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Articles

Zwitterionic Phosphodiester-Substituted Neoglycoconjugates as Ligands for Antibodies and Acute Phase Proteins

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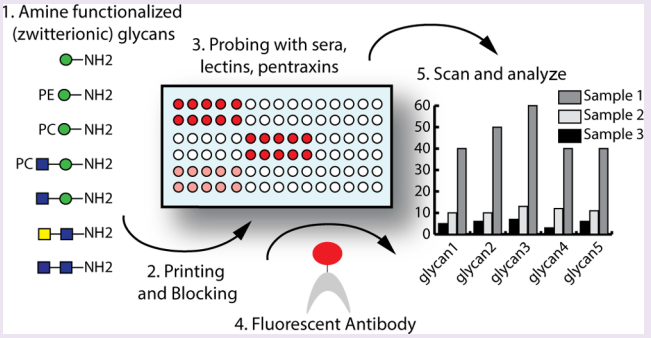
ABSTRACT: Zwitterionic modifications of glycans, such as phosphorylcholine and phosphoethanolamine, are known from a range of prokaryotic and eukaryotic species and are recognized by mammalian antibodies and pentraxins; however, defined saccharide ligands modified with these zwitterionic moieties for high-throughput studies are lacking. In this study, we prepared and tested example mono- and disaccharides 6-substituted with either phosphorylcholine or phosphoethanolamine as bovine serum albumin neoglycoconjugates or printed in a microarray format for subsequent assessment of their binding to lectins, pentraxins, and antibodies. C-Reactive protein and anti-phosphorylcholine antibodies bound specifically to ligands with phosphorylcholine, but recognition by concanavalin A was abolished or decreased as compared with that to the corresponding nonzwitterionic compounds. Furthermore, in array format, the phosphorylcholine-modified ligands were recognized by IgG and IgM in sera of either non-infected or nematode-infected dogs and pigs. Thereby, these new compounds are defined ligands which allow the assessment of glycan-bound phosphorylcholine as a target of both the innate and adaptive immune systems in mammals.



Glycans present in cell-surface glycoprotein and glycolipids have been shown to play a major role in the immune cross-talk between parasites and their hosts, leading to immunomodulating effects.[1,2] Particular examples are N- or lipid-linked glycans modified with phosphorylcholine (PC), which are a conserved signature of nematodes,[3−6] a phylum with many parasitic species. These may have adopted PC-modified glycans as a means of improving their chances of survival in the host by modulating vertebrate immune systems, possibly via interactions involving the Toll-like receptor TLR4.[6,7] In another parasite, the immunodominant Ag5 antigen of *Echinococcus granulosus* (a cestode) carries PC residues on its biantennary N-glycans,[8] while recently, PC has been found on the N-glycans of glycoproteins originating from moths and moth cell lines.[9] PC is also present on various fungal glycoconjugates such as N-glycans of *Penicillium nordicum*, the peptidophosphogalactomannan of *Penicillium charlesii*, and glycolipids of *Aspergillus fumigatus* or *Acremonium* sp.[10−13] Finally, PC is a modification not only of annelid (earthworm) glycolipids[14] but of the lipopolysaccharides of bacteria such as *Haemophilus influenzae* and *Pasteurella multocida*, pilin glycans of *Neisseria meningitidis*, or the teichoic acid of *Streptococcus pneumoniae*.[15−18] In *Neisseria* and *Haemophilus* species, on−off switching of PC expression occurs depending on whether the bacteria reside in the upper respiratory tract (where PC is advantageous for adhesion) or in systemic sites (where PC may be recognized by the immune system).[19,20] The nonmethylated form of PC, phosphoethanolamine (PE), is also a modification, e.g., of lipopolysaccharide from *N. meningitidis*, glycolipids of dipteran insects, all eukaryotic glycosylphosphatidylinositol (GPI) anchors, and N-glycans of various *Penicillium* species, the sexually transmitted parasite *Trichomonas vaginalis*, and the N-glycans of honeybee royal jelly, venom, and larvae.[12,21−26] Such PC/PE-modified structures are distinct from certain immunogenic bacterial zwitterionic glycans, such as the *S. pneumoniae* serotype 1 polysaccharide (Sp1) with free amino and carboxyl functions on different monosaccharide units.[27]

In bacteria, PC and PE are found attached to different hydroxyls of various hexose, heptose, or *N*-acetylhexosamine residues depending on the species, whereas in eukaryotes, zwitterionic modifications are typically on the 6-OH of either GlcNAc or Man.[28] Phosphorylcholine−carbohydrate conjugates are efficient inducers of hapten-specific antibodies and are specific epitopes of IgA myeloma proteins,[29] while both PC and PE are ligands for human "short" pentraxins; in particular, C-reactive protein, an acute phase protein often measured as

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an inflammation marker in diagnostic tests, is known to recognize phosphorylcholine and to mediate complement activation, while serum amyloid P binds phosphoethanolamine.[30−32]

Considering the biological context, we have set out to provide a series of reagents for detection and characterization of immunologically relevant binding to these phosphodiester-substituted carbohydrates. Herein, we describe the synthesis of the respective 6-*O*-PC- and 6-*O*-PE-substituted mannoside ligands as well as the disaccharide ligand 6-*O*-PC-*β*Glc*p*NAc-(1→2)-*α*-Man*p*, which correspond to part of the structures identified, e.g., in N-glycans of various fungi or protists (PE or PC modifications of mannose residues) or of free-living and parasitic nematodes [PC modification of GlcNAc*β*1,2Man motifs (see Figure 1A)]; these conjugates were either printed in a glycan microarray format or converted into BSA neoglycoconjugates, prior to subsequent testing with antibodies and pentraxins. Thereby, in comparison to conjugates such as PC directly conjugated to BSA,[33] these novel reagents better mimic the interaction of components of the innate and adaptive immune systems to glycans carrying zwitterionic modifications than those based on the PC or PE alone. Therefore, these zwitterionic glycosides were tested for binding to either lectins, pentraxins, monoclonal antibodies, or antibodies in sera of infected animals.

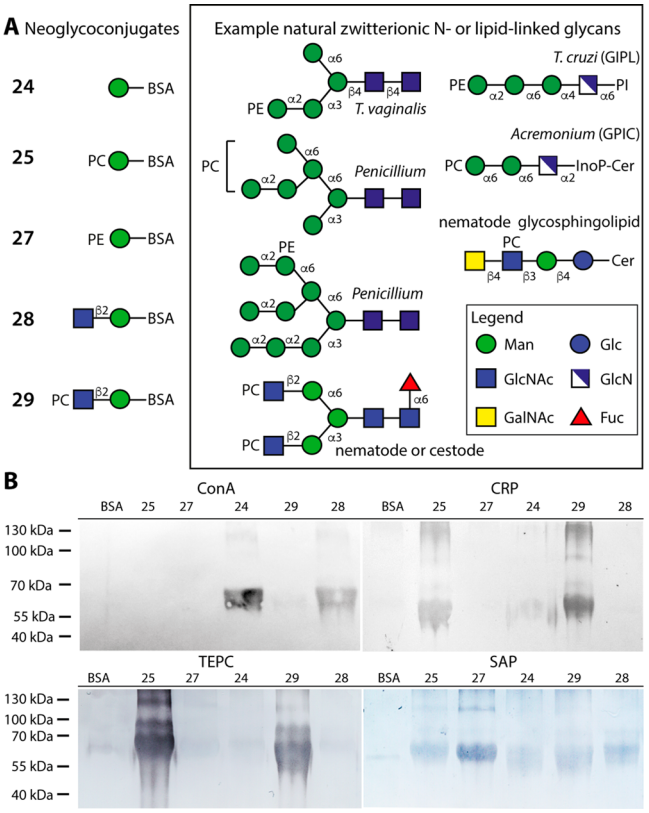

Figure 1. Example zwitterionic glycan structures and Western blotting using neoglycoconjugates. (A) Structures of the three zwitterionic neoglycoconjugates (PE-6Man, PC-6Man, and PC-6GlcNAc*β*1,2-Man) are shown in the Symbol Nomenclature for Glycans format (see also symbols) alongside example natural phosphoethanolamine- and phosphorylcholine-modified glycans from *T. vaginalis* (PE-modified N-glycan), *Penicillium* (PE- and PC-modified N-glycans), *Trypanosoma cruzi* [PE-modified glycosylphosphatidylinositol lipid (GIPL)], *Acremonium* sp. no. 413 [PC-modified glycosylphosphoinositolceramide (GPIC)], nematodes, and cestodes (PC-modified biantennary N-glycan and nematode glycosphingolipid). (B) Five BSA neoglycoconjugates (Man, PC-6Man, PE-6Man, GlcNAc*β*1,2-Man, and PC-6GlcNAc*β*1,2Man; i.e., compounds **24**, **25**, and **27**−**29**, respectively) as well as the native BSA were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis followed by Western blotting with either concanavalin A (ConA), C-reactive protein (CRP), TEPC15, or serum amyloid P (SAP). Coomassie Blue staining and MALDI-TOF MS data for these conjugates are shown in the Supporting Information (page S41).

RESULTS AND DISCUSSION

Preparation of the PC- and PE-Mannoside Ligands and Conjugates. To test the interactions of proteins with zwitterionically modified saccharides, the initial focus was on mimicking PC/PE-Man motifs of fungal and trichomonad glycans. These mannoside ligands were equipped with a 2-(2-azidoethoxy)ethyl spacer group, suitable for coupling to proteins and solid surfaces after formation of the corresponding *ω*-amino group. The known[34] mannoside **1** was first deprotected via Zemplén transesterification to give **2**, followed by reduction of the azido group to provide the non-phosphorylated glycoside **3** as the control ligand in 96% yield (Scheme 1). To address position 6 for selective phosphorylation, tetraol **2** was treated with *t*BDPSCl/imidazole in DMF to afford the 6-*O*-silyl ether derivative **4** in 93% yield. Subsequent benzylation with benzyl bromide/NaH in DMF gave the tri-*O*-benzyl derivative **5** in 80% yield followed by fluoride treatment to produce primary alcohol **6** in 87% yield. Several approaches have been reported for the introduction of phosphorylcholine residues into carbohydrates, capitalizing on phosphoramidite-based coupling reactions or acylation with $POCl_3$ followed by reaction with choline tosylate.[35−39] In addition, PC groups have been generated via reaction of a primary alcohol with 2-chloro-2-oxo-1,3,2-dioxaphospholane followed by ring opening with dry trimethylamine or via formation of a 2-bromoethyl phosphate with subsequent nucleophilic substitution with aqueous Me_3N.[40−43]

In this study, we employed activation of **6** with $POCl_3$ followed by coupling with dry choline tosylate in pyridine; this proved to be straightforward and furnished the zwitterionic phosphodiester **7** in 75% yield. Hydrogenation of **7** had to be performed under strongly acidic conditions to completely remove the benzyl groups and to reduce the terminal azido group. By this approach, the target ligand **8** was obtained in 95% yield.

For the synthesis of the phosphoethanolamine (2-aminoethylphosphate) derivative, Boc protection of the 2-aminoethyl group was required to have access to the terminal amino spacer for conjugation applications, without touching the aminoethylphosphate moiety.[44] The phosphodiester derivative **9** was obtained from the primary alcohol **6** using coupling with benzyl-(2-*tert*-butoxycarbonylaminoethyl)-*N*,*N*-isopropylphosphorodiamidite reagent followed by oxidation with *m*CPBA. Compound **9** was thus obtained as an ∼3:2 diastereoisomeric mixture in 64% yield. Hydrogenation of **9** with 10% Pd-C furnished the target spacer ligand **10** in 92% yield suitable for ensuing conjugation reactions.

Synthesis of GlcNAc-(1→2)-Man Disaccharide Ligands and Conjugates. To test the binding of antibodies or pentraxins to a common motif of nematode and cestode N-glycans, a PC-GlcNAc*β*1,2Man ligand was prepared. For the preparation of the underlying *β*-D-Glc*p*NAc-(1→2)-*α*-D-Man*p*

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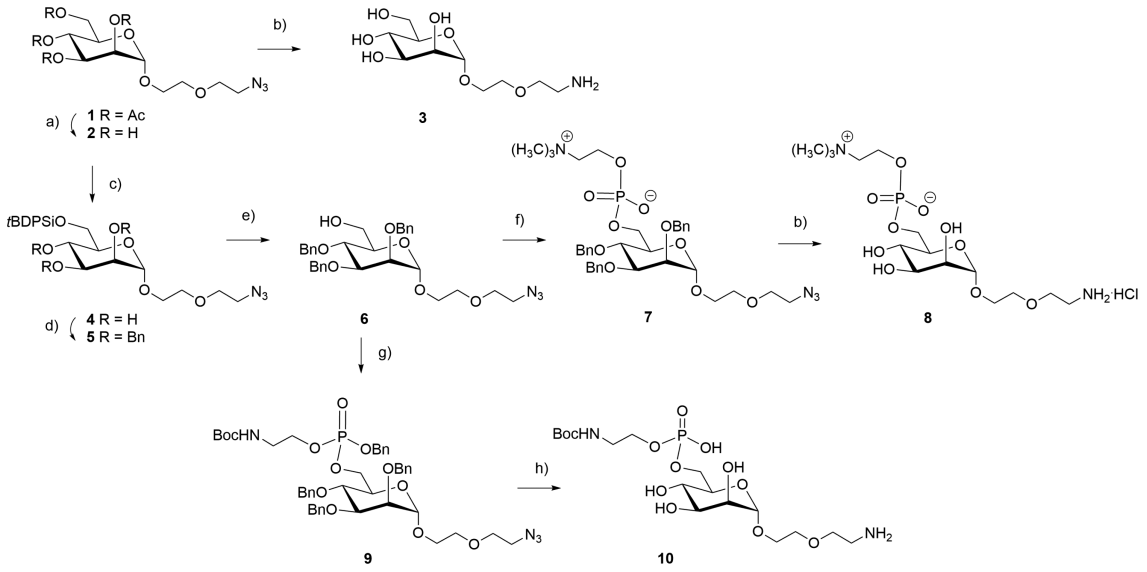
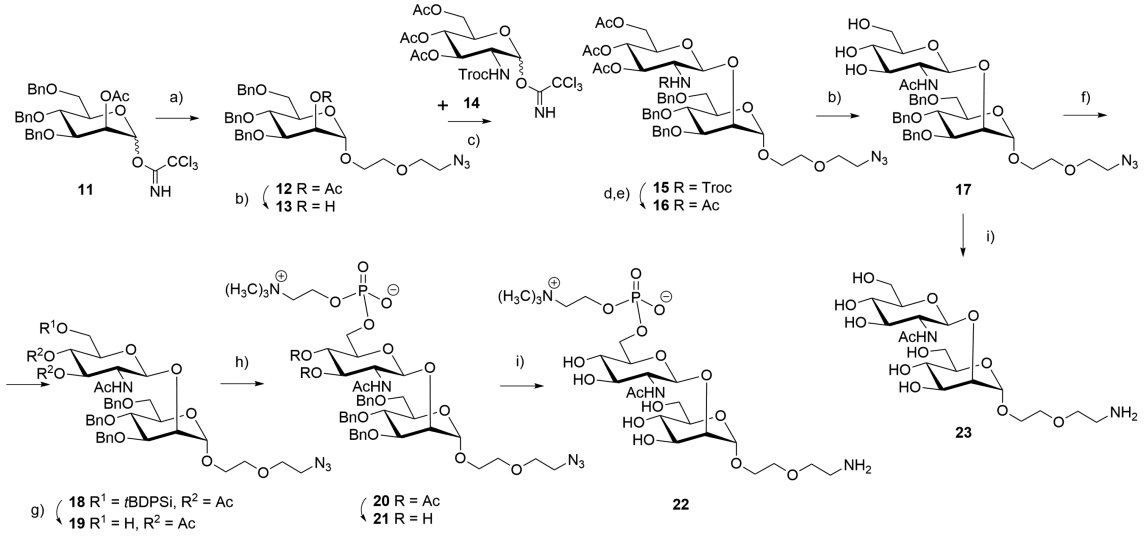
Scheme 1. Synthesis of Spacer Mannosides[a]



[a]Reagents and conditions: (a) 0.1 M NaOMe, MeOH, room temperature (rt), 2 h, 89%; (b) 10% Pd-C, H_2, MeOH, 12 h, rt, 96% for **3**, 10% Pd-C, H_2, 0.06 M HCl in MeOH, 12 h, rt, 95% for **8**; (c) *t*BDPSCl, imidazole, DMF, 93%; (d) BnBr, NaH, DMF, 12 h, rt, 80%; (e) 1 M Bu_4NF, THF, 12 h, rt, 87%; (f) $POCl_3$, Et_3N, 3 Å molecular sieves, DCM, then choline tosylate, pyr, 12 h, rt, 75%; (g) $(iPr)_2NP(OBn)O(CH_2)_2NHBoc$, 1*H*-tetrazole, MeCN, 3 h, rt, then *m*CPBA, DCM, −80 °C to rt, 1 h, 64%; (h) 10% Pd-C, MeOH, 12 h, rt, 92%.

Scheme 2. Synthesis of Disaccharide Ligands[a]



[a]Reagents and conditions: (a) TMSOTf (0.05 equiv), DCM, 4 Å molecular sieves, −40 °C, 5 min, 96%; (b) 0.1 M NaOMe, MeOH, rt, 7 h, 84% for **13** or K_2CO_3, MeOH, rt, 17 h, 98% for **13**, 85% for **17**, 98% for **21**; (c) TMSOTf (0.077 equiv), DCM, 4 Å molecular sieves, −40 °C for 6.5 h, 4 °C for 12 h, 53%; (d) 1 M LiOH, THF; (e) Ac_2O, DMAP, pyr, 15 h, rt, 75% (two steps for **16**); (f) *t*BDPSCl, DIPEA, DMF, then Ac_2O, DMAP, pyr, 2 h, rt, 80%; (g) AcOH, DMF, then 1 M TBAF, THF, 15 h, rt, 62%; (h) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, DCM, 4 Å molecular sieves, 30 min, then choline tosylate, 100 min, then *m*CBPA, 2 h, 49%; (i) 10% Pd-C, H_2, THF/water, 1% AcOH, 2 daus, rt, 64% for **22**, 59% for **23** under microflow conditions.

disaccharide, the 2-*O*-acetyl-protected mannosyl trichloroacetimidate donor **11** was prepared according to the literature.[45] Reaction of **11** with 2-(2-azidoethoxy)-ethanol in the presence of catalytic TMS-triflate and 4 Å molecular sieves in dichloromethane at −40 °C then gave an excellent yield (96%) of *α*-glycoside **12** (Scheme 2).

Deprotection of the 2-*O*-acetyl group under Zemplén conditions afforded the glycosyl acceptor **13** in 84% yield. Better yields (98%) were obtained using K_2CO_3 as the base in methanol. Coupling of the mannoside acceptor **13** with known[46−48] *N*-Troc-protected trichloroacetimidate donor **14** according to Schmidt[49] was carried out at −40 °C in the presence of TMS-triflate as the promoter. The Troc group provided the neighboring group participation to produce the *β*-linked disaccharide in 53% yield. The *β*-anomeric configuration of the GlcN residue of the (1→2)-linked disaccharide **15** was confirmed by the value of the homonuclear coupling constant $J_{1',2'}$ (8.3 Hz). Next, the Troc group had to be exchanged for the *N*-acetyl group without affecting the spacer-terminating azido function. Exchange of the protecting group[50,51] was

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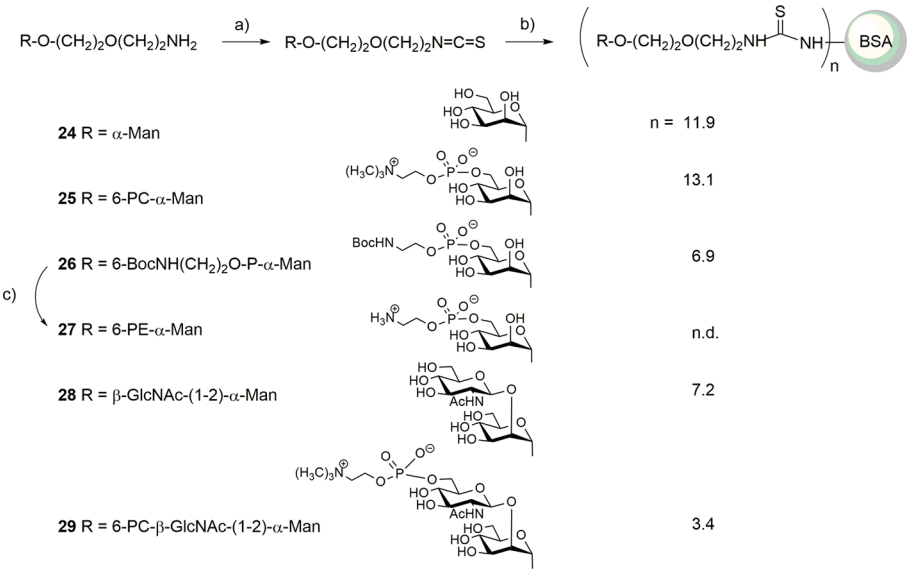
Scheme 3. Conjugation with Bovine Serum Albumin[a]



[a](a) $CSCl_2$, $CHCl_3$, 0.1 M $NaHCO_3$, 3 h, rt; (b) BSA, 0.3 M NaCl, 0.1 M $NaHCO_3$; (c) 0.35 mM TFA, 30 min, rt.

achieved in two steps by first removing all acetyl groups via alkaline hydrolysis with LiOH followed by reacetylation under conventional conditions to produce the *N,O*-acetylated disaccharide derivative **16** in 75% yield. The ester-linked acetates were subsequently removed by treatment with K_2CO_3 in methanol to give **17** in 85% yield, suitable for global deprotection to give disaccharide **23** as well as introduction of the 6-*O*-phosphorylcholine moiety. For the regioselective introduction of the 6-*O*-phosphorylcholine appendage, the primary alcohol group was protected as a temporary *tert*-butyldiphenylsilyl ether followed by acetylation to afford **18** in 80% yield.

Removal of the *t*BDPS group was effected by treatment with TBAF but was accompanied by minor 4′-*O*-acetyl migration to position 6′. Separation of the latter regioisomer from **19** was achieved by chromatography and eventually gave the pure primary alcohol **19** in 62% isolated yield. In contrast to the PC-modified monosaccharide ligand **8**, the introduction of the phosphorylcholine group using $POCl_3$ and choline tosylate could not be accomplished. Therefore, sequential coupling of the alcohol **19** was performed with 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite in dichloromethane in the presence of 1*H*-tetrazole, followed by reaction of the intermediate phosphomonoamidite with choline tosylate; subsequent oxidation with *m*CPBA was successful and gave the phosphodiester derivative **20** in 49% isolated yield after alkaline cleavage of the cyanoethyl protecting group. Removal of the protecting groups was effected by alkaline cleavage of the 3′- and 4′-*O*-acetyl groups using K_2CO_3 in MeOH to afford **21** in near theoretical yield, followed by hydrogenolysis of the benzyl groups and reduction of the terminal azide. Hydrogenation reactions in methanol or ethanol solutions, however, resulted in the simultaneous formation of reductive amination products, which could not be separated from **22**. This side reaction has been noted in the literature.[52] Finally, the hydrogenation was carried out in a THF/water mixed solvent containing 1% acetic acid. Purification of the product was achieved via immobilization of **22** to a cation-exchange resin, followed first by elution with water and eventually by elution with aqueous ammonia to release the product in 64% yield. As a control, the disaccharide ligand **23** was obtained in 59% yield by hydrogenation of **17** on Pd-C under microflow conditions in a THF/water mixture containing 1% acetic acid.

The structures of the PE- and PC-substituted ligands were fully confirmed by assignment of the NMR spectra, which showed $^{31}P/^{13}C$ heteronuclear spin coupling interactions of C-6 and C-5 of the mannoside derivatives **8** and **10** as well as for C-6 and C-5 of the GlcNAc unit in the disaccharide **22**. Additional coupling interactions were also observed for the methylene groups involved in the phosphodiester linkage.

Synthesis of Neoglycoconjugates. Conjugation of ligands **3**, **8**, **10**, **22**, and **23** was achieved via *in situ* activation as an isothiocyanate derivative by reaction with thiophosgene followed by reaction with bovine serum albumin (Scheme 3).[53] The neoglycoconjugates **24**−**29** were obtained by exhaustive dialysis. Acidic cleavage of the Boc group was first elaborated for the model compound **10**. Cleavage of the Boc group using aqueous 1.2 mM TFA at room temperature led to a complete removal of the Boc group within the 10 min reaction time. The reaction was continued for 15 h, and TLC monitoring confirmed that the PE group was still intact. Milder conditions (0.35 mM TFA, 30 min reaction time) were then chosen for hydrolyzing the Boc group of the ligands in BSA conjugate **26** to give **27**. The ligand:protein ratios of the glycoconjugates were assessed by MALDI-TOF MS (see the Supporting Information, page S41) and gave 11.9 for **24**, 13.1 for **25**, 6.9 for **26**, 7.2 for **28**, and 3.4 for **29**. Cleavage of the Boc group of conjugate **26** to give the final PE conjugate **27** was supported by MALDI-TOF MS data, indicating a shift of the average molecular mass from 70.2 to 70.0 kDa.

Western Blotting and Microarray Experiments. The BSA neoglycoconjugates were then tested for binding to a monoclonal antibody (TEPC15) known for its ability to bind (i) *Pneumococcus* C-polysaccharide as well as various other phosphorylcholine-containing glycoconjugates[29] and (ii) the human pentraxins C-reactive protein and serum amyloid P,[30−32] which recognize phosphorylcholine and/or phosphoethanolamine (Figure 1B). TEPC15 bound both PC-Man and

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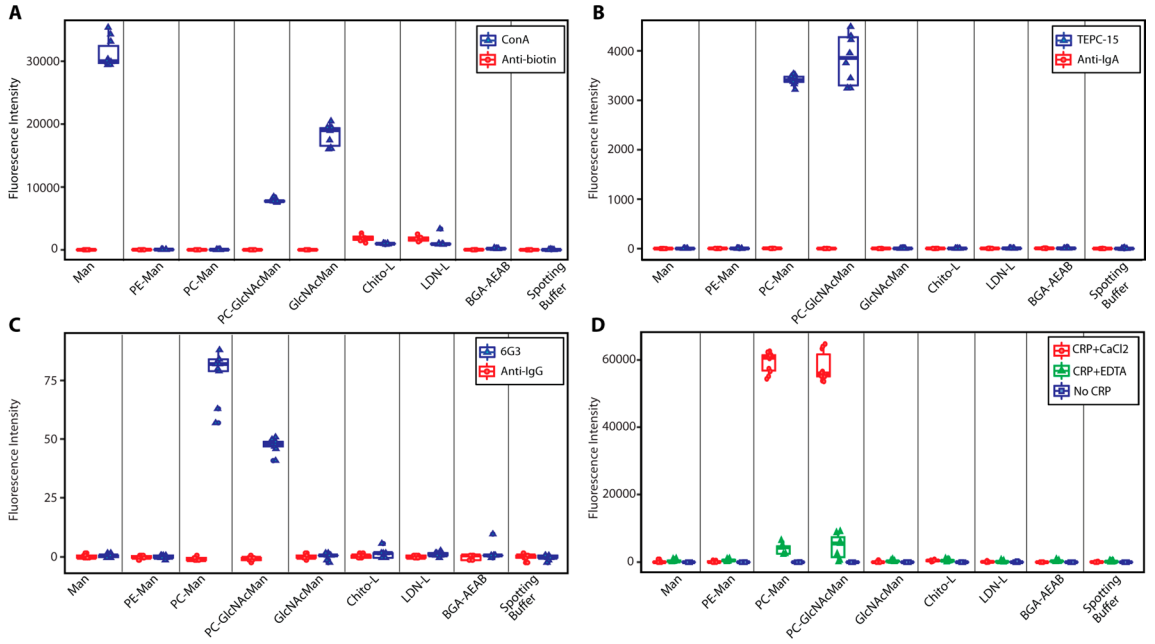

Figure 2. Glycan array data for binding to concanavalin A, anti-phosphorylcholine antibodies, and human C-reactive protein. (A) Array data for binding of the biotinylated lectin concanavalin A (10 μg/mL; detection with anti-biotin) to synthetic compounds **3** (Man), **8** (PC-Man), **10** (in deprotected form; PE-Man) **22** (PC-GlcNAcβ1,2Man), and **23** (GlcNAcβ1,2Man); previously synthesized aminooxy conjugates of chitobiose (Chito-L) and LacdiNAc (LDN-L) as well as 2-amino-*N*-(2-aminoethyl)-benzamide-labeled blood group A (BGA-AEAB) were used as controls (40 fmol per spot). ConA strongly recognizes the mannose compound; however, this interaction is completely blocked by the zwitterionic modifications phosphoethanolamine (PE) or phosphorylcholine (PC), whereas the level of binding to GlcNAcβ1,2Man was reduced by ~60% if the PC modification was present. No binding to Con A was detected for chitobiose, LacdiNAc, or blood group A; neither AAL, WGA, RCA-I, GSL-II, nor LCA recognized any of the compounds (data not shown). (B) The mouse myeloma IgA anti-PC TEPC 15 antibody (detection with fluorescent anti-mouse IgA) binds specifically to PC-modified compounds. (C) A similar binding pattern could be detected for the anti-PC 6G3 antibody (detection with fluorescent anti-mouse IgG, which is cross-reactive to rat antibodies), but the binding was weaker as judged by the lower fluorescence intensity. (D) Human C-reactive protein (detected with anti-CRP, followed by fluorescent anti-mouse IgG) binds specifically and in a calcium-dependent manner, as shown by comparison to incubation in the presence of 5 mM EDTA, to the PC-modified synthetic compounds. The charts represent the uncorrected mean fluorescence value with standard deviations (n = 10 spots) as well as a negative control (spotting buffer). No unspecific binding to anti-biotin, anti-mouse IgA Alexa Fluor 647, or anti-CRP antibody was detected.

PC-GlcNAcβ1,2Man, but not the Man-, PE-Man-, and GlcNAcβ1,2Man-BSA conjugates. On the other hand, C-reactive protein recognized best the PC-GlcNAcβ1,2Man conjugate, but the PC-Man-BSA to a lesser extent, while serum amyloid P only significantly bound PE-Man-BSA, while ConA did not bind the PC/PE-modified conjugates.

For the microarray experiments, concentrations of compounds **3**, **8**, **10**, **22**, and **23** were first normalized by quantification using HILIC/ELSD (see the Supporting Information, pages S13 and S14) to ensure that equimolar amounts of the 2-(2-aminoethoxy)ethyl saccharides were printed before assessing their binding to plant lectins. Similarly to the blotting data, it could be shown that the interaction of concanavalin A with Man was abolished or that with GlcNAcβ1,2Man decreased when the Man or GlcNAc was substituted with either PE or PC, respectively (Figure 2A). No binding to five other tested lectins was observed [AAL, WGA, RCA-I, GSL-II, and LCA (data not shown)].

The microarrays were then also tested with human C-reactive protein[30] as well as two monoclonal anti-phosphorylcholine antibodies: the aforementioned murine TEPC15[29] as well as the 6G3 derived from rats infected with *Trichinella*.[54] Although the absolute recorded intensities differed widely, the pattern of binding for all three was identical in that only PC-Man and PC-GlcNAcβ1,2Man were recognized in a specific manner, whereby the binding to C-reactive protein as detected by anti-CRP binding was, as expected, dependent on the presence of Ca(II) ions; however, in this format, testing arrays with the combination of serum amyloid P and anti-SAP showed nonspecific high background values (data not shown).

Next, the five immobilized 2-(2-aminoethoxy)ethyl saccharides were tested with sera from one control and two nematode-infected dogs (naturally infected with *Dirofilaria immitis* microfilariae), whereby both IgM and IgG reactivities were assessed (Figure 3A,B). As part of a previous study, we concluded that dogs had "natural" antibodies to chitobiose regardless of the infection status,[55] a result that appears to be reproducible. Here, we now observe significant binding to especially PC-Man (an epitope found in fungi) and, to a lesser extent, PC-GlcNAcβ1,2Man (an epitope known from nematodes), even with the sera of the control dog. Interestingly, when array experiments were performed with native N-glycans from *Dirofilaria*, it was the pool of glycans from which phosphorylcholine had been removed (using hydrofluoric acid) to which binding of dog antibodies was stronger than the native ones.[55] In contrast, one of two *Ascaris*-infected pigs and two of three *Oesophagostomum*-infected pigs displayed IgG and IgM reactivity with either of the phosphorylcholine-modified compounds, with the highest fluorescence intensities recorded for binding of IgM for one *Ascaris*-infected animal; interestingly, IgG recognized only the PC-GlcNAcβ1,2Man compound, while IgM bound both the PC-GlcNAcβ1,2Man and PC-Man conjugates (Figure 3C–F). In the case of both

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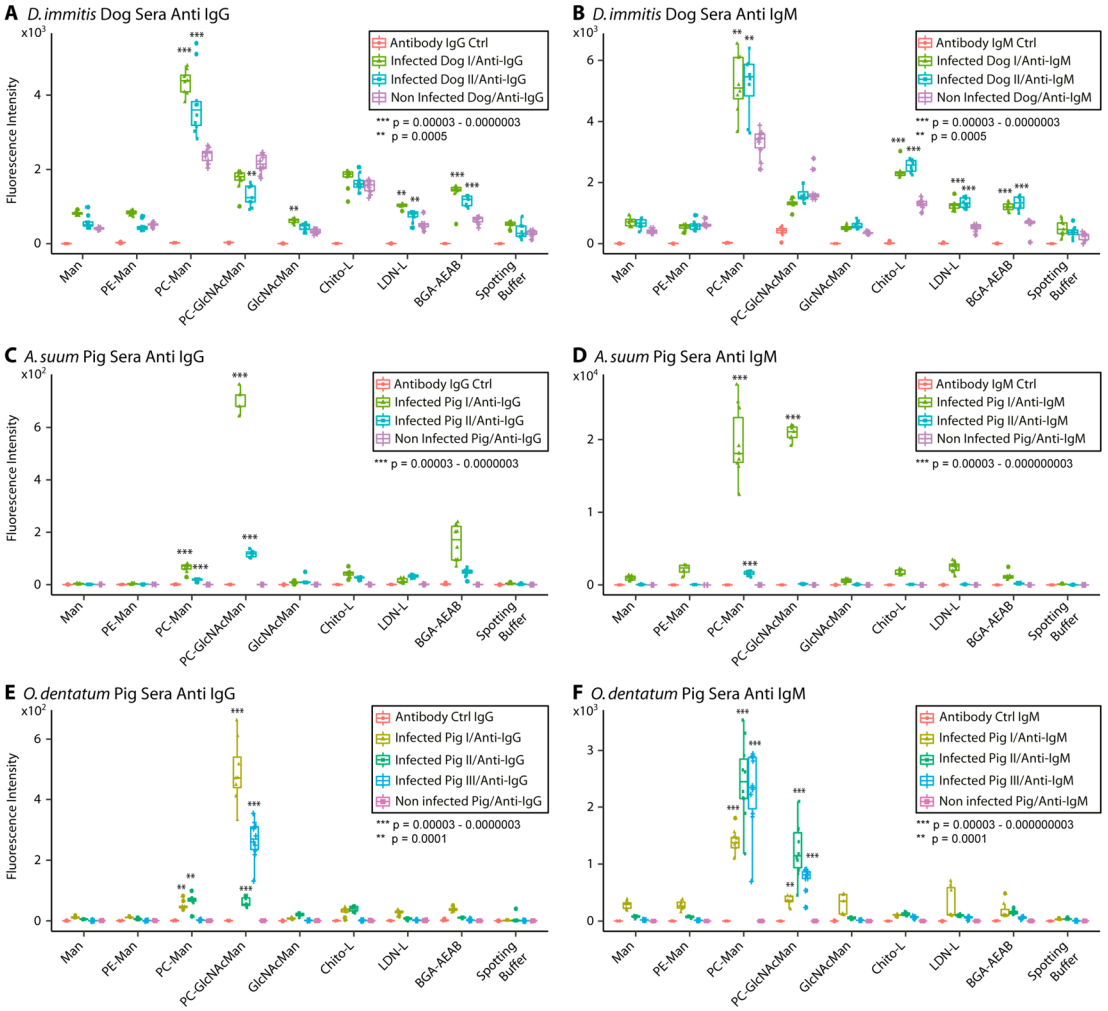

Figure 3. Binding of dog and pig IgG and IgM antibodies to synthetic glycans. (A and B) The binding IgG and IgM antibodies in three different dog sera (1:250 diluted; non-infected dog, *Dirofilaria*-infected dog I, and *Dirofilaria*-infected dog II) were tested with respect to synthetic compounds. In all three dog sera, IgG as well as IgM antibodies that strongly interact with PC-modified compounds (**8** and **22**) are found; especially sera from both dogs infected with *D. immitis* show statistically significantly larger amounts of antibodies toward the PC-Man compound but not the PC-GlcNAcMan. In all three sera, there was no significant binding detected to mannose (Man; **3**), phosphoethanolamine-mannose (PE-Man; **10**), or GlcNAcβ1,2Man (GlcNAcMan; **23**). As already shown by Martini et al.,[55] dog antibodies bind the chitobiose (Chito-L) and LacdiNAc (LDN-L) compounds. In this array also, the blood group A antigen was included, which showed some interaction with dog IgG as well as IgM antibodies. (C–F) Similar experiments were also performed with sera of pigs infected with *Ascaris suum* or *Oesophagostomum dentatum* and show statistically significant interactions of IgM and IgG from infected animals with PC-GlcNAcβ1,2Man (**22**) and IgM also to PC-Man (**8**) as compared to non-infected animals.

control pigs, there is no detectable reactivity to phosphorylcholine.

Although the differences between the two *Dirofilaria*-infected and one non-infected dog for binding to PC-Man are significant ($p \leq 0.0005$), the binding to PC-GlcNAcMan for all three sera is similar; thus, it is possible that the anti-phosphorylcholine antibodies in dog sera are "natural", i.e., result from exposure to environmental bacteria or fungi, and are not related to the infection with the parasitic nematode. This could agree with the concept that nematodes are in part "invisible" to the immune system in some host/parasite systems,[56] despite being immunogenic in other cases.[57] It is also compatible with the aforementioned results with C-reactive protein, as the interaction of this pentraxin with the PC on N-glycans has been proposed not to result in activation of the complement cascade despite binding to C1q, due to the inherent flexibility of N-glycans as compared to bacterial polysaccharides.[58]

Conclusion. The synthesis of glycans modified with phosphorylcholine and phosphoethanolamine is inherently challenging; there are few examples of their chemical construction and none targeted at the production of glycan arrays. Using a phosphoramidite-based approach or activation with phosphorus oxychloride as previously employed to construct PC-Gal-based glycodendrimers akin to annelid glycolipids or PE-heptosyl-modified fragments of bacterial lipopolysaccharides, we have succeeded in synthesizing zwitterionically substituted mono- and disaccharides. The ligands had been equipped with spacer groups containing terminal amino groups for ready conversion into BSA conjugates and glycan arrays. Biologically significant is the fact that the zwitterionically modified Man- or GlcNAcβ1,2-Man-mimicking motifs of N- and lipid-linked glycans of various lower eukaryotes were shown to interact with antibodies, lectins, and pentraxins. Reagents of this type have potential value as "molecular diagnostics" for parasite

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infections, in vaccination trials to combat helminth infections, or as tools to investigate the innate immune system or mechanisms of immunomodulation.

METHODS

Chemical Syntheses. Descriptions of the syntheses of the 2-(2-aminoethoxy)ethyl glycosides **3**, **8**, **10**, **22**, and **23** as well as of the BSA conjugates **24**, **25**, and **27**−**29** are given in the Supporting Information.

Western Blotting. BSA conjugates (**24**, **25**, and **27**−**29**) were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane via semidry blotting. This membrane was then incubated with blocking buffer [Tris-buffered saline containing Tween 20; TTBS supplemented with 0.5% (w/v) BSA] for 1 h, prior to incubation with either biotinylated concanavalin A (Vector), TEPC15 primary antibody (Sigma), human C-reactive protein (CRP; MPBio), or human serum amyloid P (SAP; Merck) for 45 min. Thereafter, the membrane was washed three times with TTBS before incubation with either alkaline phosphatase-conjugated anti-biotin (Sigma), alkaline phosphatase-conjugated anti-mouse IgA secondary antibody (Sigma), rabbit anti-CRP, or rabbit anti-SAP for 45 min; in the case of the detection of pentraxins, alkaline phosphatase- or peroxidase-conjugated anti-rabbit IgG was used. The membrane was again washed three times with TTBS and then stained with either SigmaFast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium or SigmaFast 3,3-diaminobenzidine in the dark. (All steps were performed at room temperature; see the Supporting Information, page S42, for dilutions.)

Glycan Array-Based Lectin, Pentraxin, and Antibody Screening. Solutions of 2-(2-aminoethoxy)ethyl glycosides (**3**, **8**, **10**, **22**, and **23**; ∼1 mg mL^{-1}) were quantified by HILIC (Merck ZIC; 150 mm × 4.6 mm, 3.5 μm) using a Shimadzu HPLC-MS-ELSD system and a gradient from 95% to 40% aqueous acetonitrile. ELSD peak areas were used to quantify ligand solutions; for the calibration curves, xylotriose, xylotetraose, and α-cyclodextrin were used as reference materials (see the Supporting Information, page S42). Ligand solutions were then diluted with spotting buffer [300 mM sodium phosphate (pH 7.5) and 0.005% Tween 20] to a final concentration of 50 μM and then spotted (n = 10) by noncontact printing (Scienion Flexarrayer S1) onto NHS-derivatized Nexterion H glass slides (Schott). After hybridization for 16 h, slides were blocked [50 mM ethanolamine in 50 mM sodium borate (pH 9.0)] for 1 h at room temperature and washed serially with TSM [20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$] with 0.05% (w/v) Tween 20 (TSM wash buffer, TSMWB), TSM alone, and water prior to drying and storage at −20 °C before use.[59] The slides were incubated with dilutions in TSMWB of either one control and two *Dirofilaria*-infected dog sera,[55] *Ascaris*- or *Oesophagostomum*-infected pig sera, biotinylated Con A, or other lectins (Vector Laboratories), murine IgA monoclonal TEPC15 (Sigma), monoclonal rat anti-phosphorylcholine antibody 6G3 (raised in *Trichinella*-infected rats; provided by J. Appleton),[54] or natural human C-reactive protein (MPBio) followed by the relevant secondary and/or tertiary antibodies (see the Supporting Information for concentrations). After the first incubation, the slides were washed serially with TSMWB (i.e., Tris-buffered saline supplemented with $CaCl_2$, $MgCl_2$, and Tween), TSM, and water. For detection of lectins or TEPC15, the directly labeled anti-biotin FITC conjugate (Sigma), anti-mouse IgA FITC conjugate (Invitrogen), or anti-mouse IgG AF 647 conjugate (Invitrogen) was added in binding buffer and the slides were again washed as described above. For detection of CRP or antibodies in sera, unconjugated murine anti-CRP (biotechne), rabbit anti-dog, or rabbit anti-pig IgG/IgM (Sigma) was used; the slides were washed again as described above and dried prior to adding the tertiary antibody anti-mouse IgG AF 647 conjugate and anti-rabbit AF 647 conjugate (Invitrogen). Slides were scanned with an Agilent G2565CA Microarray Scanner [multiple photomultiplier tube (PMT) gain values from 10 to 100%], and raw image files were analyzed by GenePix 7 software. The negative controls (spotting buffer or no primary reagent) show fluorescence due to either the labels themselves or nonspecific binding of the fluorescent secondary antibodies. As controls for lectin and antibody binding, additional synthetic compounds were included in the array: chitobiose linker (Chito-L), LacdiNAc linker (LDN-L), and AEAB [2-amino-*N*-(2-aminoethyl)-benzamide]-labeled blood group A tetrasaccharide (BGA-AEAB). The chitobiose and LacdiNAc compounds were synthesized on an aminoxy-based linker as previously described.[55,60] The fluorescence values (green for FITC and red for Alexa Fluor 647) were used to calculate the mean and standard deviation from all 10 spots; data were analyzed for significant differences of the means by Welch's t test using the R progamming language for statistical computing, whereby data were grouped by chemoenzymatically synthesized compounds and test samples. Plots were visualized using the ggboxplot function of the ggplot2 library.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.9b00794.

General synthetic methods; syntheses of compounds and BSA conjugates; further information regarding antibodies, pentraxins, and sera; and HPLC, NMR, and MALDI-TOF MS data (PDF)

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Notes

The authors declare no competing financial interest.

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