ACS Infectious Diseases

Article

pubs.acs.org/journal/aidcbc

A Synthetic Disaccharide Analogue from *Neisseria meningitidis* A Capsular Polysaccharide Stimulates Immune Cell Responses and Induces Immunoglobulin G (IgG) Production in Mice When Protein-Conjugated

Silvia Fallarini,[†] Benedetta Buzzi,[‡] Sara Giovarruscio,[†] Laura Polito,[§] Giulia Brogioni,[⊥] Marta Tontini,[⊥] Francesco Berti,[⊥] Roberto Adamo,[⊥] Luigi Lay,[‡,∥] and Grazia Lombardi*[,†,∥]

[†]Department of Pharmaceutical Sciences, University of "Piemonte Orientale, A. Avogadro", Largo Donegani 2, 28100 Novara, Italy

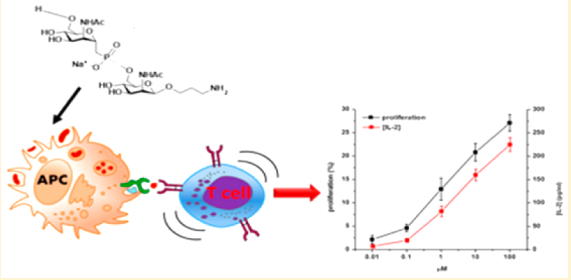
[‡]Department of Chemistry and CRC "Materiali Polimerici" (LaMPo), University of Milan, Via Golgi 19, 20133 Milan, Italy

[§]Institute of Molecular Science and Technologies, ISTM-CNR, Via G. Fantoli 16/15, 20138 Milan, Italy

[⊥]Novartis Vaccines and Diagnostics, Via Fiorentina 10, 53100 Siena, Italy

Supporting Information

ABSTRACT: Some new phosphonoester-linked oligomers, stabilized analogues of the corresponding phosphate-bridged oligomers of *Neisseria meningitidis* A (MenA) capsular polysaccharide (CPS), were conjugated to human serum albumin (HSA), as a protein carrier model, and studied for immunological activities. We determined (i) in vitro, their biocompatibility (CAM test) and activity in inducing both T cell proliferation (CFSE method) and IL-2 release (ELISA), and (ii) in vivo, their ability to stimulate specific IgG antibody production (ELISA). All HSA-conjugated compounds induce T cell proliferation (40% of proliferation at 10^2 μM), whereas only the phosphonodisaccharide was effective (28% of proliferation at 10^2 μM) among the unconjugated forms. IL-2 release confirmed these results. In addition, the HSA-conjugated showed in vivo the capacity of eliciting the production of specific IgG antibodies. In conclusion, we obtained novel biocompatible, water-stable, and immunoactive MenA CPS analogues. A short disaccharide fragment showed the unusual behavior of triggering T cell proliferation in vitro.



KEYWORDS: *vaccines, Neisseria meningitidis, zwitterionic polysaccharides, glycoconjugates, immune response*

Bacterial meningitis is a severe infection with a high mortality rate (over 135,000 deaths/year) and morbidity (20% of survivors with minor or major sequelae).[1] Among the variety of pathogens in humans, *Neisseria meningitidis* (Men), an aerobic diplococcal Gram-negative microorganism endowed with a polysaccharide capsule (CPS), causes the majority of this disease. On the basis of the chemical and antigenic properties of the Men CPS, 13 distinct serogroups of Men have been so far classified. Groups A, B, C, Y, W, and X account for 90% of global diseases,[2] whereas serogroup A (MenA) seems to be prevalent in a region of Africa known as the "meningitis belt". This hyperendemic region includes 22 countries of sub-Saharan Africa, and it is characterized by seasonal epidemics of meningococcal disease, approximately every 8−10 years.[3,4]

Prevention remains the most effective way to protect people against meningitis, and unmodified CPS-based vaccines have been available since 1970 to prevent disease and to stop epidemics. It is widely accepted, however, that carbohydrates are essentially T cell-independent antigens, stimulating B cell activation and specific immunoglobulin M (IgM) production, but incapable of inducing T cell priming, class switching from IgM to IgG antibodies, and immunological memory.[5] The immunogenicity and clinical efficacy of CPS vaccines are well established: the current WHO position paper on the meningococcal vaccines states that in schoolchildren and adults a single dose of group A CPS vaccine provides protection for at least 3 years, whereas in children under 4 years of age the antibody response declines rapidly over the first 2−3 years.[6] In addition, these vaccines are not easy to handle and not fully effective, as demonstrated by the meningococcal epidemics still occurring in Africa.[7]

Exceptions among carbohydrate immunogens are some zwitterionic polysaccharides (ZPS), that are, bacterial polysaccharides containing both positive and negative charge centers within a repeating unit. ZPS behave like traditional T cell-dependent antigens, triggering in vitro antigen presenting cells (APC) and $CD4^+$ T cells through TLR2- and MHC II-dependent pathways.[8−12]

Received: June 10, 2015
Published: August 10, 2015

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

The weak immunogenicity of saccharide antigens can be strongly enhanced by conjugation to a variety of immunogenic carrier proteins.[13] At the end of 20th century some tetravalent (groups A, C, W, and Y) meningococcal conjugate vaccines (Menactra, Menveo, and Nimenrix) were introduced in the developed world. These vaccines are immunogenic in infancy, provide long-lasting protection, reduce the rate of carriage leading to herd immunity, and induce a booster response to subsequent doses.[14] As a result of the Meningitis Vaccine Project, a low-cost monovalent serogroup A−tetanus toxoid (TT) conjugate vaccine (MenAfriVac), specifically designed for African infections, was licensed.[15] This vaccination campaign led to a significant drop in the disease with a remarkable decrease in the percentage of patient deaths.[16]

The MenA CPS structure consists of (1→6)-linked 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate repeating units, with 80% of O-acetylation at 3-OH.[17,18] This polysaccharide tends to hydrolyze significantly in water because of the intrinsic chemical lability of the phosphodiester linkages involving the anomeric position of each repeating unit, further increased by the presence of the axial *N*-acetyl group at C-2 of mannosamine, which can assist the breaking of the C1−O1 bond with subsequent removal of a phosphomonoester group.[19,20] The successful delivery of effective anti-MenA conjugate vaccines in liquid formulation is therefore strictly dependent on the efficiency of the "cold chain" system in the specific country, that is, the maintenance of an optimal temperature during transport, storage, and handling.

Taking into account all of these concepts, the design of new MenA CPS analogues, stable in liquid formulation, resistant to temperature stress, and endowed with immunological properties comparable to those of the natural CPS, remains of great importance.

We recently reported the synthesis of phosphonoester-linked oligomers (compounds **1**−**3**, Figure 1) of MenA CPS as

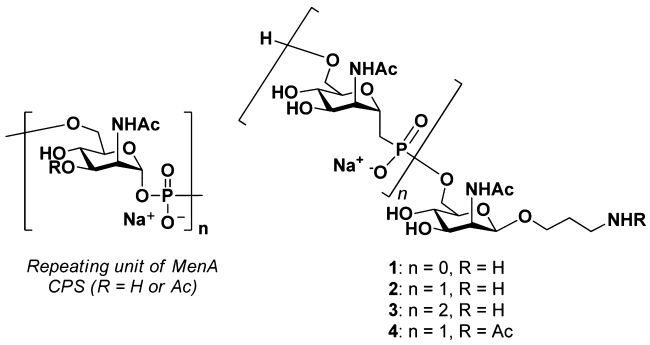


Figure 1. Structures of the repeating unit of MenA CPS and phosphono oligomers **1**−**4**.

stabilized analogues of the corresponding phosphate-bridged natural oligomers. In these compounds, containing an aminopropyl spacer suitable for protein conjugation, the replacement of the anomeric oxygen with a methylene group led to improved stability in water. The newly synthesized compounds were biologically active, because they are recognized by a human anti-MenA polyclonal antibody with EC_{50} values on the order of 10^{-3} mg/mL and efficacies dependent on the chain length of saccharide molecules.[21]

Here, we first conjugated the phosphonoester-linked oligomers to human serum albumin (HSA), as a model of protein carrier, and then we studied the biocompatibility and ability of compounds **1**, **2**, and **3** and their conjugated counterparts **8**, **9**, and **10** to stimulate T cells (proliferation and IL-2 release) in vitro. The immunoactive compounds were then injected in mice to study their ability to stimulate the production of specific IgG antibodies, a hallmark of T cell-dependent immune responses.

RESULTS AND DISCUSSION

Synthesis of Neo-glycoconjugates. The conjugation of oligomers **1**−**3** to HSA was achieved by means of the squarate ester coupling protocol.[22−24] Squaric acid esters, in fact, can react selectively with primary or secondary amines under mild conditions, in the presence of carboxy and alcoholic (or phenolic) hydroxy groups. Squaric acid ester therefore displays enormous synthetic benefits as a molecular bridge for connecting two amino compounds. Thus, compounds **1**−**3** were transformed into the corresponding squarate ester amide derivatives **5**−**7**, as illustrated in Scheme 1. The subsequent conjugation to HSA was successfully carried out by incubation of each activated intermediate with the protein in a borate buffer solution (pH 9) at room temperature. The progress of each conjugation reaction was monitored by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).[25]

Each conjugation was allowed to proceed until a significant change of the molecular weight was no longer observed, hence assuming that no more oligosaccharide units could be incorporated in the carrier. In this way we obtained glycoconjugates **8a**, **9a**, and **10a** with the maximal saccharide/protein molar ratio (full-sugar loading) attainable in our experimental conditions (Table 1; see the Supporting Information for MALDI MS spectra). We then planned the synthesis of a second series of glycoconjugates (**8b**, **9b**, and **10b**, Table 1) with a saccharide/protein molar ratio corresponding to approximately half of the value achieved in our first set of experiments (half-sugar loading).

Initially, we plotted the progress of each conjugation reaction as saccharide/protein molar ratio versus the reaction time so that we could extrapolate the incubation time needed to obtain the new series of glycoconjugates (see the Supporting Information). Accordingly, the conjugation reactions with activated intermediates **5**−**7** were stopped after 6, 2.5, and 2 h, respectively. The MALDI MS analysis confirmed that the experimental saccharide/protein molar ratios of glycoconjugates **8b**−**10b** were in very good agreement with the predicted values (see the Supporting Information), that are, very close to half of the maximal values (see Table 1).

In Vitro Evaluation of the Biological Activity of the Synthetic MenA Analogues and Their Protein-Conjugated Derivatives. *ELISA Determination.* To determine whether the new synthetic compounds were mimicking fragments of the native repeating units, the activities of **1**−**3**[21] were compared with those of the corresponding conjugates (**8**−**10**). We evaluated the ability of each compound to inhibit the binding between the native MenA CPS, coated onto plates, and the mouse anti-MenA polyclonal antibody in a classical competitive ELISA. This murine serum was obtained after three immunizations with a MenA-CRM_{197} conjugate (commercial vaccine).[20]

The inhibition curves, shown in Figure 2, allowed the calculation of the maximum inhibition that each compound elicited in this system (relative efficacy), whereas the concentration that produces 50% of the maximum inhibition

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

Scheme 1. Conjugation of Phosphono Oligomers to HSA

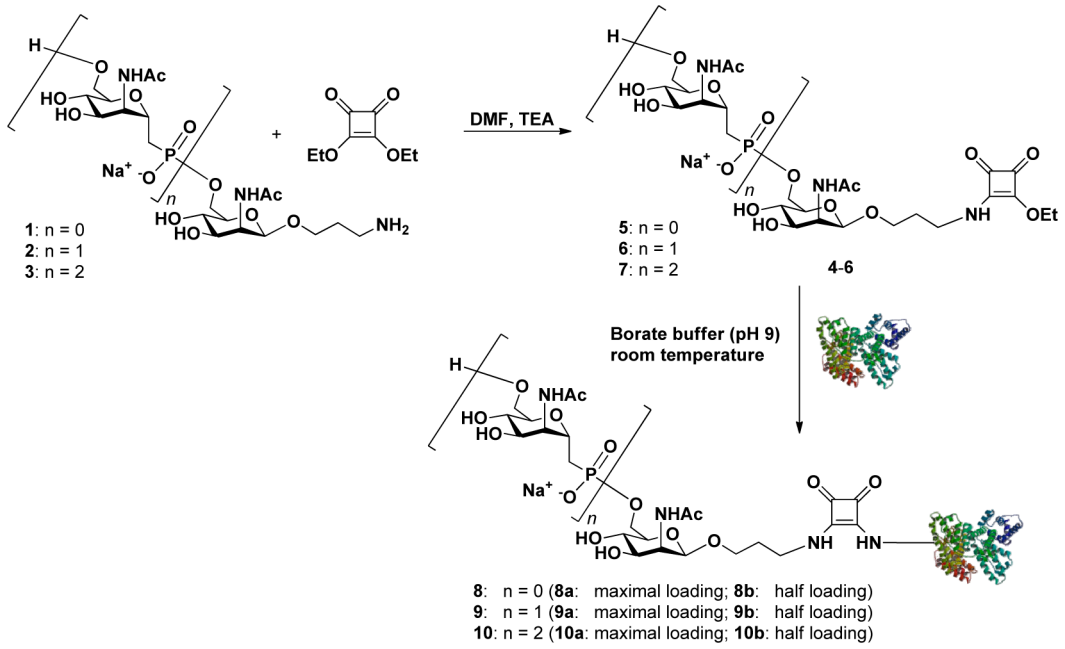


Table 1. Sugar Loading on HSA of the Synthesized Glycoconjugates

HSA/oligomer conjugation	reaction time	sugar loading
monomer 1		
compd **8b**	6 h	12.8
compd **8a**	5 days	26.5
dimer 2		
compd **9b**	2.5 h	8.4
compd **9a**	7 days	20.2
trimer 3		
compd **10b**	2 h	6.5
compd **10a**	6 days	14.2

Table 2. Results of the Competitive ELISA

compd	IC_{50} ± SEM (mg/mL)	max inhibition[a] (%)
MenA	$(3.58 \pm 0.3) \times 10^{-5}$	100 ± 3
1	$(4.36 \pm 0.2) \times 10^{-1}$	33 ± 3
8b	$(2.21 \pm 0.3) \times 10^{-4}$	49 ± 3
8a	$(2.54 \pm 0.2) \times 10^{-4}$	57 ± 3
2	$(3.21 \pm 0.9) \times 10^{-1}$	32 ± 3
9b	$(4.72 \pm 0.5) \times 10^{-4}$	52 ± 3
9a	$(1.03 \pm 0.7) \times 10^{-4}$	65 ± 3
3	$(2.35 \pm 0.1) \times 10^{-1}$	34 ± 3
10b	$(1.61 \pm 0.2) \times 10^{-4}$	58 ± 3
10a	$(1.11 \pm 0.7) \times 10^{-4}$	67 ± 3

[a]Measured at 1 mg/mL.

(IC_{50}) was taken as an indirect index of its relative potency (Table 2).

Analysis of the results showed that all of the synthesized compounds were recognized by the polyclonal anti-MenA serum: the native MenA CPS, as expected, exhibited the highest potency (IC_{50} = 3.58 × 10^{-5} mg/mL) and efficacy (100% of inhibition at 10^{-2} mg/mL). The HSA-conjugated compounds showed both a higher potency (IC_{50} values at 10^{-4} mg/mL) and a greater efficacy (50% of inhibition at 10^{0} mg/mL) than

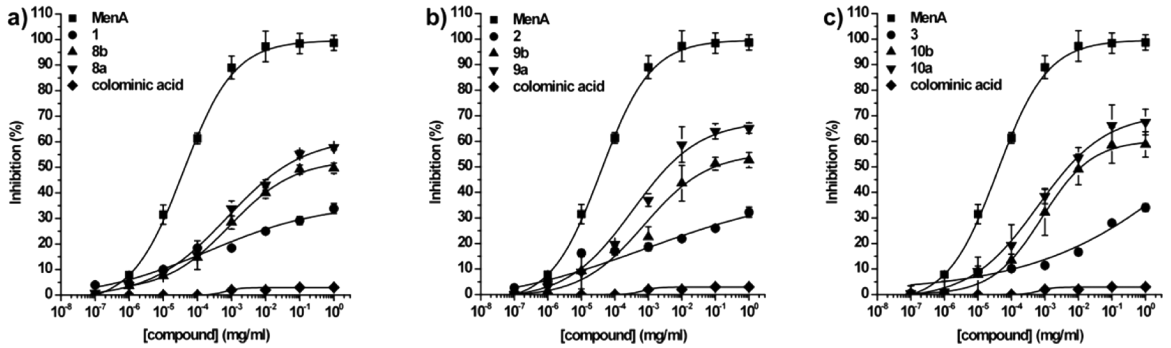


Figure 2. Compounds' ability to bind to specific anti-MenA antibody: (a) HSA-conjugated monosaccharides **8b** and **8a** and unconjugated monosaccharide **1**; (b) HSA-conjugated disaccharides **9b** and **9a** and unconjugated disaccharide **2**; (c) HSA-conjugated trisaccharides **10b** and **10a** and unconjugated trisaccharide **3**. Concentration−response curves of compounds are shown for the inhibition of the binding between MenA, coated onto the plates, and the anti-MenA mouse polyclonal antibody evaluated by competitive ELISA. The data represent the mean ± SEM of five experiments run in triplicate, and they reach statistical significance ($P \leq 0.05$) versus the negative control (colominic acid) starting from 10^{-4} mg/mL.

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

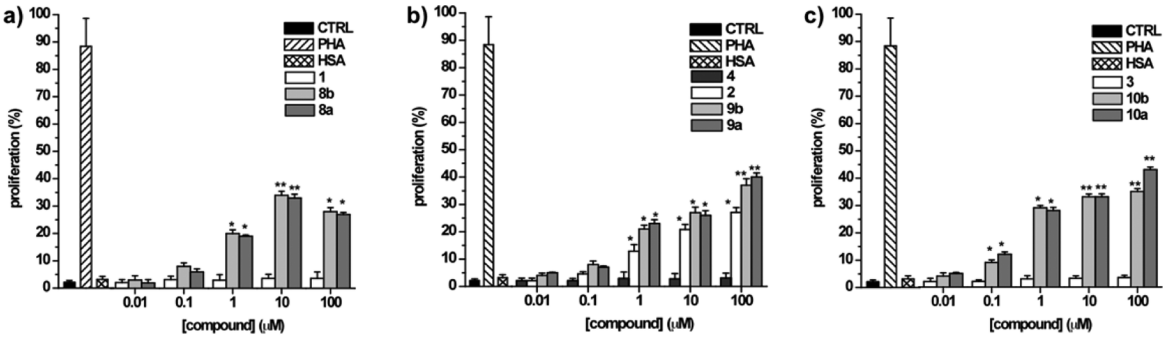


Figure 3. Effects of tested compounds on T cell proliferation: (a) HSA-conjugated monosaccharides **8b** and **8a** and unconjugated monosaccharide **1**; (b) HSA-conjugated disaccharides **9b** and **9a** and unconjugated disaccharides **2** and **4**; (c) HSA-conjugated trisaccharides **10b** and **10a** and unconjugated trisaccharide **3**. T-cell proliferation was analyzed by FACS and expressed as mean percentage ± SEM of proliferated T cells. Cells treated with 1 mg/mL phytohemeagglutinin (PHA), a widely used mitogen able to trigger cell division in T-lymphocytes, were considered as positive controls, whereas HSA was used as protein control and compound-untreated cells (CTRL) as negative controls. (∗)$P < 0.05$ and (∗∗) $P < 0.01$ versus compound-untreated controls.

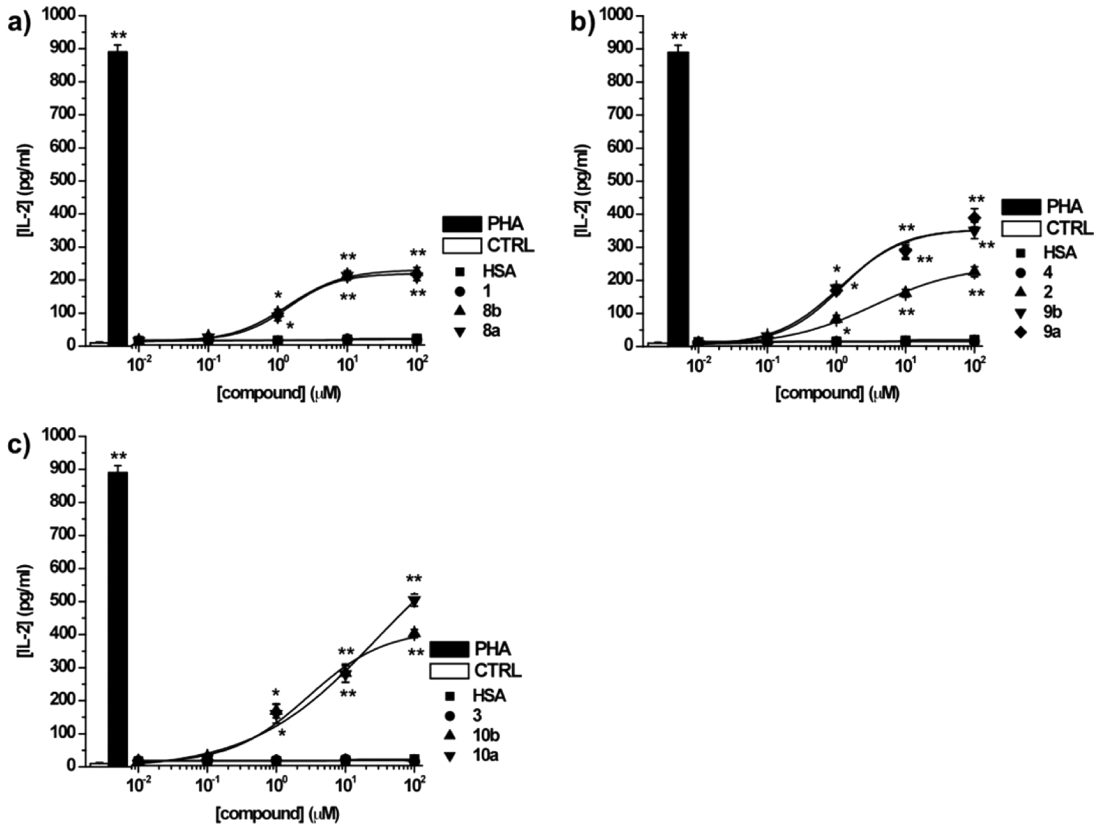


Figure 4. Effects of tested compounds on IL-2 release: (a) HSA-conjugated monosaccharides **8b** and **8a** and unconjugated monosaccharide **1**; (b) HSA-conjugated disaccharides **9b** and **9a** and unconjugated disaccharides **2** and **4**; (c) HSA-conjugated trisaccharides **10b** and **10a** and unconjugated trisaccharide **3**. The levels of IL-2 in the supernatants were measured by standard ELISA. The data represent the mean ± SEM of at least three independent experiments. Cells treated with 1 mg/mL phytohemeagglutinin (PHA), a widely used mitogen able to trigger T cell division and IL-2 release, were considered as positive controls, whereas HSA was used as protein control and compound-untreated cells (CTRL) as negative controls. (∗) $P < 0.05$ and (∗∗) $P < 0.01$ versus compound-untreated controls.

the unconjugated oligomers (IC_{50} values at 10^{-1} mg/mL and 30% of inhibition at 10^{0} mg/mL) (Table 2). In addition, the sugar loading seems to affect the antibody binding, because the conjugates with the higher sugar loading (**8a−10a**) were slightly more effective at 10^{0} mg/mL (around 60% of inhibition) than the corresponding lower sugar loading conjugates (**8b−10b**) (50% of inhibition), although all compounds exhibited the same potency.

The low efficacy of the synthetic analogues in comparison to natural antigen is not surprising because an anti-MenA polyclonal antibody, which is expected to recognize a number of different MenA CPS epitopes, some of which are longer than the synthetic fragments employed in this study, was used for ELISA. This observation is consistent with previous literature data reporting that the chain length of saccharide antigens is an essential characteristic for the efficacy.[26]

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

Our results also suggest that the phosphonester-linked MenA CPS analogues are able to mimic the natural saccharide epitopes recognized by the anti-MenA antibody and that these epitopes are preserved after conjugation to the protein. Moreover, the protein conjugation can confer a saccharide distribution/localization more suitable for antibody binding.[27]

Immunological Activity. To rule out the possibility that the immune cell responses were due to the presence of endotoxin contaminations, such as lipopolysaccharide (LPS), introduced into the system through chemicals, materials or equipment, each compound was carefully evaluated for purity. A limulus amebocyte lysate (LAL) endochrome assay was thus performed in four different batches of each compound, and the concentration of LPS was measured. LPS content was always below the value of 0.01 ng/mL, sufficient to stimulate immune cell responses,[28] suggesting that the compounds under testing were LPS-free and appropriate for biological evaluation (data not shown).

Then, the biocompatibility of all the new synthetic compounds was determined. CAM-labeled peripheral blood mononuclear cells (PBMC) were treated with increasing concentrations (10^{-2}–10^{2} μM) of each compound for 24 h, and cell viability was analyzed by FACS. No toxicity was measured at all concentrations considered (see the Supporting Information, Figure S4), indicating that all of the compounds under evaluation are biocompatible and suitable for other biological tests.

Because the activation and clonal expansion of both antigen-specific cytotoxic $CD8^+$ T lymphocytes and T helper $CD4^+$ cells are essential for mediating protective immunity and immunological memory,[29] the ability of the monovalent oligomers **1**–**3** and their corresponding conjugates **8**–**10** to activate T cells was determined. Human monocytes were pulsed with increasing concentrations (10^{-2}–10^{2} μM) of each compound, cocultured (6 days) with carboxyfluorescein succinimidyl ester (CFSE)-labeled human PBMC, and antigen-specific T cell proliferation was measured by FACS.

All HSA-conjugated compounds induced T cell proliferation in a concentration-dependent manner (Figure 3). Conjugates **9** and **10** stimulated approximately the same level of T-cell proliferation (40% of T cell proliferation vs controls at 10^{2} μM), and this effect was significantly ($P \leq 0.05$) higher than that of conjugates **8** (28% of T cell proliferation vs controls at the same concentration). The phosphonodisaccharide **2** resulted the only unconjugated compound able to stimulate T cells. Its effectiveness was only weakly inferior (−10% of T cell proliferation at 10^{2} μM) to that of the corresponding conjugates **9a** and **9b** (Figure 3b). The IL-2 levels measured in the cell culture medium by ELISA were consistent with the proliferative responses (Figure 4).

The mechanism underlying this effect remains to be clarified, even if it should be emphasized that only the phosphonodisaccharide **2** shows a peculiar charge pattern in its molecule. Compound **2** contains, in fact, a zwitterionic motif in its structure, deriving from the negatively charged phosphonoester group and the positively charged protonated nitrogen of the linker moiety. This characteristic could, therefore, confer the unique immunological properties of ZPS. The removal of the positive charge by N-acetylation of compound **2**, leading to phosphonodisaccharide **4** (see Figure 1), abrogated this activity and supports our hypothesis that the preservation of the zwitterionic motif is essential for biological activity.[11,30]

In the corresponding conjugates (**9**) the zwitterionic character of compound **2** is lost, as the amino group of the spacer is engaged in the amide bond with the squarate linker. We anticipated that the conjugation with an immunogenic protein carrier (HSA) could compensate for this loss.

Finally, our data are in agreement with those from Gallorini et al.,[31] who reported that the distribution, the spatial relationship, and the net charge of ZPS may influence the biological activity. In our experiments, in fact, the negatively charged phosphonotrisaccharide **3** did not exhibit the same biological activity as compound **2**. We argued that the presence of one positive and two negative charges not alternatively distributed on the surface of the trisaccharide and/or the negative net charge could account for the lack of immunoactivity.

In Vivo Evaluation of HSA Conjugates. Having demonstrated that conjugates **8**–**10** and monovalent compound **2** are able to stimulate in vitro T cell responses, we then tested in vivo their ability to elicit the production of specific antibodies against MenA CPS and to protect against infections. Specific IgG production to meningococcal polysaccharides is generally considered an important prerequisite in the selection of vaccine candidates.[32] Groups of eight BALB/c mice were thus immunized with three doses, 2 weeks apart, of 2 μg (based on saccharide) of each conjugated compound (**8**–**10**) or with 2 or 20 μg of phosphonodisaccharide **2**. The MenA–CRM_{197} conjugate, prepared as previously reported,[20] and the N-acetylated counterpart **4** were also injected as controls. All compounds were formulated with aluminum phosphate, an adjuvant commonly used for vaccine studies.[33]

Figure 5 shows ELISA data obtained with mouse sera collected after the third dose of each compound administration.

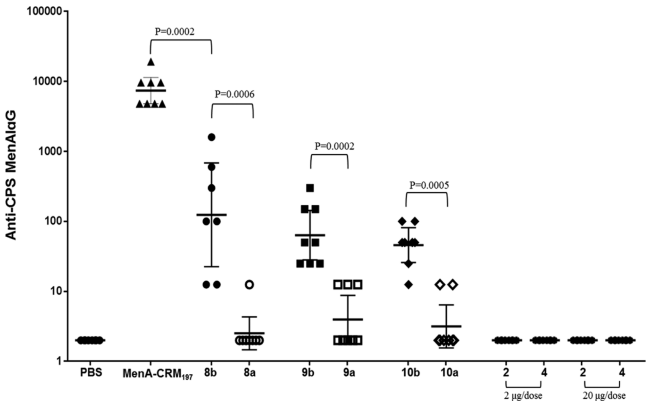


Figure 5. IgG levels detected at OD = 1 in individual sera (collected 2 weeks after the third immunization) of BALB/c mice immunized at 2 μg saccharide dose of antigen against native polysaccharide coating. MenA–CRM_{197} conjugate obtained from native CPS (commercial vaccine) was used as control in the study. Each point represents an individual mouse serum; horizontal bold bars indicate geometric mean titers (GMT) of each group with 95% statistical confidence intervals.

All of the conjugates with half-sugar loading (**8b**–**10b**) exhibited a similar effectiveness in inducing the production of specific anti-MenA CPS IgG in mice. In agreement with our previous findings, the immune responses were lower than those achieved with MenA–CRM_{197}, obtained from MenA native CPS.[20] This might be due to the fact that the synthetic structures are not sufficiently long to cover the entire MenA CPS epitope.

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487–496

The immune response appeared not to be dependent on the oligomer length, as the conjugated monomer elicited similar IgG titers compared to conjugated dimer or trimer. In our previous studies titers were elicited only against a carba trimer conjugated to CRM_{197}, which gave a GMT of 173, whereas lower length fragments were not immunogenic. Here the IgG level induced by the three conjugated synthetic structures was comparable to the level achieved with the carba trimer−CRM_{197} conjugate.[20] This could indicate that the ManNAc repeating unit is the epitope primarily recognized. Furthermore, each conjugate with half-sugar loading (**8b−10b**) was more immunoactive than the corresponding full-sugar loading compound (**8a−10a**). From these results the relative antigenicity of each compound appeared to be dependent on the degree of saccharide incorporation. The effect of saccharide incorporation on the immunogenicity of carbohydrate antigens is controversial and case-dependent. Two interconnected parameters, which are the sugar length and the density of loading, seem to play important roles. Whereas for some carbohydrate antigens the highest immune response might benefit from a high level of glycan incorporation,[34] in other examples a low sugar loading seems to be necessary.[35]

Our compounds could, thus, display a high number of organized epitopes on the protein surface, and this pattern could improve antigen presentation and uptake, which in turn lead to T cell stimulation through the multivalent exposition of the MenA analogues.[34] Compounds **8b−10b** could be more effective in stimulating B cell receptor (BCR) cross-linking and, consequently, in inducing B cell activation and IgG antibody production,[36] whereas very high density incorporation could be detrimental for that effect.

We also considered the possibility that the change of the net charge occurring during the conjugation process correlates with the low immunogenicity determined in glycoconjugates **8−10**. This supports data from other groups reporting that protein phosphorylation has negative effects on the binding properties.[37] We, therefore, measured in the same conditions of vaccine formulation (PBS, pH 7.2) the electrophoretic mobility and the *Z* potential of all glycoconjugates **8−10** using the electrophoretic light scattering (ELS) technique. We found that all of the conjugates exhibit a *Z* potential more negative than that of HSA, as consequence of conjugation. In addition, glycoconjugates **8a−10a** have closely related values of *Z* potential and electrophoretic mobility, and this held true also for compounds **8b−10b**. However, the full-sugar loading conjugates **8a−10a** have more negative values than compounds **8b−10b**, suggesting that the *Z* potential of glycoconjugates **8−10** is primarily affected by the number of saccharide units incorporated in the carrier, rather than the chain length. Consequently, we cannot exclude that the decrease of the net charge occurring in the full-sugar loading conjugates **8a−10a**, in comparison to compounds **8b−10b**, is partially responsible of their lower immunogenicity.

We chose HSA for conjugation of the synthetic molecules, because this protein is easily available and widely employed as a model carrier; however, we cannot exclude that the use of more immunogenic proteins, such as CRM_{197} or TT, could lead to more effective glycoconjugates, capable of raising high titers of bactericidal IgG antibodies. Moreover, other structural features have been described to play a major role in the antigenic determinants of MenA CPS, and they could be targeted to improve the immunogenicity of the glycoconjugates. For example, recent studies provided evidence that O-acetylation, occurring predominantly at 3-OH on native MenA CPS, is extremely important to the immunogenic epitopes of the polysaccharide and positively correlates with host protection.[38] The introduction of a phosphomonoester group at 6-OH, occurring in some licensed vaccines as a consequence of the acidic hydrolysis of the native polysaccharide, could also be beneficial.[19] Studies in this direction are currently ongoing in our laboratory, and results will be reported in due course.

When the unconjugated phosphonodisaccharide **2** was injected, no change in the IgG levels was measured. This result, even if not consistent with the in vitro experiments shown above (see Figure 3), confirms previous literature data, reporting that short zwitterionic oligosaccharides can prime in vitro T cell activation without inducing protection against abscess formation in vivo.[12] The overall observations suggest that a disaccharide analogue would be too small to retain in vivo the immunoactivity of ZPS observed in vitro.

An alternative hypothesis is that the zwitterionic compound **2** is not per se immunogenic, but it could rather act as an adjuvant. This hypothesis agrees with the observation of Gallorini and co-workers[39] that ZPS, chemically generated by modification of anionic polysaccharide antigens, display adjuvant properties, being able to activate APC in a TLR2-dependent manner, but do not induce polysaccharide-specific IgG production.

Conclusions. Phosphonoester-linked mono-, di-, and trisaccharide analogues of MenA CPS were synthesized, conjugated to the carrier protein HSA, and tested both in vitro and in vivo for their ability to induce immune responses using as control a MenA−CRM_{197} obtained from the native CPS. The newly synthesized compounds conjugated to the carrier protein induced in vitro T cell activation and in vivo IgG production, demonstrating that the chemical modifications introduced do not prevent an immune response. The half-sugar loading compounds (**8b−10b**) seem to display a pattern of organized epitope on the protein surface more suitable than that of the full-sugar loading compounds (**8a−10a**) for B cell activation and IgG production. Conversely, the length of the synthetic molecules does not appear to be important for the induction of high anti-MenA titers.

Our experiments also show that the phosphonodisaccharide MenA CPS analogue **2**, not conjugated to a carrier protein, possesses the physicochemical properties for triggering in vitro T cell activation. The same compound, on the contrary, did not induce anti-MenA IgG antibodies in mice, probably because it is too small to elicit in vivo immunogenicity. On the basis of structure analysis we might speculate that the zwitterionic character of compound **2** is essential for its in vitro immunoactivity. Additional efforts are currently directed to the synthesis of new oligosaccharide MenA CPS analogues, with a well-defined acetylation pattern, higher molecular weight and anchored to the protein without loss of the zwitterionic character, to determine whether their immunological properties might be improved.

MATERIALS AND METHODS

Carbohydrates Conjugation to HSA Protein. *1-(N-Propyl-2-acetamido-2-deoxy-β-D-mannopyranosidyl)-2-ethoxycyclobutene-3,4-dione (**5**).* A solution of diethyl squarate (39.45 μL, 0.27 mM) and TEA (25 μL, 0.18 mM) in dry DMF (2 mL) was added to a solution of **1** (50 mg, 0.18 mM) in dry DMF (2 mL). The reaction was stirred at room temperature for 3 h by monitoring the reaction course on TLC

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

(MeOH/$CHCl_3$ 2:8). The solvent was evaporated to give a crude residue that was purified by flash-silica gel chromatography (MeOH/$CHCl_3$ 2:8) affording **5** as a white foam (61 mg, 85%): ^{1}H NMR (400 MHz, CD_3OD, 50 °C), δ 4.75 (m, 1H, OCH_2CH_3); 4.65 (d, $J_{1,2}$ = 1.3 Hz, 1H, H-1); 4.47 (bd, $J_{2,3}$ = 3.3 Hz, 1H, H-2); 3.96−3.81 (m, 3H, H-7a, H-6a, H-6b); 3.68−3.64 (m, 3H, H-7b, H-3, H-9a); 3.52−3.47 (m, 2H, H-9b, H-4); 3.34−3.32 (m, 1H, H-5); 2.02 (s, 3H, CH_3CO); 1.92−1.85 (m, 2H, H-8a, H-8b); 1.48 (t, 3H, OCH_2CH_3). ^{13}C NMR (100.6 MHz, CD_3OD, 50 °C), δ 99.39 C-1; 77.02 C-5; 73.21 C-3; 69.33 OCH_2CH_3; 67.25 C-4; 65.86 C-7; 60.84 C-6; 53.51 C-2; 41.32 C-9; 30.20 C-8; 21.23 CH_3CO; 14.66 OCH_2CH_3. HRMS (ESI), *m*/*z* (%) 425.15240 (80) $[M + Na]^+$, 827.31786 (20) $[2M + Na]^+$.

*1-[(N-Aminopropyl (2-acetamido-2-deoxy-β-D-mannopyranosidyl) C-(2-acetamido-2-deoxy-α-D-mannopyranosyl)-methanephosphonate sodium salt]-2-ethoxycyclobutene-3,4-dione (**6**).* A solution of diethyl squarate (9.4 μL, 0.06 mM) and TEA (6 μL, 0.04 mM) in dry DMF (1 mL) was added to a solution of **2** (25 mg, 0.04 mM) in dry DMF/MeOH 1:1 (1 mL). The reaction was stirred at room temperature for 3 h by monitoring the reaction course on TLC (MeOH/$CHCl_3$ 1:1). The solvent was evaporated to give a crude residue that was purified by flash-silica gel chromatography (MeOH/$CHCl_3$ 1:1) affording a white foam, which was dissolved in H_2O/MeOH and first eluted through a column filled with Dowex 50W X8 resin (H^+ form) and then through a column filled with the same resin in Na^+ form. The eluate was lyophilized to afford **6** as a foam (21 mg, 70%): ^{1}H NMR (400 MHz, D_2O, 50 °C), δ 4.96 (m, 3H, H-1, OCH_2CH_3); 4.69 (m, 1H, H-2); 4.57 (m, 1H, H-2); 4.45−4.40 (m, 1H, H-1′); 4.40−4.32 (m, 2H, H-6a, H-6b); 4.23−4.20 (m, 1H, H-3′); 4.16−4.11 (m, 1H, H-7b); 4.11−3.99 (m, 3H, H-6a, H-3, H-6b); 3.95−3.92 (m, 1H, H-7a); 3.91−3.71 (m, 5H, H-5′, H-4′, H-4, H-9a, H-9b); 3.70−3.68 (m, 1H, H-5); 2.38−2.30 (m, 2H, H-7a, H-7b); 2.28 (s, 3H, CH_3CO); 2.26 (s, 3H, CH_3CO); 2.15−2.09 (m, 2H, H-8a, H-8b); 1.68−1.64 (m, 3H, OCH_2CH_3). ^{13}C NMR (100.6 MHz, D_2O, 50 °C), δ 99.61 C-1; 75.84 C-5;74.47 C-5′; 72.94 C-1′; 72.23 C-3; 70.95 OCH_2CH_3; 69.71 C-3′; 67.83 C-4′; 67.30 C-7; 67.04 C-4; 63.33 C-6; 60.91 C-6′; 53.37 C-2; 53.24, 53.14 C-2′; 41.99 C-9; 29.94 C-8; 28.56, 28.04 C-7′; 22.39 CH_3CO; 15.35 OCH_2CH_3. ^{31}P NMR (162 MHz, D_2O, 45 °C), δ 23.67 Hz. HRMS (ESI), *m*/*z* (%) 682.22091 (100) $[M - Na]^-$.

*1-[(N-Aminopropyl (2-acetamido-2-deoxy-β-D-mannopyranosidyl) C-(2-acetamido-2-deoxy-α-D-mannopyranosyl)-methanephosphonyl C-(2-acetamido-2-deoxy-α-D-mannopyranosyl) sodium salt methanephosphonate sodium salt]-2-ethoxycyclobutene-3,4-dione (**7**).* A solution of diethyl squarate (7.44 μL, 0.05 mM) and TEA (4.67 μL, 0.03 mM) in dry DMF/MeOH 1:1 (1 mL) was added to a solution of **3** (30 mg, 0.03 mM) in dry DMF/MeOH 1:1 (1 mL). The reaction was stirred at room temperature for 24 h by monitoring the reaction course on TLC (MeOH/$CHCl_3$/H_2O 5:5:2). The solvent was evaporated to give a crude residue that was purified by flash-silica gel chromatography ((MeOH/$CHCl_3$/H_2O 5:5:1) affording a white foam, which was dissolved in H_2O/MeOH, first eluted through a column filled with Dowex 50W X8 resin (H^+ form), and then through a column filled with the same resin in Na^+ form. The eluate was lyophilized to afford **1** as a foam (22 mg, 65%): HRMS (ESI), *m*/*z* (%) 481.14024 (70) $[M - 2Na]^{2-}$; 985.27051 (30) $[M - Na]^-$; 936.28817 (15) $[M - 2Na + H]^-$.

*General Procedure for Squarate-Mediated Coupling of Oligomers 5−7 to Human Serum Albumin, HSA (**8**−**10**).* To a solution of HSA (29.9 mg, 0.45 μM) in a borate buffer solution (190 μL, pH 9) was added the squarate ester-linked oligomer (**5**, **6**, or **7**) (9 μM) to form a 50 mM solution with respect to the squarate ester derivative. The mixture was stirred at 25 °C, and the progress of conjugation was monitored by MALDI-TOF-MS (see the Supporting Information). When the desired loading was achieved (see Results and Discussion), the reaction was stopped by addition of a borate buffer solution (10 mL, pH 7). The mixture was then concentrated with an Amicon ultrafiltration cell (50 mL), equipped with a YM-10 membrane, using five changes of water followed by freeze-drying to afford a white solid.

Competitive ELISA. Ninety-six-well flat-bottom plates were incubated overnight at 4 °C with a mixture of MenA CPS (1 mg/mL) and methylated HSA (mHSA; 1 mg/mL) (MenA CPS and mHSA were generous gifts from Novartis Farma SpA, Siena, Italy). A solution of 5% fetal bovine serum (FBS) (Lonza, Visp, Switzerland) in phosphate-buffered saline (PBS) supplemented with Brij-35 (0.1%) and sodium azide (0.05%) was added to each well to block unspecific binding sites. The plates were incubated overnight at 4 °C with a solution (1:400) of mouse anti-MenA, used as reference serum. This serum was obtained after three imunizations with MenA−CRM_{197} vaccine (commercial vaccine), as described below. When compounds were tested, they were added to each well just before the addition of the reference serum. The plates were stained with *p*-nitrophenyl phosphate (*p*-NPP), and the absorbance was measured at 405 nm with an Ultramark microplate reader (Bio-Rad, Milan, Italy). Experiments were repeated in triplicate. Control experiments to verify unspecific binding were performed by coating the ELISA plates with colominic acid from *Escherichia coli*.

PBMC Isolation. PBMC were isolated by gradient centrifugation onto Ficoll−Hystopaque (Lonza) of venous blood obtained from healthy volunteers after their informed consent.[28] PBMC were suspended in RPMI-1640 medium containing 100 mg/mL kanamycin (Sigma-Aldrich, Milan, Italy), 10% heat-inactivated FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% MEM amino acid solution, and 0.01 mM 2-mercaptoethanol (Lonza) for immediate use. Human monocytes were isolated from heparinized venous blood of healthy volunteers, and their purity was assessed with the pan-leukocyte anti-CD45 (HLE-1) and the anti-CD14 (Leu-M3) monoclonal antibodies (BioLegend, San Diego, CA, USA) as previously described.[28] Cells were used on the day of isolation under endotoxin-free conditions. Before each experiment, the viable cell count was assessed (always >95%) by Trypan blue staining, and cells were seeded in complete fresh medium. Monocytes and PBMC used for coculture experiments were always isolated from the same healthy donor.

Viability Assay. PBMC were labeled with 1 μM Calcein-AM (CAM) (Life Technologies, Monza, Italy) in serum-free PBS for 15 min at 37 °C in the dark. After being washed, labeled cells were seeded in 24-well plates and treated with increasing concentrations (from 1×10^{-2} to 1×10^{2} μM) of each compound for 24 h at 37 °C in a 5% CO_2 humidified incubator. After incubation, the cells of each well were harvested, washed, and labeled with propidium iodide (PI), and the viability was measured by flow cytometry (FACS-Vantage, Becton Dickinson, Milan, Italy). Live cells were identified as CAM^{high}/PI^- population, whereas dead cells were

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

CAM^{low}/PI^{+}. Viability was calculated by FACSDiva software and expressed as the percentage of CAM^{high}/PI^{-} population relative to untreated cells.

Proliferation Assay. Monocytes (1×10^5 cells/well), used as APCs, were pulsed with increasing concentrations (from 1×10^{-2} to 1×10^2 μM) of each compound for 24 h. Responder PBMC (1×10^6 cells/mL) were labeled with 0.25 mM CFSE in serum-free PBS for 30 min at 37 °C. Filtered FBS was then added to stop the reaction, and cells were washed several times with RPMI-1640. CFSE-labeled responder T cells (5×10^4) were cocultured with antigen-pulsed or unpulsed homologous monocytes for 6 days at 37 °C and 5% CO_2 in a humidified incubator. Phytohemagglutinin (PHA) (1 mg/mL) (Sigma-Aldrich) was used as a positive control of PBMC proliferation, whereas compound-untreated cells were considered as negative controls. After 6 days of coculture, cells were harvested, washed three times with warmed PBS, resuspended in fresh PBS, and analyzed by FACS. Cell proliferation was measured as the reduction of CFSE fluorescence intensity due to cell division by using FACSDiva sftware.

ELISA. Monocytes (1×10^5 cells/well), used as APCs, were pulsed with increasing concentrations (from 1×10^{-2} to 1×10^2 μM) of each compound for 24 h. Responder PBMC (1×10^6 cells/well) were cocultured with antigen-pulsed or unpulsed homologous monocytes for 6 days at 37 °C and 5% CO_2 in a humidified incubator. PHA (1 mg/mL) was added in positive control wells, whereas compound-untreated cells were considered as negative controls. After 6 days of coculture, the supernatants were collected and stored at −20 °C until assays. The levels of IL-2 were measured by ELISA using a mouse anti-human IL-2 monoclonal antibody and quantified with a recombinant IL-2 standard. Absorbance was measured at 450 nm using an Ultramark microplate reader (Bio-Rad), and the concentration of IL-2 in each sample was determined by extrapolation from reference standard curves. The detection limit of this method was 4 pg/mL.

Immunization of Mice. Vaccines were formulated in PBS (pH 7.2) buffer in a volume of 200 μL per dose. An injection volume of 200 μL per mouse was used. Aluminum phosphate ($AlPO_4$) was used as adjuvant in an amount of 0.12 mg (expressed as Al^{3+}) per dose. MenA vaccines were administered to mice in 2 or 20 μg per dose based on saccharide content. Mice were immunized subcutaneously at days 1, 14, and 28. Bleedings were performed at day 0 (pre immune), day 27 (post 2), and day 42 (post 3). Control groups received PBS with adjuvant. All animal studies were carried out in compliance with current Italian legislation on the care and use of animals in experimentation (Legislative Decree 116/92) and with the Novartis Animal Welfare Policy and Standards. Protocols were approved by the Italian Ministry of Health and by the local Novartis Animal Welfare Body (authorization AWB 201008).

ELISA Analysis. The antibody response induced by the unconjugated and glycoconjugated antigens against the MenA polysaccharide was measured by ELISA. Plates were coated with the polysaccharide by adding 100 μL/well of a 5 μg/mL polysaccharide solution in PBS (pH 8.2) buffer followed by incubation overnight at 4 °C. Coating solutions were removed from the plates by washing each well three times with PBS buffer containing 0.05% Tween 20 (Sigma-Aldrich) (TPBS). A blocking step was performed by adding 100 μL of BSA solution at 3% in TPBS and incubating the plates for 1 h at 37 °C. Blocking solution was removed from the plates by washing three times per well with TPBS. Then 200 μL of prediluted serum (1:25 for pre immune, 1:200 for a reference serum, 1:50−1:100 for test sera) was added to the first well of each column of the plate, whereas 100 μL of TPBS was dispensed into the remaining wells. Eight 2-fold serial dilutions along each column were then performed by transferring from well to well 100 μL of serum solutions. After primary antibody dilution, plates were incubated for 2 h at 37 °C. After three washings with TPBS, 100 μL of TPBS solution of secondary antibody alkaline phosphate conjugates (anti-mouse IgG 1:10000, Sigma-Aldrich) was added, and the plates were incubated for 1 h at 37 °C. Three more washes with TPBS were performed, and 100 μL/well of a 1 mg/mL of *p*-NPP (Sigma-Aldrich) in a 0.5 M diethanolammine buffer (pH 9.6) was added. After 30 min of incubation at room temperature, plates were read at 405 nm using a Bio-Rad plate reader. Raw data acquisition was performed by Microplate Manager Software (Bio-Rad). Serum titers were expressed as the reciprocal of serum dilution corresponding to a cutoff OD = 1. Each immunization group is represented as the geometrical mean (GMT) of the single-mouse titers. The statistical and graphical analysis was performed by GraphPad 5.0 software.

Statistical Analysis. Results are expressed as means ± SEM of at least five different experiments run in triplicate. Statistical significance was calculated by Student's *t* test for unpaired varieties. Differences were considered statistically significant when $P \leq 0.05$. Data were fitted as sigmoidal concentration−response curves and analyzed with a four-parameter logistic equation by using Origin version 6.0 software (Microcal Software, Northampton, MA, USA). The IC_{50} value was the concentration (mg/mL) of synthetic compound that inhibits the binding of the native MenA CPS to the specific anti-MenA antibody by 50%, and it was calculated using the same software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00071.

NMR spectra of compounds **5**−**7**; plots of the progress of conjugation reactions; MALDI-TOF spectra and SDS-PAGE of glycoconjugates **8**−**10**; cell viability curves (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*(G.L.) E-mail: grazia.lombardi@uniupo.it. Phone: (0039) 0321 375824.

Author Contributions

‖G.L. and L.L. contributed equally.

Author Contributions

G.L. and L.L. designed the research; L.L., B.B., and L.P. synthesized compounds; S.F. and S.G. performed in vitro experiments; G.B. and M.T. performed in vivo experiments; S.F., L.P., and F.B. analyzed data; G.L., S.F., L.L., F.B., and R.A. wrote the paper.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Antonella Vallario for flow cytometry measurements. This work was supported by MIUR-Italy (PRIN 2010−

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

2011, prot. 2010JMAZML_003) and Cariplo Foundation Italy (NANOVAC project, rif. 2013-0624).

REFERENCES

(1) Zahlanie, Y. C., Hammadi, M. M., Ghanem, S. T., and Dbaibo, G. S. (2014) Review of meningococcal vaccines with updates on immunization in adults. *Hum. Vaccin. Immunother. 10*, 995−1007.
(2) Stephens, D. S., Greenwood, B., and Brandtzaeg, P. (2007) Epidemic meningitis, meningococcaemia, and *Neisseria meningitides*. *Lancet 369*, 2196−2210.
(3) Rouphael, N. G., and Stephens, D. S. (2012) Neisseria meningitidis: biology, microbiology, and epidemiology. *Methods Mol. Biol. 799*, 1−20.
(4) Harrison, L. H., Trotter, C. L., and Ramsay, M. E. (2009) Global epidemiology of meningococcal disease. *Vaccine 27* (Suppl. 2), B51−B63.
(5) Segal, S., and Pollard, A. J. (2005) Vaccines against bacterial meningitis. *Br. Med. Bull. 72*, 65−81.
(6) WHO. Meningococcal vaccines: WHO position paper, November 2011. *Wkly. Epidemiol. Rec.* 2011, *86*, 521−540.
(7) Maiden, M. C. (2013) The impact of protein-conjugate polysaccharide vaccines: an endgame for meningitis? *Philos. Trans. R. Soc. Lond. B. Biol. Sci. 368*, 0147.
(8) Stephen, T. L., Niemeyer, M., Tzianabos, A. O., Kroenke, M., Kasper, D. L., and Kalka-Moll, W. M. (2005) Effect of B7-2 and CD40 signals from activated antigen-presenting cells on the ability of zwitterionic polysaccharides to induce T-Cell stimulation. *Infect. Immun. 73*, 2184−2189.
(9) Wang, Q., McLoughlin, R. M., Cobb, B. A., Charrel-Dennis, M., Zaleski, K. J., Golenbock, D., Tzianabos, A. O., and Kasper, D. L. (2006) A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2. *J. Exp. Med. 203*, 2853−2863.
(10) Trück, J., Lazarus, R., Clutterbuck, E. A., Bowman, J., Kibwana, E., Bateman, E. A., and Pollard, A. J. (2013) The zwitterionic type I *Streptococcus pneumoniae* polysaccharide does not induce memory B cell formation in humans. *Immunobiology 218*, 368−372.
(11) Tzianabos, A. O., Onderdonk, A. B., Rosner, B., Cisneros, R. L., and Kasper, D. L. (1993) Structural features of polysaccharides that induce intra-abdominal abscesses. *Science 262*, 416−419.
(12) Kalka-Moll, W. M., Tzianabos, A. O., Wang, Y., Carey, V. J., Finberg, R. W., Onderdonk, A. B., and Kasper, D. L. (2000) Effect of molecular size on the ability of zwitterionic polysaccharides to stimulate cellular immunity. *J. Immunol. 164*, 719−724.
(13) Costantino, P., Rappuoli, R., and Berti, F. (2011) The design of semi-synthetic and synthetic glycoconjugate vaccines. *Expert Opin. Drug Discov. 6*, 1045−1066.
(14) Baltimore, R. S. (2006) Recent trends in meningococcal epidemiology and current vaccine recommendations. *Curr. Opin. Pediatr. 18*, 58−63.
(15) Hedari, C. P., Khinkarly, R. W., and Dbaibo, G. S. (2014) Meningococcal serogroups A, C, W-135, and Y tetanus toxoid conjugate vaccine: a new conjugate vaccine against invasive meningococcal disease. *Infect. Drug. Resist. 7*, 85−99.
(16) Trotter, C. L., Yaro, S., Njanpop-Lafourcade, B. M., Drabo, A., Kroman, S. S., Idohou, R. S., Sanou, O., Bowen, L., Findlow, H., Diagbouga, S., Gessner, B. D., Borrow, R., and Mueller, J. E. (2013) Seroprevalence of bactericidal, specific IgG antibodies and incidence of meningitis due to group A *Neisseria meningitidis* by age in Burkina Faso 2008. *PLoS One 8*, e55486.
(17) Bundle, D. R., Smith, I. C., and Jennings, H. J. (1974) Determination of the structure and conformation of bacterial polysaccharides by carbon 13 nuclear magnetic resonance. Studies on the group-specific antigens of *Neisseria meningitides* serogroups A and X. *J. Biol. Chem. 249*, 2275−2281.
(18) Lemercinier, X., and Jones, C. (2000) An NMR spectroscopic identity test for the control of the capsular polysaccharide from *Haemophilus influenzae* type b. *Biologicals 28*, 175−183.
(19) Berti, F., Romano, M. R., Micoli, F., Pinto, V., Cappelletti, E., Gavini, M., Proietti, D., Pluschke, G., MacLennan, C. A., and Costantino, P. (2012) Relative stability of meningococcal serogroup A and X polysaccharides. *Vaccine 30*, 6409−6415.
(20) Gao, Q., Tontini, M., Brogioni, G., Nilo, A., Filippini, S., Harfouche, C., Polito, L., Romano, M. R., Costantino, P., Berti, F., Adamo, R., and Lay, L. (2013) Immunoactivity of protein conjugates of carba analogues from *Neisseria meningitidis* a capsular polysaccharide. *ACS Chem. Biol. 8*, 2561−2567.
(21) Torres-Sanchez, M. I., Zaccaria, C., Buzzi, B., Miglio, G., Lombardi, G., Polito, L., Russo, G., and Lay, L. (2007) Synthesis and biological evaluation of phosphono analogues of capsular polysaccharide fragments from *Neisseria meningitidis* A. *Chem.−Eur. J. 13*, 6623−6635.
(22) Kamath, V. P., Diedrich, P., and Hindsgaul, O. (1996) Use of diethyl squarate for the coupling of oligosaccharide amines to carrier proteins and characterization of the resulting neoglycoproteins by MALDI-TOF mass spectrometry. *Glycoconjugate J. 13*, 315−319.
(23) Zhang, J., Yergey, A., Kowalak, J., and Kováč, P. (1998) Studies towards neoglycoconjugates from the monosaccharide determinant of *Vibrio cholera* O:1, serotype Ogawa using the diethyl squarate reagent. *Carbohydr. Res. 313*, 15−20.
(24) Hou, S., Saksena, R., and Kováč, P. (2008) Preparation of glycoconjugates by dialkyl squarate chemistry revisited. *Carbohydr. Res. 343*, 196−210.
(25) Chernyak, A., Karavanov, A., Ogawa, Y., and Kováč, P. (2001) Conjugating oligosaccharides to proteins by squaric acid diester chemistry: rapid monitoring of the progress of conjugation, and recovery of the unused ligand. *Carbohydr. Res. 330*, 479−486.
(26) Costantino, P., Norelli, F., Giannozzi, A., D'Ascenzi, S., Bartoloni, A., Kaur, S., Tang, D., Seid, R., Viti, S., Paffetti, R., Bigio, M., Pennatini, C., Averani, G., Guarnieri, V., Gallo, E., Ravenscroft, N., Lazzeroni, C., Rappuoli, R., and Ceccarini, C. (1999) Size fractionation of bacterial capsular polysaccharides for their use in conjugate vaccines. *Vaccine 17*, 1251−1263.
(27) Pillai, S., Ciciriello, S., Koster, M., and Eby, R. (1991) Distinct pattern of antibody reactivity with oligomeric or polymeric forms of the capsular polysaccharide of *Haemophilus influenzae* type b. *Infect. Immun. 59*, 4371−4376.
(28) Fallarini, S., Paoletti, T., Battaglini, C. O., Ronchi, P., Lay, L., Bonomi, R., Jha, S., Mancin, F., Scrimin, P., and Lombardi, G. (2013) Factors affecting T cell responses induced by fully synthetic glyco-gold-nanoparticles. *Nanoscale 5*, 390−400.
(29) Williams, M. A., and Bevan, M. J. (2007) Effector and memory CTL differentiation. *Annu. Rev. Immunol. 25*, 171−192.
(30) Tzianabos, A., Wang, J. Y., and Kasper, D. L. (2003) Biological chemistry of immunomodulation by zwitterionic polysaccharides. *Carbohydr. Res. 338*, 2531−2538.
(31) Gallorini, S., Berti, F., Parente, P., Baronio, R., Aprea, S., D'Oro, U., Pizza, M., Telford, J. L., and Wack, A. (2007) Introduction of zwitterionic motifs into bacterial polysaccharides generates TLR2 agonists able to activate APCs. *J. Immunol. 179*, 8208−8215.
(32) Qiao, W., Ji, S., Zhao, Y., and Hu, T. (2015) Conjugation of β-glucan markedly increase the immunogencity of meningococcal group Y polysaccharide conjugate vaccine. *Vaccine 17*, 2066−2072.
(33) Vecchi, S., Bufali, S., Skibinski, D. A., O'Hagan, D. T., and Singh, M. (2012) Aluminum adjuvant dose guidelines in vaccine formulation for preclinical evaluations. *J. Pharm. Sci. 101*, 17−20.
(34) Pozsgay, V., Chu, C., Pannell, L., Wolfe, J., Robbins, J. B., and Schneerson, R. (1999) Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from *Shigella dysenteriae* type 1. *Proc. Natl. Acad. Sci. U. S. A. 96*, 5194−5197.
(35) Adamo, R., Hu, Q., Torosantucci, A., Crotti, S., Brogioni, G., Allan, M., Chiani, P., Bromuro, C., Quinn, D., Tontinia, M., and Bertia, F. (2014) Deciphering the structure−immunogenicity relationship of anti-*Candida* glycoconjugate vaccines. *Chem. Sci. 5*, 4302−4311.
(36) Bachmann, M. F., and Zinkernagel, R. M. (1997) Neutralizing antiviral B cell responses. *Annu. Rev. Immunol. 15*, 235−270.

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496

(37) Nishi, H., Hashimoto, K., and Panchenko, A. R. (2011) Phosphorylation in protein-protein binding: effect on stability and function. *Structure 19*, 1807−1815.
(38) Berry, D. S., Lynn, F., Lee, C. H., Frasch, C. E., and Bash, M. C. (2002) Effect of O acetylation of *Neisseria meningitidis* serogroup A capsular polysaccharide on development of functional immune responses. *Infect. Immun. 70*, 3707−3713.
(39) Gallorini, S., Berti, F., Mancuso, G., Cozzi, R., Tortoli, M., Volpini, G., Telford, J. L., Beninati, C., Maione, D., and Wack, A. (2009) Toll-like receptor 2 dependent immunogenicity of glyco-conjugate vaccines containing chemically derived zwitterionic polysaccharides. *Proc. Natl. Acad. Sci. U. S. A. 106*, 17481−17486.

DOI: 10.1021/acsinfecdis.5b00071
ACS Infect. Dis. 2015, 1, 487−496