Letters

Phosphorylcholine−Carbohydrate−Protein Conjugates Efficiently Induce Hapten-Specific Antibodies Which Recognize Both *Streptococcus pneumoniae* and *Neisseria meningitidis*: A Potential Multitarget Vaccine against Respiratory Infections

Sylvie Bay,*[,§] Valérie Huteau,[§,||] Maria-Leticia Zarantonelli,[‡] René Pires,[‡] Joël Ughetto-Monfrin,[§] Muhamed-Kheir Taha,[‡] Patrick England,[⊥] and Pierre Lafaye[†]

Unité de Chimie Organique URA CNRS 2128, Unité de Génétique et Biochimie du Développement, Unité des Neisseria, and Plate-forme de Biophysique des Macromolécules et de leurs Interactions, Institut Pasteur, Paris, France

Received February 9, 2004

Abstract: Phosphorylcholine (ChoP) is commonly expressed at the surface of pathogens of the respiratory tract, including *Streptococcus pneumoniae* and *Neisseria meningitidis*. We designed a synthetic hapten comprising ChoP and part of its native carrier structure in *S. pneumoniae*, i.e. *N*-acetyl-D-galactosamine (GalNAc). Protein conjugates of this hapten induced GalNAc−ChoP-specific antibodies which recognized ChoP on both *S. pneumoniae* and *N. meningitidis*. GalNAc−ChoP could therefore lead to the rational design of a novel multipurpose vaccine against respiratory infections.

Neisseria meningitidis and *Streptococcus pneumoniae* are major causative bacterial agents of invasive respiratory infections and meningitis. These bacterial species are genetically and antigenically variable, and therefore currently available vaccines are far from satisfactory.[1,2] Moreover, an increasing number of *S. pneumoniae* strains are resistant to various antibiotics, and the emergence of *N. meningitidis* strains with diminished susceptibility to β-lactams becomes a matter of concern.[3] Antibody-based therapies could therefore gain renewed interest for the prophylaxis and treatment of these respiratory infections.[4]

Phosphorylcholine (ChoP) is frequently incorporated in the surface antigens of several prokaryotes (*Haemophilus*, *Streptococcus*, *Neisseria*, *Mycoplasma*, *Salmonella*, and *Pseudomonas*) and eukaryotes (pathogenic helminths and nematodes) (for review, see ref 5).

In respiratory infections, ChoP is thought to be directly involved in the steps of adhesion and colonization of the respiratory epithelium, as well as in the inflammatory process leading to the invasion of the host. Through ChoP, *H. influenzae* and *S. pneumoniae* are able to bind to the platelet activating factor receptor (PAF-receptor), thus mimicking endogenous processes of cellular signaling.[6,7] Furthermore, several proteins of *S. pneumoniae*, which are important for its virulence, are attached to the cell wall via ChoP.[8] ChoP also appears to be responsible for the triggering of innate immune reactions against *H. influenzae*, mediated by the C-reactive protein (CRP), which is the natural ligand for ChoP in blood and acts as a complement-binding opsonin.[9]

Therefore, ChoP is an attractive target for the development of immunotherapies directed against these major bacterial infections of the respiratory tract.[10] It has been shown previously that ChoP-specific T15-idiotype antibodies, despite their low affinity, are protective when used in passive immunizations.[11,12] Likewise, the injection of ChoP coupled to a protein carrier induced the production of high-affinity specific antibodies in mice.[13] Moreover, intranasal[14,15] or parenteral[16] immunization experiments with a similar ChoP−protein conjugate protected mice against a lethal challenge with *S. pneumoniae*.

However, the epitope recognized by the induced antibodies is not always limited to ChoP but also includes the covalent link used for the coupling with the carrier.[13] In the first studies, ChoP−protein conjugates used for the immunizations contained a diazophenyl linker between ChoP and the tyrosine and histidine residues of the protein carrier, resulting in immunodominant responses directed against aromatic rings. To minimize this irrelevant immune response, efforts have been made to replace the aromatic rings by an aliphatic linker. The resulting immunogen provided total protection of Xid mice against a lethal challenge with *S. pneumoniae*, whereas the diazophenyl-containing conjugate did not.[17,18]

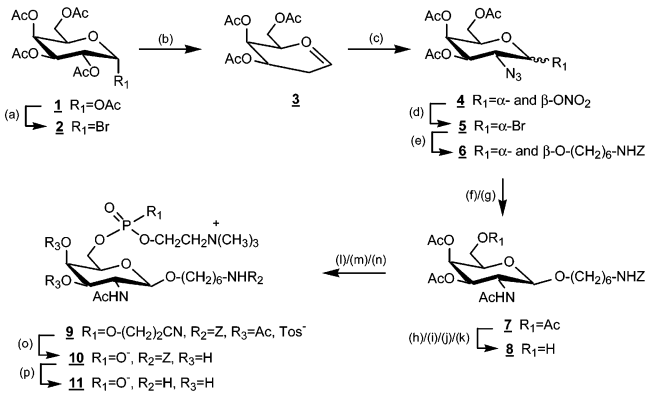
These results suggest that the nature of the linker between ChoP and the protein carrier can be critical for the induction of high affinity protective antibodies intended for antibacterial therapies.

According to the pathogen, ChoP is coupled to a variety of bacterial cell structures. In the case of *N. meningitidis*, ChoP is linked to the glycoproteins of the pili, but its molecular carrier (saccharide or amino acid) has not yet been clearly identified.[19,20] In *H. influenzae*, ChoP is grafted to the surface lipopolysaccharides.[21] In *S. pneumoniae*, ChoP is part of the C-polysaccharide (teichoic acid) and F-antigen (lipoteichoic acid).[22,23] Despite this diversity in the native macromolecular backbone, it appears that, at least for *H. influenzae* and *S. pneumoniae*, ChoP is presented in position 6 of an hexose or an hexosamine, respectively. Whether this carbohydrate part of the epitope is involved in the pathogenic process remains an open question.

* Corresponding author: Unité de Chimie Organique, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15; tel: (33) 01-45-68-83-97; fax: (33) 01-45-68-84-04; e-mail: sbay@pasteur.fr.
§ Unité de Chimie Organique URA CNRS 2128.
† Unité de Génétique et Biochimie du Développement.
‡ Unité des *Neisseria*.
⊥ Plate-forme de Biophysique des Macromolécules et de leurs Interactions.
|| Present address: PF7, Synthèse d'oligonucléotides longs à haut débit, Institut Pasteur, Paris, France.

10.1021/jm040783p CCC: $27.50
Published on Web 07/07/2004

Scheme 1. Synthesis of GalNAc−ChoP[a]



[a] Reagents and conditions: (a) 30% HBr in CH_3COOH, RT, 1 h 30 min (99%); (b) Zn, NMI, dry AcOEt, reflux, 2 h; (c) $(NH_4)_2$-$Ce(NO_3)_6$, NaN_3, CH_3CN, −25 °C, 3 h (two steps, 44%); (d) LiBr, dry CH_3CN, RT, 3 h (99%); (e) HO−$(CH_2)_6$−NHZ, AgOTf, collidine, dry CH_2Cl_2, −40 °C, 15 h; (f) $NaBH_4$, H_3BO_3, $NiCl_2$, EtOH, RT, 1 h 30 min; (g) Ac_2O, EtOH, RT, 1 h (three steps, 54%); (h) MeONa, MeOH, RT, 10 min; (i) DMTrCl, Pyr, RT, 2 h; (j) Ac_2O, Pyr, RT, 3 h; (k) 2% ABS in CH_2Cl_2/MeOH:7/3, 0 °C, 2 min (four steps, 60%); (l) chloro 2-cyanoethyl (*N*,*N*-diisopropyl)phosphoramidite, DIEA, dry CH_3CN, RT, 20 min; (m) Choline$^+$ Tos$^-$, tetrazole, CH_3CN, RT, 24 h; (n) I_2/Pyr/THF/H_2O, CH_3CN, RT, 15 min (three steps, 31%); (o) MeONa, MeOH, RT, 15 min; (p) H_2, Pd/C, EtOH, RT, 1 h (two steps, 55%).

In this study, we aimed at raising high-affinity antibodies against ChoP in its bacterial context in order to target several pathogens of the respiratory tract. By mimicking the *S. pneumoniae* model, we synthesized two carbohydrate−ChoP (GalNAc−ChoP) protein conjugates, and we showed that these immunogens induce hapten-specific antibodies which recognize two major bacterial pathogens of the respiratory tract: a Gram-positive bacterium, *S. pneumoniae*, and a Gram-negative bacterium, *N. meningitidis*.

Synthesis of the Bacterial Hapten GalNAc−ChoP (Scheme 1). In *S. pneumoniae*, one or two ChoP molecules are linked at the position 6 of the *N*-acetyl-D-galactosamine residues within the repeating unit of the C-polysaccharide [-6)-*β*-D-Glc*p*-(1−3)-α-AAT*p*-(1−4)-α-D-Gal*p*NAc−(1−3)-*β*-D-Gal*p*NAc-(1−1)-D-ribitol-5-P-(O-] (AAT = 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose).[22,23] Fragments of this repeating unit have been synthesized for structural[24] and immunological[25] studies. However, none of them bear a ChoP residue.

To mimic the bacterial environment of the ChoP, we designed a synthetic antigen comprising both ChoP and part of its native carrier structure, i.e. the 6-substituted *N*-acetyl-D-galactosamine residue.

The synthesis is summarized in Scheme 1. Starting from 1,2,3,4,6-penta-*O*-acetyl-*β*-D-galactopyranoside **1**, a succession of bromination, reductive dehalogenation, azidonitration, bromination, Koenigs−Knorr reaction with 6-(benzyloxycarbonyl)hexanol linker, reduction−acetylation of the azido group, and then selective deprotections/protections gave the 6-OH key intermediate **8** (nine steps, overall yield 14%).

The ChoP was then coupled at the position 6 of the GalNAc residue with the phosphoramidite method. By using ^{31}P NMR, we could follow the completion of the different reactions, and the three following steps were sequentially performed one-pot. **8** was reacted with chloro 2-cyanoethyl (*N*,*N*-diisopropyl)phosphoramidite in the presence of DIEA. After disappearance of the ^{31}P signal assigned to the starting material (δ 182.41 ppm) and concomitant appearance of the new phosphoramidite signals (δ 150.63 and 150.21 ppm), phosphitylation was performed by adding choline tosylate to the reaction mixture together with tetrazole. Reaction was completed after 24 h as shown by ^{31}P NMR. The resulting new ^{31}P signals account for the two phosphite triester diastereoisomers (δ 141.62 and 141.41 ppm). After oxidation, the formation of the phosphotriester diastereoisomers **9** was indicated by new ^{31}P signals in the spectrum (δ −1.96 and −2.03 ppm) (three steps, overall yield 31%).

The carbohydrate moiety, the cyanoethyl group, and the linker were deprotected to afford the hapten **11** (two steps, overall yield 55%).

Synthesis of the GalNAc−ChoP−Protein Conjugates. The conjugation of **11** to the tetanus toxoid protein (TT) or the Alpaga Serum Albumin (ASA) through activation with the EDC/SulfoNHS method yielded the expected GalNAc−ChoP−protein conjugates. The ChoP:protein ratio of the conjugates TT−GalNAc−ChoP and ASA−GalNAc−ChoP were estimated at, respectively, 17:1 and 29:1 by a microphosphate assay.[26]

Induction of GalNAc−ChoP-Specific Antibodies Which Recognize *S. pneumoniae* and *N. meningitidis*. Biozzi mice were immunized either with TT−GalNAc−ChoP or with ASA−GalNAc−ChoP, and the immune sera were tested for reactivity with the parental immunogen after boost injections. Each animal developed either a strong anti-TT−GalNAc−ChoP response or a strong ASA−GalNAc−ChoP response (data not shown). For each immunogen, further experiments were performed with the serum showing the highest reactivity. The specificity of the serum for ChoP was assessed in inhibition assays using TT, ASA, GalNAc−ChoP, GalNAc alone, or *p*-nitro-phenyl-ChoP. The 50% inhibition concentrations (IC_{50}) are shown in Table 1. The results suggest that immunization with both antigens generates antibodies specific for ChoP. Moreover, these antibodies have a stronger avidity toward GalNAc−ChoP ($IC_{50} = 1 \times 10^{-6}$ M for TT−GalNAc−ChoP and $IC_{50} = 0.15 \times 10^{-6}$ M for ASA−GalNAc−ChoP) than toward ChoP alone ($IC_{50} = 20 \times 10^{-6}$ M for TT−GalNAc−ChoP and $IC_{50} = 7 \times 10^{-6}$ M for ASA−GalNAc−ChoP).

To further characterize the quality of the murine anti-ChoP antibody response, immune sera were tested

Table 1. ELISA Binding Profile of Immune Sera to Coated *S. pneumoniae*[a]

	competitor, IC_{50} (M)				
immunogen	TT	ASA	GalNAc	GalNAc−ChoP	*p*-nitrophenyl-ChoP
TT−GalNAc−ChoP	$>10^{-4}$	ND[b]	$>10^{-4}$	1×10^{-6}	20×10^{-6}
ASA−GalNAc−ChoP	ND	$>10^{-4}$	$>10^{-4}$	0.15×10^{-6}	7×10^{-6}

[a] The specificity of the sera for ChoP was assessed by measuring the 50% inhibition concentration (IC_{50}) using different antigens carrying or not ChoP. [b] ND: not determined.

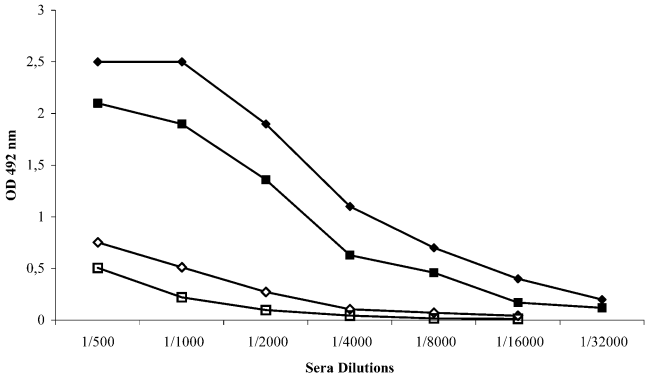


Figure 1. ELISA measurement of heat-inactivated *S. pneumoniae* cells reactivity for murine anti-TT−GalNAc−ChoP (◆) and ASA−GalNAc−ChoP (■) immune sera. (◇ and □): corresponding sera of nonimmunized animals.

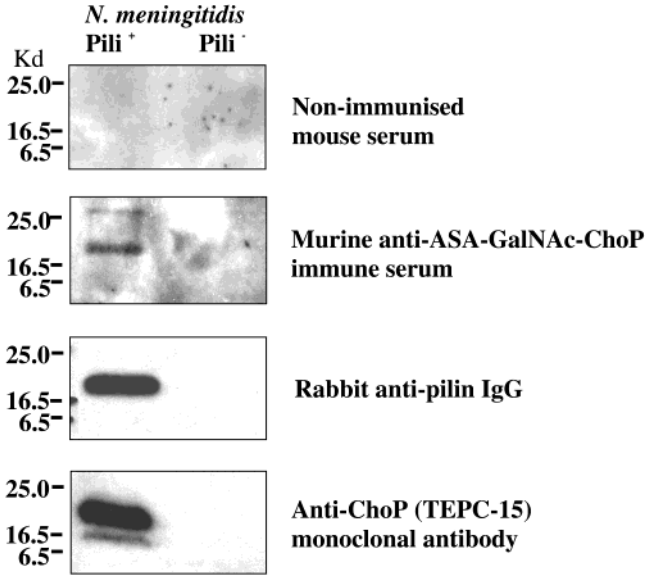


Figure 2. Immunoblot of anti-ASA−GalNAc−ChoP immune serum with *N. meningitidis* extracts. A wild-type pilin-positive ($Pili^+$), ChoP-positive serogroup C strain, was tested in parallel with its isogenic *pilE* mutant (devoid of pilin, $Pili^-$). The specific monoclonal anti-ChoP antibody TEPC-15 was used as positive control, and a polyclonal rabbit anti-pilin IgG was used to colocalize associated ChoP in *N. meningitidis*.

against heat-inactivated *S. pneumoniae* (Figure 1). The results suggest that both sera contain antibodies that are able to recognize *S. pneumoniae*. The specificity of these responses was assessed in inhibition assays with *S. pneumoniae* cell-wall polysaccharide (C−Ps) because this structure contains specifically ChoP linked to GalNAc. The IC_{50} was about 35 ng/mL and 20 ng/mL for the TT−GalNAc−ChoP and for the ASA−GalNAc−ChoP sera, respectively (data not shown).

Binding of the immune sera to *N. meningitidis* pilin-associated ChoP was tested by immunoblotting on whole bacterial cell extracts.[27] As shown in Figure 2, sera obtained by immunization with synthetic ASA−GalNAc−ChoP specifically recognized pilin-associated ChoP in the wild-type *N. meningitidis* strain but not in the pilin-defective mutant. Cross-reactive recognition of the *S. pneumoniae* uncapsulated ChoP-positive strain R6 (ATCC 39937) was also confirmed by these assays (data not shown).

This study demonstrates the ability of ChoP−carbohydrate−protein conjugates to raise a strong hapten-specific antibody response against two phylogenetically unrelated bacteria, *S. pneumoniae* and *N. meningitidis*, that express ChoP at their surface. The efficacy of the immune response is currently investigated in animal models.

Taken together, our results highlight the potential of the ChoP−carbohydrate epitope as a model antigen for the rational design of a wide spectrum vaccine which could confer cross-protection against several pathogens of the respiratory tract. An additional advantage of such ChoP−carbohydrate conjugates vaccine is their safety. Indeed, since they closely mimic the native bacterial antigen, they should induce more specific immune response and prevent potential cross-reactivities with phosphatidylcholine-bearing macromolecules of the host.

Acknowledgment. We are grateful to J.-M. Alonso for helpful discussions, constant support, and for critical reading of the manuscript. We thank M. Leduc for valuable advice and preparation of heat-inactivated *S. pneumoniae*. This work was supported by a grant from the Institut Pasteur (Programme Transversal de Recherche no. 67).

Supporting Information Available: Experimental procedures for the preparation of **6**−**11** and of the protein conjugates TT−GalNAc−ChoP and ASA−GalNAc−ChoP. Characterization data for compounds **6**β, **7**, **8**, and **11**. Methods for immunizations and ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Lipsitch, M. Bacterial vaccines and serotype replacement: lessons from *Haemophilus influenzae* and prospects for *Streptococcus pneumoniae*. *Emerg. Infect. Dis.* **1999**, *5*, 336−345.
(2) Swartley, J. S.; Marfin, A. A.; Edupuganti, S.; Liu, L. J.; Cieslak, P.; Perkins, B.; Wenger, J. D.; Stephens, D. S. Capsule switching of *Neisseria meningitidis*. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 271−276.
(3) Neu, H. C. The crisis in antibiotic resistance. *Science* **1992**, *257*, 1064−1073.
(4) Casadevall, A.; Scharff, M. Return to the past: the case for antibody-based therapies in infectious diseases. *Clin. Infect. Dis.* **1995**, *21*, 150−161.
(5) Trolle, S.; Andremont, A.; Fattal, E. Towards a multipurpose vaccination using phosphorylcholine as a unique antigen. *STP Pharma. Sci.* **1998**, *8*, 19−30.
(6) Cundell, D. R.; Gerard, N. P.; Gerard, C.; Idanpaan-Heikkila, I.; Tuomanen, E. I. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature* **1995**, *377*, 435−438.
(7) Swords, W. E.; Buscher, B. A.; Ver Steeg Ii, K.; Preston, A.; Nichols, W. A.; Weiser, J. N.; Gibson, B. W.; Apicella, M. A. Nontypeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol. Microbiol.* **2000**, *37*, 13−27.
(8) Rosenow, C.; Ryan, P.; Weiser, J. N.; Johnson, S.; Fontan, P.; Ortqvist, A.; Masure, H. R. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol. Microbiol.* **1997**, *25*, 819−829.
(9) Lysenko, E.; Richards, J. C.; Cox, A. D.; Stewart, A.; Martin, A.; Kapoor, M.; Weiser, J. N. The position of phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* affects binding and sensitivity to C-reactive protein-mediated killing. *Mol. Microbiol.* **2000**, *35*, 234−245.
(10) Musher, D. M. How contagious are common respiratory tract infections? *New Engl. J. Med.* **2003**, *348*, 1256−1266.
(11) Briles, D. E.; Forman, C.; Crain, M. Mouse antibody to phosphocholine can protect mice from infection with mouse-virulent human isolates of Streptococcus pneumoniae. *Infect. Immun.* **1992**, *60*, 1957−1962.
(12) Yother, J.; Forman, C.; Gray, B. M.; Briles, D. E. Protection of mice from infection with *Streptococcus pneumoniae* by anti-phosphocholine antibody. *Infect. Immun.* **1982**, *36*, 184−188.

(13) Brown, M.; Schumacher, M. A.; Wiens, G. D.; Brennan, R. G.; Rittenberg, M. B. The structural basis of repertoire shift in an immune response to phosphocholine. *J. Exp. Med.* **2000**, *191*, 2101−2112.
(14) Trolle, S.; Chachaty, E.; Kassis-Chikhani, N.; Wang, C.; Fattal, E.; Couvreur, P.; Diamond, B.; Alonso, J.-M.; Andremont, A. Intranasal immunization with protein-linked phosphorylcholine protects mice against a lethal intranasal challenge with *Streptococcus pneumoniae*. *Vaccine* **2000**, *18*, 2991−2998.
(15) Fattal, E.; Pecquet, S.; Couvreur, P.; Andremont, A. Biodegradable microparticles for the mucosal delivery of antibacterial and dietary antigens. *Int. J. Pharm.* **2002**, *242*, 15−24.
(16) Wallick, S.; Claflin, J. L.; Briles, D. E. Resistance to *Streptococcus pneumoniae* is induced by a phosphocholine-protein conjugate. *J. Immunol.* **1983**, *130*, 2871−2875.
(17) Kenny, J. J.; Guelde, G.; Fischer, R. T.; Longo, D. L. Induction of phosphocholine-specific antibodies in X-linked immune deficient mice: *in vivo* protection against a *Streptococcus pneumoniae* challenge. *Int. Immunol.* **1993**, *6*, 561−568.
(18) Fischer, R. T.; Longo, D. L.; Kenny, J. J. A novel phosphocholine antigen protects both normal and X-linked immune deficient mice against Streptococcus pneumoniae. Comparison of the 6-O-phosphocholine hydroxyhexanoate-conjugate with other phosphocholine-containing vaccines. *J. Immunol.* **1995**, *154*, 3373−3382.
(19) Serino, L.; Virji, M. Phosphorylcholine decoration of lipopolysaccharide differentiates commensal *Neisseriae* from pathogenic strains: identification of licA-type genes in commensal *Neisseriae*. *Mol. Microbiol.* **2000**, *35*, 1550−1559.
(20) Warren, M. J.; Jennings, M. P. Identification and characterization of *pptA*: a gene involved in the phase-variable expression of phosphorylcholine on pili of *Neisseria meningitidis*. *Infect. Immun.* **2003**, *71*, 6892−6898.
(21) Schweda, E. K.; Brisson, J. R.; Alvelius, G.; Martin, A.; Weiser, J. N.; Hood, D. W.; Moxon, E. R.; Richards, J. C. Characterization of the phosphocholine-substituted oligosaccharide in lipopolysaccharides of type b *Haemophilus influenzae*. *Eur. J. Biochem.* **2000**, *267*, 3902−3913.
(22) Fischer, W.; Behr, T.; Hartmann, R.; Peter-Katalini, J.; Egge, H. Teichoic acid and lipoteichoic acid of *Streptococcus pneumoniae* possess identical chain structures. A reinvestigation of teichoid acid (C polysaccharide). *Eur. J. Biochem.* **1993**, *215*, 851−857.
(23) Karlsson, C.; Jansson, P. E.; Skov Sorensen, U. B. The pneumococcal common antigen C-polysaccharide occurs in different forms. Mono-substituted or di-substituted with phosphocholine. *Eur. J. Biochem.* **1999**, *265*, 1091−1097.
(24) Qin, H.; Grindley, T. B. Determination of the configuration of ribitol in the C-polysaccharide of *Streptococcus pneumoniae* using a synthetic approach. *Can. J. Chem.* **1999**, *77*, 481−494.
(25) Smid, P.; de Zwart, M.; Jörning, W. P. A.; van der Marel, G. A.; van Boom, J. H. Stereoselective synthesis of a tetrameric fragment of Streptococcus pneumoniae type 1 containing an α-linked 2-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranose (SUG*p*) unit. *J. Carbohydr. Chem.* **1993**, *12*, 1073−1090.
(26) Bartlett, G. R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **1958**, *234*, 466−468.
(27) Kolberg, J.; Hoiby, E. A.; Jantzen, E. Detection of the phosphorylcholine epitope in *streptococci*, *Haemophilus* and pathogenic *Neisseriae* by immunoblotting. *Microb. Pathog.* **1997**, *22*, 321−329.

JM040783P