1H), 5.57 (d, $J = 6.4$ Hz, 1H), 5.35 (m, 1H), 5.07 (s, 2H), 4.89 (d, $J = 10.6$ Hz, 1H), 4.87 (d, $J = 10.6$ Hz, 1H), 4.79–4.68 (m, 4H), 4.67 (d, $J = 10.6$ Hz, 1H), 4.55 (d, $J = 11.1$ Hz, 1H), 4.45 (d, $J = 11.1$ Hz, 1H), 4.43 (d, $J = 11.1$ Hz, 1H), 4.26 (m, 1H), 4.14–3.51 (m, 9H), 3.33 (m, 4H), 2.79 (q, $J = 7.2$ Hz, 6H), 2.04, 1.99 (2 s, 6H), 1.10 (t, $J = 7.2$ Hz, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.1$, 170.6, 156.3, 138.5, 138.2, 138.1, 137.8, 136.3, 128.3–127.4, 99.7, 95.2 (d, $J = 5.7$ Hz), 77.6, 77.3, 75.0, 74.9, 74.3, 73.7, 72.5, 71.1 (d, $J = 4.0$ Hz), 70.9, 70.8, 66.7, 64.8 (d, $J = 5.1$ Hz), 64.2, 61.7, 50.1, 50.0, 48.8, 45.9, 40.6, 25.4, 23.3, 20.1, 10.3; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = -2.8$; HRMS (FAB): calcd for $C_{54}H_{63}N_3O_{16}P_1$ $[M - Et_3NH]^-$: 1040.3946; found: 1040.3928.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate 6-(6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate)], bis-triethylammonium salt (19): This compound was obtained by the condensation of **10** (0.246 g, 0.416 mmol) and **18** (0.476 g, 0.416 mmol) in pyridine (5.0 mL) in the presence of trimethylacetyl chloride (77 μL, 0.624 mmol) for 5 min, followed by oxidation with iodine (0.211 g, 0.832 mmol) in pyridine/H_2O (95:5, 5 mL). The reaction mixture was processed as described for the preparation of **16**. Column chromatography (MeOH/CH_2Cl_2 0:1 → 7:93+1% Et_3N) afforded **19** (0.536 g, 74%) as an amorphous solid. $R_f = 0.52$ (MeOH/CH_2Cl_2 1:9+1% Et_3N); $[\alpha]_D = +20$ ($c = 1.4$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.93$ (d, $J = 9.4$ Hz, 1H), 7.72 (d, $J = 8.8$ Hz, 1H), 7.32–7.27 (m, 35H), 5.54 (d, $J = 6.4$ Hz, 1H), 5.35 (d, $J = 7.6$ Hz, 1H), 5.09 (s, 2H), 4.92 (d, $J = 11.1$ Hz, 1H), 4.86 (d, $J = 2.3$ Hz, 1H), 4.82–4.62 (m, 7H), 4.61 (d, $J = 11.1$ Hz, 1H), 4.56 (d, $J = 11.1$ Hz, 1H), 4.45 (d, $J = 10.5$ Hz, 1H), 4.43 (d, $J = 11.1$ Hz, 1H), 4.29–3.93 (m, 6H), 3.85 (m, 1H), 3.80–3.62 (m, 3H), 3.47–3.24 (m, 4H), 3.02 (q, $J = 7.6$ Hz, 12H), 2.05, 2.03, 1.96 (3s, 9H), 1.23 (t, $J = 7.6$ Hz, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.1$, 171.0, 170.9, 156.3, 138.6–136.5, 128.4–127.4, 99.7, 95.6 (d, $J \approx 5$ Hz), 95.5 (d, $J \approx 4$ Hz), 79.4, 77.2, 75.0, 74.9, 74.8, 73.7, 73.6, 72.1, 71.0, 70.8, 70.3, 66.5, 65.1 (d, $J = 4.3$ Hz), 64.8 (d, $J = 3.9$ Hz), 62.6, 61.3, 61.2, 49.2, 48.6, 45.5, 40.5, 22.9, 20.7, 8.5; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = -2.7$, -3.3; HRMS (FAB): calcd for $C_{76}H_{86}N_6Na_1O_{24}P_2$ $[M - 2Et_3NH + Na]^-$: 1551.5066; found: 1551.5052.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside 6-[2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate 6-(2-acetamido-6-*O*-acetyl-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate)], bis-triethylammonium salt (20): Sodium borohydride (0.267 g, 7.06 mmol) was added slowly to an ice-cooled solution of **19** (1.53 g, 0.882 mmol) and nickel chloride hexahydrate (1.00 g, 4.24 mmol) in MeOH (50 mL) while maintaining the temperature below 10 °C. After 1 h, acetic anhydride (3.3 mL, 35.3 mmol) was added dropwise to the reaction mixture which was stirred at 5 °C. After 30 min, the reaction mixture was processed as described for the preparation of **13**. Column chromatography (MeOH/CH_2Cl_2 1:8+0.3% Et_3N) afforded **20** (1.23 g, 80%) as an amorphous solid. $R_f = 0.42$ (MeOH/CH_2Cl_2 1:8+1% Et_3N); $[\alpha]_D = +15$ ($c = 0.7$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 8.00$ (d, $J = 9.4$ Hz, 1H), 7.87 (d, $J = 9.4$ Hz, 1H), 7.34–7.22 (m, 35H), 6.08 (m, 1H), 5.58 (m, 2H), 5.09 (s, 2H), 4.92 (d, $J = 9.9$ Hz, 1H), 4.89 (d, $J = 10.5$ Hz, 1H), 4.81–4.71 (m, 5H), 4.67 (d, $J = 10.5$ Hz, 1H), 4.63 (d, $J = 11.1$ Hz, 1H), 4.53 (d, $J = 11.1$ Hz, 1H), 4.47 (d, $J = 10.5$ Hz, 1H), 4.45 (d, $J = 11.7$ Hz, 1H), 4.33–3.53 (m, 25H), 3.31 (m, 4H), 2.73 (q, $J = 7.2$ Hz, 12H), 2.05, 2.04, 2.02, 1.96 (4s, 12H), 1.12 (t, $J = 7.2$ Hz, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.2$, 170.8, 169.9, 156.4, 138.8–136.6, 128.4–127.4, 99.9, 95.9, 95.3 (d, $J = 5.0$ Hz), 75.2, 74.9, 74.8, 73.7, 72.2, 71.3, 71.1, 70.9, 69.3, 66.6, 65.1 (d, $J \approx 3$ Hz), 65.0, 64.8, 63.1, 50.1, 50.0, 49.2, 48.6, 45.8, 40.6, 30.9, 23.4, 23.1, 23.0, 20.7, 10.1; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = -2.5$, -3.3; HRMS (FAB): calcd for $C_{79}H_{90}N_4Na_2O_{25}P_2$ $[M - 2Et_3NH + Na]^-$: 1567.5267; found: 1567.5294.

2-(Benzyloxycarbonyl)aminoethyl 2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranoside 6-[2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate 6-(2-acetamido-3,4-di-*O*-benzyl-2-deoxy-α-D-mannopyranosyl phosphate)], bis-triethylammonium salt (21): Compound **17**

(1.12 g, 0.640 mmol) in MeOH (20 mL) was treated with a solution of 25% NaOMe/MeOH (0.45 mL) at room temperature. After 30 min, the reaction mixture was processed as described for the preparation of **14**. Column chromatography ($MeOH/CH_2Cl_2$ 1:5+1% Et_3N) afforded **21** (1.08 g, 99%) as an amorphous solid. $R_f = 0.41$ ($MeOH/CH_2Cl_2$ 1:8+1% Et_3N); $[\alpha]_D = +21$ ($c = 1.0$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.71$ (d, $J = 10.0$ Hz, 1H), 7.63 (d, $J = 9.1$ Hz, 1H), 7.34–7.22 (m, 35H), 6.11 (d, $J = 7.6$ Hz, 1H), 5.58 (d, $J = 6.4$ Hz, 1H), 5.48 (d, $J = 7.0$ Hz, 1H), 5.36 (m, 1H), 5.08 (s, 2H), 4.90 (d, $J = 10.5$ Hz, 1H), 4.89 (d, $J = 10.5$ Hz, 1H), 4.82–4.56 (m, 7H), 4.46 (d, $J = 11.1$ Hz, 1H), 4.44 (d, $J = 11.1$ Hz, 1H), 4.43 (m, 1H), 4.21–3.84 (m, 10H), 3.71 (m, 4H), 3.55 (m, 7H), 3.31–3.22 (m, 3H), 2.61 (q, $J = 6.9$ Hz, 12H), 2.05, 2.06, 1.97 (3s, 9H), 1.07 (t, $J = 6.9$ Hz, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.0, 170.8, 170.9, 156.3, 138.6–136.5, 128.4–127.4, 99.7, 95.6$ (d, $J = 4.9$ Hz), 94.5 (d, $J = 4.3$ Hz), 79.4, 77.2, 75.0, 74.9, 74.8, 73.9, 73.7, 73.6, 72.3, 72.1, 71.0, 70.8, 70.2, 66.5, 65.0 (d, $J = 7.8$ Hz), 62.7, 61.3 (d, $J = 7.8$ Hz), 49.0, 48.6, 45.6, 40.5, 27.5, 23.0, 20.7, 8.6; ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = -2.9, -3.4$; MS (MALDI-TOF): calcd for $C_{76}H_{88}N_4Na_1O_{24}P_2$ $[M - 2Et_3NH + Na]^-$: 1524.5; found: 1524.6; HRMS (FAB+, CsI): calcd for $C_{76}H_{88}Cs_3N_4O_{24}P_2$ $[M - 2Et_3NH + 3Cs]^+$: 1901.2427; found: 1901.2386.

2-Aminoethyl 2-acetamido-2-deoxy-α-D-mannopyranoside (1): A stirred mixture of compound **14** (0.390 g, 0.674 mmol), EtOH (10 mL), and 10% Pd/C (Degussa type E101 NE/W, 0.800 g) was subjected to hydrogen pressure (200 psi) for 5 d. The mixture was filtered over Celite 521 (Aldrich), the residue washed with EtOH, and concentrated under reduced pressure to give an oil which was lyophilized from water to afford crude **5** (0.146 g, 82%). A portion of this material was purified by C-18 reverse-phase silica gel chromatography ($MeOH/H_2O$ 1:1) affording pure **1** as an amorphous solid. $R_f = 0.42$ (EtOAc/MeOH/H_2O/AcOH 3:3:1:3); $[\alpha]_D = +23$ ($c = 1.2$, MeOH); HRMS (FAB): calcd for $C_{10}H_{21}N_2O_6$ $[M+H]^+$: 265.1400; found: 265.1404.

2-Aminoethyl 2-acetamido-2-deoxy-6-*O*-phosphate-α-D-mannopyranoside, bis-triethylammonium salt (2): A stirred mixture of compound **15** (0.380 g, 0.453 mmol), EtOH (10 mL), triethylammonium acetate buffer (pH 7, 2.0 mL), and 10% Pd/C (Degussa type E101 NE/W, 0.800 g) was subjected to hydrogen pressure (200 psi). After 6 d, the reaction mixture was processed as described for the preparation of **1** to afford **2** (0.140 g, 100%) as a crude material. A portion of this material was purified through a Biogel P-2 column (pretreated with 0.1% aq AcOH, then 0.1% aq Et_3N) which was eluted with 0.01% aq Et_3N to afford **2** as a white, amorphous solid. $[\alpha]_D = +27$ ($c = 0.36$, H_2O); MS (MALDI-TOF): $C_{22}H_{51}N_4O_9P_1$ $[M]^+$: 546.3; found: 546.4; HRMS (FAB+, CsI): calcd for $C_{10}H_{19}Cs_3N_2O_9P_1$ $[M - 2Et_3NH + 3Cs]^+$: 546.1700; found: 546.1693

2-Aminoethyl 2-acetamido-2-deoxy-α-D-mannopyranoside 6-(2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate), triethylammonium salt (3): A stirred mixture of compound **18** (0.250 g, 0.219 mmol), EtOH (10 mL), triethylammonium acetate buffer (pH 7.0, 2.0 mL), and 10% Pd/C (Degussa type E101 NE/W, 0.500 g) was subjected to hydrogen pressure (200 psi). After 6 d, the reaction mixture was processed as described for the preparation of **1** to afford **3** (0.125 g, 100%) as a crude material. A portion of this material was purified through a Biogel P-2 column (pretreated with 0.1% aq AcOH, then 0.1% aq Et_3N) which was eluted with 0.01% aq Et_3N to afford **3** as a white, amorphous solid. $[\alpha]_D = +34$ ($c = 0.3$, H_2O); HRMS (FAB): calcd for $C_{18}H_{33}N_3O_{14}P_1$ $[M - Et_3NH]^?$: 546.1700; found: 546.1693.

2-Aminoethyl 2-acetamido-2-deoxy-α-D-mannopyranoside 6-[2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate 6-(2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate)], bis-triethylammonium salt (4): A stirred mixture of compound **21** (0.250 g, 0.146 mmol), EtOH (10 mL), triethylammonium acetate buffer (pH 7.0, 2.0 mL), and 10% Pd/C (Degussa type E101 NE/W, 0.500 g) was subjected to hydrogen pressure (200 psi). After 6 d, the reaction mixture was processed as described for the preparation of **1** to afford crude **4** (0.150 g, 100%). A portion of this product was purified through a Biogel P-4 column (pretreated with 0.1% aq AcOH, then 0.1% aq Et_3N) which was eluted with 0.01% aq Et_3N to afford **4** as a white, amorphous solid. $[\alpha]_D = +29$ ($c = 0.4$, H_2O); MS (FAB): calcd for $C_{26}H_{47}N_4O_{22}P_2$ $[M - Et_3NH]^-$: 829.2; found: 829.2; HRMS (FAB+, CsI): calcd for $C_{26}H_{46}Cs_3N_4O_{22}P_2$ $[M - 2Et_3NH + 3Cs]^+$: 1226.9242; found: 1226.9229.

General procedure for the cation exchange of triethylammonium salts for sodium salts for 1–4: Dowex MSC-1 (Na^+ form, 7 mL) resin (pre-washed with H_2O) was added to a stirred solution of saccharide (50 mg) in H_2O (5 mL) and the mixture was stirred for 24 h and filtered. The filtrate was freeze-dried to yield an amorphous white solid.

General procedure for the acylation of the aminoethyl glycosides 1–4 with *N*-hydroxysuccinimidyl hexa-4,6-dienoate: *N*-Hydroxysuccinimidyl hexa-4,6-dienoate (10 mg) in methanol (1 mL) was added to a stirred solution of the 2-aminoethyl glycoside (10 mg) in methanol (1 mL) at 0 °C. After 10 min, the solution was concentrated under reduced pressure. The residue was stirred in water (2 mL) for 5 min followed by filtration. The solids were discarded. The filtrate was applied to a column (3 cm × 0.5 cm) of C-18 reverse-phase silica gel that was eluted with a gradient of methanol in water. The fractions containing saccharide (Dubois-assay) were pooled and concentrated under reduced pressure. After removal of MeOH, the aqueous solution was freeze-dried to yield an amorphous white solid.

General procedure for derivatization of albumin with maleimidobutyryl groups: Sulfosuccinimidyl 4-maleimidobutyrate (8.5 mg; Pierce) was added to a stirred solution of human serum albumin (10 mg) in 0.1M pH 7.5 phosphate buffer at 0 °C. After 5 min, the ice-bath was removed. After a further period of 10 min, the solution was transferred to an Amicon diafiltration apparatus equipped with a YM-10 (10 kDa cutoff) membrane. The solution was diafiltered using five changes of water (5 mL each). The final volume was approx. 0.3 mL.

General procedure for the Diels–Alder-type bioconjugation of the diene-spacer-equipped sacharides to maleimido group-functionalized albumin: The diene-equipped saccharide (12 mg) was added at 22 °C to the solution of the derivatized protein described above. The mixture was stirred for a period of 24 h followed by diafiltration through a YM-10 (10 kDa cutoff) membrane using five changes of water. After the final filtration the solution containing the conjugate was freeze-dried. The composition of the conjugate so obtained was determined by MALDI-TOF mass spectrometry. The filtrate was pooled and freeze-dried. The ^1H NMR spectrum of the residue was identical to the spectrum of the starting saccharide-diene construct.

NMR Spectroscopy: Determinations of product identity were made by means of ^1H, ^{13}C, and ^{31}P NMR spectra recorded at 299.9, 75.4, and 121.4 MHz, respectively, by using a Varian XL 300 spectrometer at ambient temperature. The solvent was $CDCl_3$, unless stated otherwise. Chemical shifts (δ) are reported in ppm downfield from internal Me_4Si for ^1H and ^{13}C NMR spectra, and downfield with respect to external H_3PO_4 for ^{31}P NMR spectra. More detailed NMR studies were conducted for compounds **1–4** by use of a Bruker DRX-500 spectrometer at 300 K, using either a 5 mm HCN triple resonance, triple field gradient (TXI) probe, or a 5 mm broad band (BBO) probe. Solutions of 10–16 mg of compound in deuterium oxide (0.45 mL, 100 atom% D) were used, including acetone as an internal reference set at 2.225 ppm for ^1H, and 31.0 ppm for ^{13}C NMR spectra. 1D ^1H NMR spectra were acquired at 500 MHz by use of 32768 point data sets, a spectral width of 2.84 kHz, a 90° pulse (7.0 μs, TXI probe), and a pulse recycle time of 8.5 s. Resolution enhancement was performed by Gaussian multiplication of the FID, using a line broadening of −1.0 to −1.5 Hz, and a Gaussian truncation fraction of 0.3. In an effort to improve the separation of some accidentally equivalent H-5, H-6, and H-6′ signals, 1D ^1H NMR spectra of compounds **3** and **4** were also acquired at 800 MHz, by means of a Bruker DRX-800 NMR spectrometer, but no dispersive improvement was observed for these signals. 1D ^{13}C and DEPT-135 ^{13}C NMR spectra were recorded at 125.8 MHz either with or without WALTZ-16, composite pulse ^1H decoupling at 500 MHz, by using 32768 point data sets zero-filled to 32768 or 131072 points (compound **4**), a spectral width of 28.25 kHz, and a 90° pulse (10.5 μs, TXI probe). A pulse recycle time of 3.2 s was used for ^{13}C NMR spectra, and 2.0 s for DEPT ^{13}C NMR spectra. In some cases, the DEPT-135 ^{13}C NMR spectra were recorded with a smaller spectral width (11.5 kHz), and a 135° ^1H read pulse of 11.8 μs was used (TXI probe). 1D ^{31}P NMR spectra were recorded at 202.5 MHz by using the BBO probe with 8192 or 16384 data points, zero-filled to 16384 points, together with a spectral width of 3.28 or 6.07 kHz, a 90° pulse (8.3 μs), and a pulse recycle time of 4–6 s. A sample of 85% H_3PO_4 containing 10% *v*/*v* of D_2O was used as an external reference (−0.73 ppm).[25]

2D TOCSY ^1H NMR spectra were acquired in phase sensitive mode with echo/anti-echo-TPPI gradient selection. A spectral width of 2.84 kHz was used, together with 16384 (t_2) × 128, 256, or 512 (t_1) point data sets zero-filled to 16384 × 512 or 2048 points, 4–32 scans, 16–128 dummy scans, and

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a ^{1}H 90° pulse width of 7.85 μs (TXI probe), or 9.3 μs (BBO probe). 2D TOCSY experiments were also performed with broadband, WALTZ-16 ^{31}P decoupling applied either during the acquisition period or throughout the pulse sequence, or with selective, homonuclear ^{1}H decoupling of H-5 during acquisition. 2D COSY-45 ^{1}H NMR spectra were recorded by continuous wave presaturation at the HOD signal, followed by switching of the observation frequency to the center of the spectrum at the end of the relaxation delay, using a two-value, frequency list. The data set sizes were 2048 points (t_2) × 800 to 1024 points (t_1), zero-filled to 2048 × 2048 points, which were used with 4 to 8 scans and 16 dummy scans. For processing, sine-bell windows shifted by π/3 rad were used in both dimensions, together with a magnitude calculation.

2D HSQC ^{1}H/^{13}C NMR spectra were acquired at 500/125.8 MHz in phase-sensitive, sensitivity-improved, echo/anti-echo-TPPI gradient selected mode, by using the TXI probe with 2048 (t_2) × 800–1024 (t_1) point data sets, zero-filled to 2048 points in each dimension, 8–48 scans, 16–64 dummy scans, ^{1}H 90° pulse 7.0 µs, ^{13}C 90° pulse 10.5 µs, together with sine-bell squared windows shifted by π/2 rad, in both dimensions. The separation of closely spaced cross-multiplets in the 2D HSQC spectrum of compound **4** was optimized by zero-filling to 8192 points in each dimension, and by using sine-bell squared windows, shifted by π/4 rad in both dimensions.

Immunodiffusion assay: Glass microscope plates (75 × 50 cm) were pre-coated with a warm solution of 0.1 % aq agarose (preboiled) and allowed to dry overnight. A solution of 0.9 % agarose in PBS buffer (≈8.5 mL, 1.7 mM KH_2PO_4, 5.0 mM Na_2HPO_4, 0.15 M NaCl) was applied to the precoated glass plates which were allowed to dry. The plates were stored at 5 °C. Wells of approx 1.2 mm diameter were cut into the plate. The wells were filled with either the antigen (≈3 µL, 1 mg mL^{-1} in PBS buffer) or antiserum (horse 49) and allowed to stand at 5 °C. After ≈12 h, the plate was washed with PBS buffer (2 h), then pressed (≈5 lbs weight, 2 h). This process was repeated ≈3 times. The plate was treated with Coomassie Blue stain (0.2 % in Destain) and washed several times with Destain (MeOH/H_2O/AcOH 9:9:2).

[1] A. J. Pollard, C. Frasch, *Vaccine* **2001**, *19*, 1327–1346.
[2] S. L. Morley, A. J. Pollard, *Vaccine* **2001**, *20*, 666–687.
[3] J. B. Robbins, D. W. Towne, E. C. Gotschlich, R. Schneerson, *Lancet* **1997**, *350*, 880–882.
[4] I. Goldschneider, E. C. Gotschlich, M. S. Artenstein, *J. Exp. Med.* **1969**, *129*, 1307–1326.
[5] H. Peltola, *Drugs* **1998**, *55*, 347–366.
[6] R. Gold, M. L. G. I. Lepow, T. F. Draper, E. C. Gotschlich, *J. Infect. Dis.* **1979**, *140*, 690–697.
[7] H. J. Jennings, C. Lugowski, *J. Immunol.* **1981**, *127*, 1011–1018.
[8] E. C. Beuvery, R. W. Van Delft, F. Miedema, V. Kanhai, J. Nagel, *Infect. Immun.* **1983**, *41*, 609–617.
[9] D. R. Bundle, I. C. P. Smith, H. J. Jennings, *J. Biol. Chem.* **1974**, *249*, 83–96.
[10] X. Lemercinier, C. Jones, *Biologicals* **2000**, *28*, 175–183.
[11] V. Pozsgay, *J. Org. Chem.* **1998**, *63*, 5983–5999.
[12] V. Pozsgay, C. Chu, L. Pannell, J. Wolfe, J. B. Robbins, R. Schneerson, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 5194–5197.
[13] V. Pozsgay, *Adv. Carbohydr. Chem. Biochem.* **2001**, *56*, 153–199.
[14] V. Pozsgay, N. E. Vieira, A. Yergey, *Org. Lett.*, in press.
[15] N. S. Utkina, G. I. Eliseyeva, A. V. Nikolaev, V. N. Shibaev, *Bioorg. Khim.* **1993**, *19*, 228–235.
[16] A. Chernyak, S. Oscarson, N. S. Utkina, Abstracts, 20th Int. Carbohydr. Symp. Hamburg, B-204. **2000**.
[17] J. Stawinski in *Handbook of Organophosphorous Chemistry* (Ed.: R. Engel), Marcel Dekker, New York, **1992**, pp. 377–434.
[18] P. Westerduin, G. H. Veeneman, G. A. van der Marel, J. H. van Boom, *Tetrahedron Lett.* **1986**, *27*, 6271–6274.
[19] Y. Ishido, N. Sakairi, *Carbohydr. Res.* **1981**, *97*, 151–155.
[20] S. Koto, K. Asami, M. Hirooka, K. Nagura, M. Takizawa, S. Yamamoto, N. Okamoto, M. Sato, H. Tajima, T. Yoshida, N. Nonaka, T. Sato, S. Zen, K. Yago, F. Tomonaga, *Bull. Chem. Soc. Jpn.* **1999**, *72*, 765–777.
[21] J. Jankowska, M. Sobkowski, J. Stawinski, A. Kraszevski, *Tetrahedron Lett.* **1994**, *35*, 3355–3358.
[22] J. Hansson, P. J. Garegg, S. Oscarson, *J. Org. Chem.* **2001**, *66*, 6234–6243.
[23] A. Sivonen, O.-V. Renkonen, J. B. Robbins, *J. Clin. Pathol.* **1977**, *30*, 834–837.
[24] F. Y. C. Lin, V. A. Ho, H. B. Khiem, D. D. Trach, P. V. Bay, T. C. Thanh, Z. Kossaczka, D. A. Bryla, J. Shiloach, J. B. Robbins, R. Schneerson, S. C. Szu, M. N. Lanh, S. Hunt, L. Trinh, J. B. Kaufman, *N-Engl. J. Med.* **2001**, *344*, 1263–1269.
[25] M. Batley, W. Redmond, *J. Magn. Reson.* **1982**, *49*, 172–174.

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  0947-6539/02/0819-4433 $ 20.00+.50/0