

Expanding the Scope of Ugi Multicomponent Bioconjugation to Produce Pneumococcal Multivalent Glycoconjugates as Vaccine Candidates

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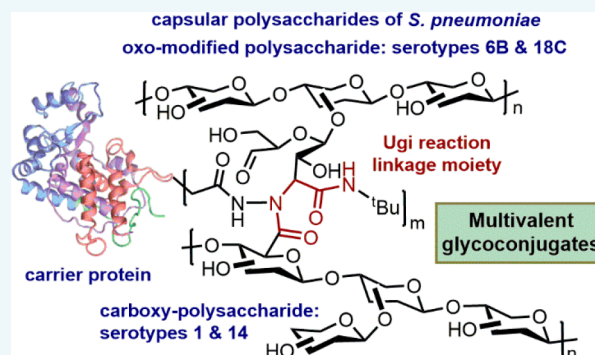


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ABSTRACT: Conjugate vaccines against encapsulated pathogens like *Streptococcus pneumoniae* face many challenges, including the existence of multiple serotypes with a diverse global distribution that constantly requires new formulations and higher coverage. Multivalency is usually achieved by combining capsular polysaccharide–protein conjugates from invasive serotypes, and for *S. pneumoniae*, this has evolved from 7- up to 20-valent vaccines. These glycoconjugate formulations often contain high concentrations of carrier proteins, which may negatively affect glycoconjugate immune response. This work broadens the scope of an efficient multicomponent strategy, leading to multivalent pneumococcal glycoconjugates assembled in a single synthetic operation. The bioconjugation method, based on the Ugi four-component reaction, enables the one-pot incorporation of two different polysaccharide antigens to a tetanus toxoid carrier, thus representing the fastest approach to achieve multivalency. The reported glycoconjugates incorporate three combinations of capsular polysaccharides 1, 6B, 14, and 18C from *S. pneumoniae*. The glycoconjugates were able to elicit functional specific antibodies against pneumococcal strains comparable to those shown by mixtures of the two monovalent glycoconjugates.



INTRODUCTION

Bacterial infections are one of the leading causes of medical concern in emerging and developed countries.¹ *Streptococcus pneumoniae*—one of the main causative agents of pneumonia, otitis media, sepsis, and meningitis—yearly accounts for more than 500 000 deaths in HIV-negative children under 5 years old.² Over the last years, vaccines have proven to be a cost-effective solution in preventing *S. pneumoniae* infections.³ Pneumococcal vaccines are mainly based on the capsular polysaccharides that protect the bacterium against phagocytosis and desiccation.⁴ Nevertheless, carbohydrate-based vaccines are unable to elicit a T-cell-dependent response and are therefore inefficient for the vaccination of risk groups including children below 2 years old and elderly and immunocompromised persons. Glycoconjugate vaccines, which are composed of polysaccharides covalently linked to protein epitopes, confer the T-cell-dependent response needed for eliciting long-lasting IgG antibodies⁵ and efficiently prevent infections caused by encapsulated bacteria such as *S. pneumoniae*.

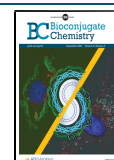
Due to *S. pneumoniae* having a diverse global distribution of serotypes, along with a high propensity for serotype

replacement, there is a need for multivalent vaccine formulations with broad serotype coverage. Multivalent vaccines, such as those employed for pneumococcal vaccination, mainly comprise the most invasive serotypes,⁵ and they have evolved from 7-⁶ to 20-valent.⁷ Heterogeneity and the complexity of the diverse capsular polysaccharides are the most challenging aspects in *S. pneumoniae* vaccine formulations. An additional issue is that such multivalent vaccines usually contain high concentrations of carrier proteins. The pre-exposure to protein carriers commonly employed in vaccination, such as diphtheria and tetanus toxoids, can interfere with the antipolysaccharide response via epitope suppression due to the pre-existing anti-carrier antibodies. Moreover, high concentrations of the same protein carrier at the site of

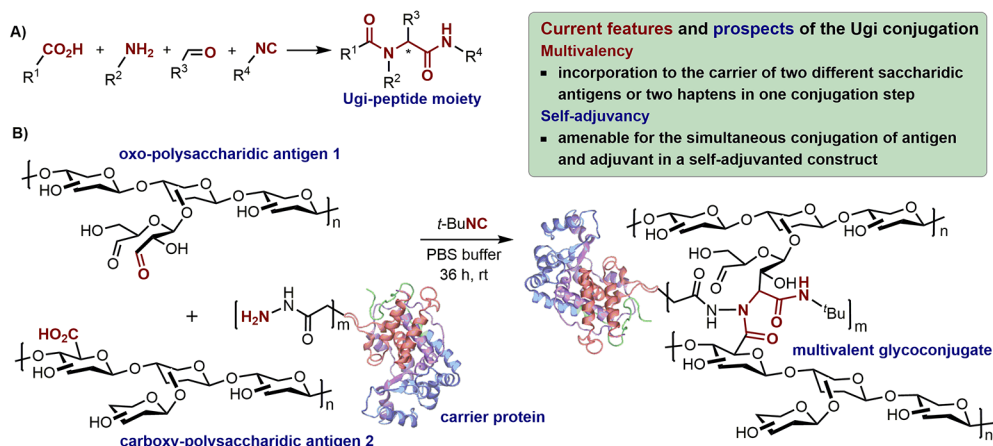
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Scheme 1. (A) Ugi Four-Component Reaction and (B) Multicomponent Conjugation Strategy to Produce Multivalent Glycoconjugates (Two Saccharidic Antigens and One Protein)^a



^aA generic protein structure is shown to represent tetanus toxoid as carrier.

injection can accentuate this phenomenon due to the competition between carrier-specific primed T lymphocytes.^{8,9}

Multivalent unimolecular glycoconjugates, where different polysaccharides are linked to a carrier protein in the same biomolecular construct, could theoretically reduce carrier epitope suppression, while still inducing potent anti-polysaccharide and anti-carrier immune responses. Although some previous attempts to build multivalent glycoconjugates were made, one of them involving capsular polysaccharides (CPs) of *S. pneumoniae* and *Neisseria meningitidis*,¹⁰ the first successful approach to an antibacterial multivalent construct, was developed by Adamo and co-workers in 2014.¹¹ This group reported the synthesis of a bivalent glycoconjugate by the sequential incorporation of the two meningococcal oligosaccharides MenA and MenC to the carrier CRM₁₉₇. The glycoconjugates showed good immunogenicity in comparison with MenA and MenC control vaccines and their combinations, thus proving the potential of joining two bacterial saccharidic antigens in a multivalent unimolecular construct.

Recently, our group investigated the efficacy of the Ugi multicomponent approach in the conjugation of large bacterial CPs to carrier proteins for accessing the corresponding glycoconjugates.¹² The Ugi four-component reaction (Ugi-4CR)¹³ combines functional groups either found in or easily incorporated into carbohydrates and proteins—i.e., a carboxylic acid, an amine, and an aldehyde—and the key isocyanide as the fourth component (Scheme 1A).¹⁴ This reaction forms a dipeptide structure that is not immunogenic *per se*,¹⁴ and its efficacy in water has been previously proven in protein conjugation.¹⁵ Before, we assessed the efficiency of the Ugi conjugation of oxo-modified CPs (e.g., 7F and 14 of *S. pneumoniae* after periodic oxidation) and the carboxylic acid-containing CPs of *Salmonella enterica* serovar Typhi to diphtheria and tetanus toxoids, always using an alkyl isocyanide as the fix component. After demonstrating the feasibility of this multicomponent protocol for obtaining monovalent glycoconjugates of two different bacteria,¹² we proved, for the first time, the possibility of conjugating two different saccharidic antigens to a carrier protein in a single step. This unique approach renders a multivalent glycoconjugate incorporating two saccharidic antigens and a protein one. Herein we broaden the scope of the Ugi multicomponent bioconjugation strategy

by demonstrating that CPs of dissimilar *S. pneumoniae* serotypes can be simultaneously conjugated to a carrier protein to assemble bivalent pneumococcal glycoconjugates.

RESULTS AND DISCUSSION

An important result of our initial study was the finding that hydrazide-activated toxoids delivered Ugi-derived glycoconjugates more efficiently than the nonactivated ones reacting *via* Lys side chains.¹⁴ Thus, the conversion of the aspartic and glutamic acid side chains into hydrazides not only incorporates additional highly nucleophilic amino components but also reduces the possibility of the protein carboxylic groups participating in the reaction. We have previously documented that Lys and Asp/Glu side chains can be cross-linked in peptides *via* an intramolecular Ugi reaction.^{16,17} In this case, the possible participation of such Asp/Glu carboxylic groups would hamper the incorporation of a carboxylic acid-containing polysaccharide. As a result, here we keep the use of hydrazide-activated tetanus toxoid (TT) as the biomolecular amino component, also taking into consideration that this carrier protein has proven to be greatly efficacious in vaccination worldwide.⁹ Scheme 1B depicts the multicomponent conjugation strategy of combining the hydrazide-activated carrier and a pair of modified CPs, serving as aldehyde and carboxylic acid components, with *tert*-butyl isocyanide as the fourth reagent. We highlight the potential of this approach to rapidly access multivalent glycoconjugates (also possible with smaller, nonsaccharidic haptens¹⁷) and envision its prospects for the construction of self-adjuvanted vaccines. Aiming at proving the versatility of the method for a variety of polysaccharides, pneumococcal CPs 1, 6B, 14, and 18C were chosen to produce the multivalent TT glycoconjugates. These serotypes have a high incidence in the world, as proven by their inclusion in the Prevnar 13¹⁸ vaccine formulation and the new Cuban pneumococcal conjugate vaccine,¹⁹ both developed using different conjugation strategies. In this regard, the pairwise sets of pneumococcal CPs 6B–1, 18C–1, and 18C–14 were chosen for the Ugi multicomponent conjugation, thus questioning if multivalent immune responses could be achieved with glycoconjugates derived from varied polysaccharide combinations. In addition

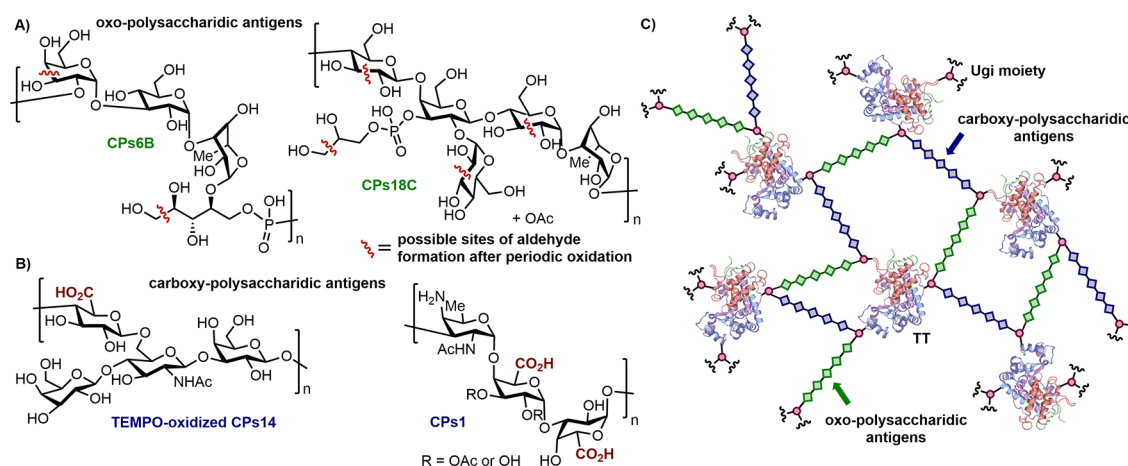


Figure 1. Structures of the repetitive units of the fragmented pneumococcal CPs employed in the Ugi bioconjugation. (A) CPs6B and CPs18C were employed as the aldehyde component after periodic oxidation. (B) CPs14 and CPs1 were employed as the carboxylic acid component (the first one after TEMPO oxidation). (C) Representation of the cross-linked lattice matrixes resulting from the multicomponent conjugation of the three polyfunctional biomacromolecules.

to CPs1—which naturally has a carboxylic acid functionality—we chose CPs14 as the carboxylic acid component due to the previous excellent results obtained in the oxidation with TEMPO.¹² However, there is no special rationale for the pairwise combinations (*i.e.*, CPs 6B–1, 18C–1, and 18C–14) besides our aim of broadening the synthetic scope and assessing the dual immunogenicity of multivalent glycoconjugates not previously evaluated. Of course, our criterium was based on the genuine possibility of assessing glycoconjugate combinations with an actual potential to proceed to biological evaluation.

To access the oxo-polysaccharides, the fragmented CPs 6B and 18C (10–30 kDa) were oxidized with sodium periodate under mild conditions (Figure 1A), resulting in the average incorporation of a carbonyl group every six repetitive units. On the other hand, CPs from pneumococcal serotypes 1 and 14 were selected as carboxy components of the Ugi reaction. Since CPs1 contains two natural uronic acids, its fragmented version (10–30 kDa, partially de-*O*-acetylated) was employed without any further modification (Figure 1B). Besides, the fragmented CPs14 (10–30 kDa) was subjected to TEMPO oxidation to generate an average of 1 carboxyl group every 4.7 repetitive units.

The Ugi multicomponent conjugation was carried out as represented in Scheme 1, using hydrazide-activated TT (around 50 hydrazide groups incorporated), as the amino component, and the water-soluble and very reactive *tert*-butyl isocyanide. Glycoconjugation including the combinations 6B–1–TT, 18C–1–TT, and 18C–14–TT were performed using 10–15 mg/mL of each CPs in phosphate-buffered saline (PBS, pH 7–7.4), with a ratio of oxo-CPs/carboxy-CPs/TT of 1.5:1.5:1 (w/w). Differently from our previous report,¹⁴ where all the components were mixed up at the beginning, herein we describe an important optimization of the conjugation setup, that is, the oxo-polysaccharide was initially added to the protein solution and the mixture was reacted overnight to allow the preformation of the corresponding imine-based biomolecular adduct. After the addition of the carboxy-polysaccharide and the isocyanide, the reaction was gently stirred for an additional 24–48 h. The overall reaction time was set to achieve high conversion into the glycoconjugates and a low amount of free protein (less than 20%), as observed

by size exclusion HPLC (SE-HPLC). Because of the polydisperse nature of the fragmented CPs and the fact that the resulting glycoconjugates occur as heterogeneous, cross-linked lattice matrixes (Figure 1C), the utilization of mass spectrometry is not an ideal tool for the characterization of such large macromolecular conjugates. Analysis of the crude and purified glycoconjugates was performed according to the WHO guidelines for the production of conjugate vaccines, which recommend SE-HPLC and nuclear magnetic resonance (NMR) for polysaccharide–protein conjugates.²⁰

The glycoconjugates were purified by diafiltration through regenerated cellulose membranes (100 kDa) and then again characterized by SE-HPLC to determine the free protein percentages (FPP). Conjugates 18C–1–TT and 18C–14–TT did not contain free protein, while the conjugate 6B–1–TT bared 7.6% free protein content (see the SI), which is acceptable according to the WHO recommendation for pneumococcal conjugate vaccines. Colorimetric techniques and quantitative NMR (qNMR) allowed determination of the total CPs content and the relative amount of each polysaccharide, respectively. The final oxo-CPs/carboxy-CPs and CPs/protein ratios are included in Table 1, showing that both CPs are not incorporated in an equimolar amount in the bivalent glycoconjugates. The rationale for this lays at the polyfunctional character of these biomacromolecules and the polydisperse nature of the CPs, which leads to the formation of macromolecular networks with different stoichiometries. Besides, while the only possible way for the Ugi reactions to take place is by the conjugation of the two polysaccharides to the protein, it is also true that a parallel Passerini three-component reaction²¹ could take place between the two CPs and the isocyanide, not incorporating the protein at that specific site. However, even if this side reaction consumes some aldehyde and carboxylic acid groups, the high availability of such functional groups at the numerous repetitive units ensures that the Ugi reaction does take place with the consequent ligation of the protein to the two different polysaccharides. Indeed, preforming the imine by mixing the hydrazide-activated TT and the oxo-CPs before the addition of the carboxy-CPs helps ensure the incorporation of the protein, as confirmed by the low amount of free protein detected (Table 1). The most intriguing case is glycoconjugate 18C–1–TT, which incorpo-

Table 1. Free Protein Percentages (FPP) and Polysaccharide/Protein Ratio of Bivalent Conjugates 6B–1–TT, 18C–1–TT, and 18C–14–TT and Their Respective Monovalent Counterparts

conjugate	FPP % ^a	concentration (mg/mL)		oxo-CPs/ carboxy-CPs ^d	CPs/protein
		protein ^b	total CPs ^c		
6B–1–TT	7.6	1.91	1.15	1:0.6	1:1.7
18C–1–TT	0	1.65	1.49	1:4.0	1:1.1
18C–14–TT	0	1.84	1.34	1:1.7	1:1.4
6B–TT	1.0	0.67	0.51		1:1.3
1–TT	3.6	0.69	0.67		1:1.0
18C–TT	0	0.59	0.58		1:1.0
14–TT	0	0.56	0.37		1:1.5

^aEstimated by HPLC with a Superose 12 column, with manual integration of the area below the curve at the protein retention time.

^bProtein quantification using Lowry's method. ^cCarbohydrate quantification using the orcinol–sulfuric acid method for CPs 14, 6B, and 18C conjugates and the *m*-hydroxybiphenyl method for CPs 1 conjugates. ^dEstimated by qNMR.

rated 4 times more CPs1 than CPs18. For a comparison purpose, Table 1 also shows the CPs/protein ratio of the monovalent glycoconjugates used as control. Both the unequivocal incorporation of the three biomolecular components and the preservation of the polysaccharide antigenic determinants were demonstrated by indirect enzyme-linked immunosorbent assays (ELISA). These were performed with the bivalent glycoconjugates along with their corresponding monovalent glycoconjugates, the natural CPs, and the nonconjugated TT. The monovalent glycoconjugates selected for comparison purposes are those used in the Cuban pneumococcal conjugate vaccine candidate.¹⁹ As shown in Figure 2, the recognition of each polysaccharide of the bivalent conjugates (dark red) by the specific serotype international reference serum was similar ($p \geq 0.05$) to those observed for the monovalent conjugates and the natural CPs, demonstrating the conservation of the polysaccharide epitope in the complex glycoconjugate structures. However, the recognition of the protein antigen by the specific polyclonal antibodies was lower in conjugates 18C–1–TT (Figure 2A) and 18C–14–TT (Figure 2B) than for the nonconjugated TT. Although in large conjugates the carrier protein epitopes are still accessible,²² previous reports of TT-conjugated bacterial polysaccharides—including 18C–TT—have shown a TT epitope recognition

from 10 to 50 times lower than for the nonmodified carrier protein.²³

The immunogenicity evaluation of the bivalent glycoconjugates was conducted in comparison with the corresponding monovalent glycoconjugates and the individual CPs, always adsorbed on AlPO₄ for adjuvation. The two conjugates incorporating CPs1, *i.e.*, 6B–1–TT and 18C–1–TT, as well as their monovalent conjugates and the natural CPs were studied with the immunization of female NZW rabbits (not in mice because of the poor immunogenicity of CPs6B- and CPs1-based glycoconjugates in these animals).²⁰ Alternatively, glycoconjugate 18C–14–TT, its two monovalent counterparts, and the two natural CPs were studied with the immunization of female Balb/c mice. Different groups containing eight Balb/c mice or three NZW rabbits were immunized with the bivalent conjugates (2 μ g/dose from the less represented CPs), with 1:1 mixtures of the two plain CPs (10 μ g/dose of each of them), and the two monovalent conjugates (equaling the amount of the corresponding CPs and the carrier protein on the bivalent conjugate). The sera extracted from the different immunized groups of animals were analyzed by ELISA to determine the specific IgG-induced response, defining the anti-CPs and anti-carrier protein IgG titers as the logarithms of the highest serum dilution giving 2- or 5-fold of the absorbance value of the preimmune sera from rabbits or mice immunization, respectively.

As shown in Figure 3, all bivalent glycoconjugates were able to elicit IgG antibodies against the two CPs included in their structures. In most cases, the IgG titers were similar to those elicited by the physical mixture of the corresponding monovalent conjugates and the titers were higher after the second and third dose. The three bivalent conjugates were able to induce anti-carrier titers comparable to those elicited by the mixtures of the monovalent conjugates, which can be explained by the administration of the same doses of carrier protein in all of the cases. Intriguingly, conjugate 18C–1–TT elicited lower anti-CPs1 IgG titers than the physical mixture containing conjugates 18C–TT and 1–TT, a behavior that was sustained after each immunization. These results are difficult to explain with the data currently available, but two different explanations could be considered. First, the lower response of anti-CPs1 IgG titers would be due to an ineffective presentation of the CPs1. In this specific case, the higher content of CPs1 with respect to CPs18C in the bivalent glycoconjugate (*i.e.*, 4:1, see Table 1) may comprise that several chains of CPs1 are conjugated to one chain of CPs18C (*via* the Passerini reaction

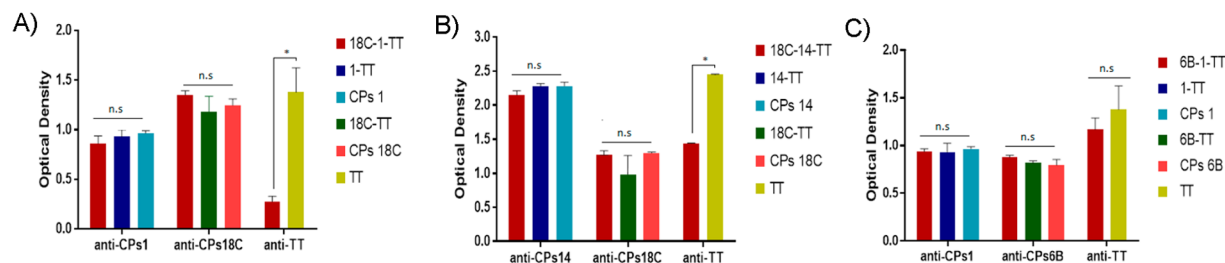


Figure 2. Recognition of specific serotype international reference serum or polyclonal anti-TT sera for the glycoconjugates 18C–1–TT (A), 18C–14–TT (B), and 6B–1–TT (C) in comparison with the monovalent glycoconjugates, both natural CPs, or TT, measured by ELISA. Bars represent the media $\pm$ SD of two different experiments. Statistical analysis was performed with the Kruskal–Wallis nonparametric test and then the uncorrected Dunn's multiple comparison test. The label ns indicates no statistical difference ($p > 0.05$) and * denotes significant statistical differences with $p \leq 0.05$.

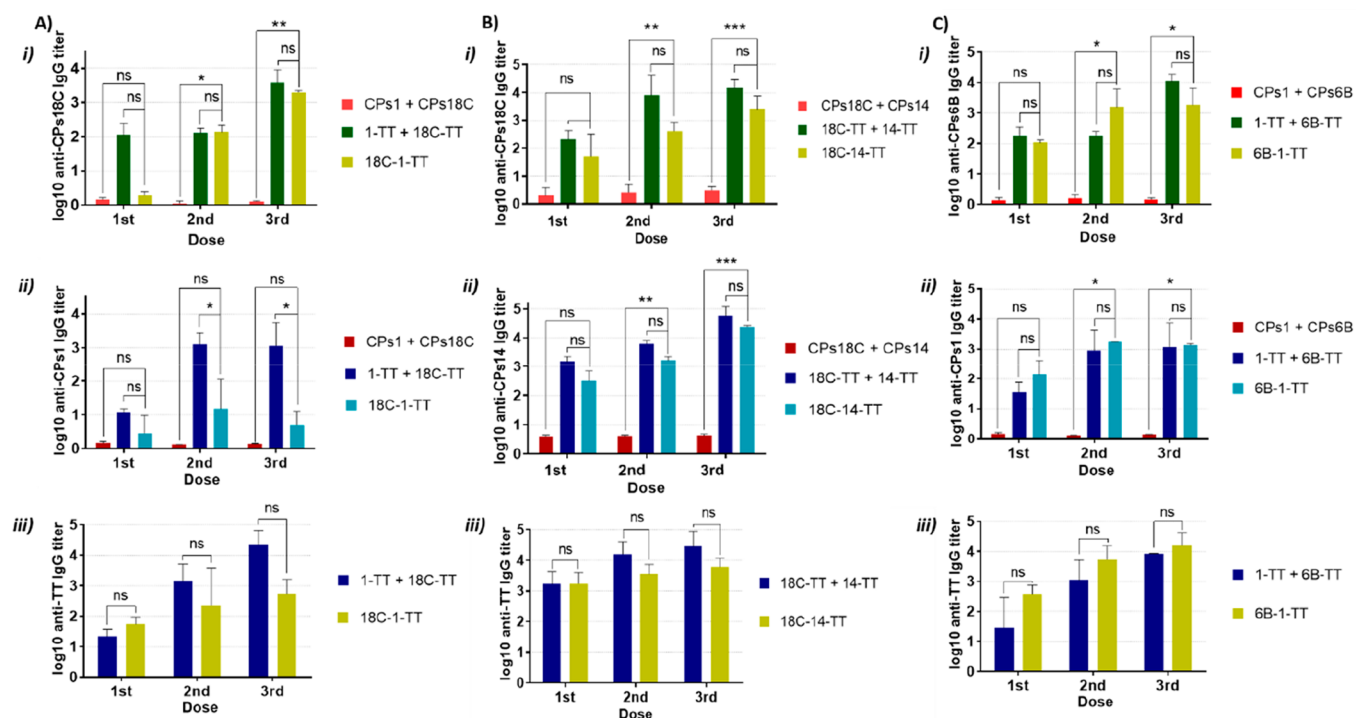


Figure 3. CPs-specific IgG elicited by the multivalent glycoconjugates compared with the individual CPs and the monovalent conjugates, as determined by indirect ELISA. (A) Conjugate 18C-1-TT (groups of three NZW rabbits), (B) conjugate 18C-14-TT (groups of eight Balb/C mice), and (C) conjugate 6B-1-TT (groups of three NZW rabbits). The results are presented as titers of specific IgG elicited against (i) the oxo-CPs, (ii) the carboxy-CPs, and (iii) the carrier protein TT, 7 days after each immunization. Statistical analysis was performed with the Kruskal–Wallis nonparametric test and then the uncorrected Dunn’s multiple comparison test. The label ns indicates no statistical difference ($p > 0.05$), and *, **, *** denote significant statistical differences with $p \leq 0.05$, 0.01, 0.001, respectively.

Table 2. OPKA Titers of Serum Samples from Rabbits or Mice Immunized with Bivalent or Monovalent Conjugates against Pneumococcal Serotypes 1, 14, 18C, and 6B^a

glycoconjugate	no. of samples	Sp1	Sp14	Sp18C	Sp6B
6B-1-TT (rabbits)	3	128 (64, 256)			19 (8, 32)
6B-TT + 1-TT (rabbits)	3	512 (512, 512)			203 (32, 512)
18C-1-TT (rabbits)	3	32 (32, 32)		256 (128, 512)	
1-TT + 18C-TT (rabbits)	3	341 (256, 512)		768 (256, 1024)	
18C-14-TT (mice)	4		144 (64, 256)	896 (512, 1024)	
14-TT + 18C-TT (mice)	4		224 (128, 256)	640 (512, 1024)	

^aValues represent the mean of OPKA titers of individual serum samples. In parentheses are the minimum and maximum values of the OPKA titer observed for each CPs. All serum samples were serially diluted in 2-fold steps starting from 1:4 (v/v), and the dilution value of 1:8 was selected as the threshold.

explained above) and not directed linked to the protein. This could mean that, despite the larger content of CPs1 in the bivalent construct, a large amount of this saccharidic antigen is not being processed and presented as a saccharide–peptide conjugate. The second potential reason for the lower immunogenicity of CPs1 in conjugate 18C-1-TT could be associated with the compromised TT antigenicity after conjugation in this particular construction. This would lead to fewer T epitopes available to achieve the necessary CPs immunogenicity, even when the impact is higher for CPs1 compared to CPs18C.

The immunogenicity of glycoconjugates is variable, and this variability has been attributed to the structure of the particular polysaccharide in a given construct and to the conjugation method used for linking the CPs to the carrier protein.^{24,25} Different mechanisms are involved in immune responses to immunization with different glycoconjugates, and the chemical

structure of the polysaccharide determines the mechanisms used by antigen-presenting cells (APCs) to offer the antigens to different subsets of CD4+ T-cells. A mechanism previously described comprises the recognition of the saccharide portion of glycoconjugate by CD4+ T-cells, when it is presented in the context of a covalently bound peptide into major histocompatibility complex class II of APCs. This saccharide–peptide presentation to carbohydrate-specific CD4+ T-cells induces polysaccharide-specific adaptive immune responses. This mechanism could explain that the variable immunogenicity previously considered as polysaccharide specific is related to the efficiency of the carbohydrate–peptide presentation. In the case of the bivalent constructs here obtained, the different presentation of the saccharide portion from each serotype could explain the differences in the IgG titers for the monovalent conjugate, in which all the polysaccharidic chains are always linked to the protein portion. Nevertheless, more

immunological studies are required with these novel glycoconjugates to better understand the mechanisms of uptake, processing, and presentation for the activation of the adaptive immune response.

Antibodies elicited by pneumococcal conjugate vaccines against different serotypes should be able to opsonize pneumococci, thus enabling phagocytosis.²⁶ The functional antibacterial antibodies elicited by the bivalent conjugates, as well as by the corresponding mixtures of monovalent conjugates against *S. pneumoniae* 1, 6B, 14 and 18C strains, were determined by opsonophagocytic killing assays (OPKAs). OPKA titers were calculated as the reciprocal of the sera dilution that caused a 50% reduction of the colony-forming unit CFU (killing) compared with the CFU from the control wells containing all reagents except for the mouse sera.²⁶ As an opsonic titer of 1:8 is the minimum value correlated with protection in vaccinated infants, it is normally used as a cutoff for OPKAs with mice or rabbit samples during the preclinical evaluation of pneumococcal vaccines.²⁷ OPKA titers shown in Table 2 confirm the functional activity of the antibodies elicited by the three bivalent glycoconjugates and their monovalent counterparts. Even when all CPs in both monovalent and multivalent conjugates elicited specific IgG antibodies with an opsonophagocytic titer over 1:8, all constructions do not have the same behavior. The OPKA titers observed for conjugate 6B–1–TT against serotype 6B and 18C–1–TT against serotype 1 were lower than those elicited by the physical mixtures of the monovalent conjugates. On the other hand, the OPKA titer against CPs18C in the multivalent conjugate is higher than that of the monovalent 18C–TT in mice. These results, together with the CPs IgG titers, confirm the variability of the immunogenicity of the conjugates. High variability in the levels of OPKA titers is reported in humans for commercial PCV vaccines. For example, in a clinical trial for the safety and immunogenicity of a 13-valent pneumococcal conjugate vaccine (PCV13), the OPKA titer for serotype 1 was 45.3, while that for serotype 14 was 1809.6.²⁸ In any case, the most relevant result is that OPKA titers over 1:8 were obtained, because this is the titer that is relevant in terms of clinical protection against pneumococci disease in humans.

CONCLUSION

Our results successfully expand the scope of the Ugi-4CR as a powerful bioconjugation method for the assembly of dissimilar multivalent glycoconjugate vaccine candidates. The reaction proved efficient at the simultaneous conjugation of different oxo-functionalized CPs, such as CPs 6B and 18C, and carboxy-functionalized CPs, such as TEMPO-oxidized CPs14 and CPs1 from *S. pneumoniae* to tetanus toxoid. However, the incorporation of the two CPs is not strictly in an equimolar amount due to the polyfunctional and polydisperse nature of these biomolecules, which works against dose control. In this regard, better control of the activation degree of the oxo- and carboxy-CPs and the use of more homogeneous CPs may contribute to achieving an oxo-CPs/carboxy-CPs ratio close to 1. Another crucial aspect of the formulation is to keep the CPs/protein ratio—for each polysaccharide incorporated—within the range for commercial antibacterial vaccines recommended by the World Health Organization (WHO).²⁰ The recognition of each antigen—including TT—by the corresponding specific antibodies demonstrates that the polysaccharide and protein epitopes are preserved during the

Ugi conjugation protocol. For conjugates 18C–1–TT and 18C–14–TT, the recognition of the protein was lower than for nonconjugated TT, but it did not compromise the immunogenicity of TT. The bivalent conjugates were also able to elicit high IgG titers, in most cases comparable to those of the physical mixtures of the corresponding monovalent conjugates, and were proved to be functional as demonstrated by OPKAs. Only conjugate 18C–1–TT showed relatively low IgG titers against CPs1, which suggests that the combination of serotypes could be optimized. All OPKA titers were in agreement with the values recommended by the WHO for formulations of pneumococcal conjugate vaccines capable of inducing a protective response against the different *S. pneumoniae* serotypes. These findings, together with our previous result on the preparation of the bivalent conjugate 7F–14–TT,¹⁴ contribute to the use of Ugi multicomponent bioconjugation as a feasible strategy to produce multivalent glycoconjugate vaccines, eventually incorporating serotypes from different bacteria and/or molecular adjuvants. This multicomponent strategy can be more cost-effective than the typical conjugation of each CPs to the carrier protein, as fewer conjugation and purification steps are required. Future immunological studies are required to assess whether formulations based on this new type of multivalent glycoconjugate can diminish the carrier-induced epitope suppression phenomenon described for those based on physical mixtures of multiple monovalent conjugates.

EXPERIMENTAL SECTION

General. Carbohydrate and protein contents were determined using different colorimetric techniques and quantitative ¹H NMR. The sulfuric acid–orcinol method was employed for the carbohydrate content determination in CPs14-, CPs6B-, and CPs18C-containing conjugates, while a *m*-hydroxybiphenyl-based method was employed for the carbohydrate content determination of CPs1 and the quantification of carboxyl groups in TEMPO-oxidized CPs14. A modified Park–Johnson method was employed for the quantification of carbonyl groups in periodate-oxidized CPs. The Hestrin method was employed for the determination of the *O*-acetyl content in CPs1. In all cases, the Lowry method was employed for protein quantification, while the TNBS method was employed for the quantification of amino/hydrazide groups. Colorimetric determinations were performed using a spectrophotometer (Jenway 6705 UV/vis). Proton nuclear magnetic resonance spectra were recorded on a Varian Mercury 400 (400 MHz) and on a Bruker Advance (600 MHz) at 25 °C. Samples of CPs as well as their conjugates were prepared by dissolving 10 mg of CPs in 0.6 mL of deuterated water (D₂O, Merck >99.8%), lyophilizing, and dissolving them again three times. All chemical shifts are reported in ppm and were determined using the residual solvent as an internal standard. Coupling constants (*J*) are reported in hertz (Hz). All the chromatography-destined solvents were distilled, dried, and degasified following the reported procedures. All the reactants employed in the synthesis, characterization, and chromatography were purchased from Merck, Fluka, and Panreac.

Carrier Protein. Tetanus toxoid (lot 7001) was produced in GMP facilities at the Finlay Institute of Vaccines and then detoxified according to production procedures recommended by the WHO.²⁹ Briefly, tetanus toxin was produced in homogeneous cultures in Latham medium. The cell suspension

resulting from the fermentation process was detoxified with 0.3% (v/v) formaldehyde at 37 ± 1 °C. Cell separation was then performed, followed by purification by saline precipitation and tangential filtrations.

Capsular Polysaccharides. Natural CPs 1 (lot 604), 6B (lot 2001), 14 (lot 1701), and 18C (lot 804) were purified from the corresponding pneumococcal strains at GMP facilities of the Finlay Institute of Vaccines, as recommended by the WHO.³¹ Briefly, the polysaccharides were produced in an industrial fermenter. The cell fraction was separated from the culture using tangential microfiltration and the clarified fraction was washed by tangential ultrafiltration with a porosity of 300 kDa. The retentate was purified of contaminants by an acidification process and ion exchange chromatography.

Procedure for the De-O-acetylation and Fragmentation of CPs1 from *S. pneumoniae*. CPs1 ($K_d = 0.153$, 88.8% O-acetyl content) was dissolved in buffer $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ (30 mmol/L, pH 9.4) to a concentration of 1 mg/mL. The mixture was stirred at 40 °C for 2 h and then diafiltrated through a regenerated cellulose membrane (30 kDa) with 10 water volumes and 4 bar of nitrogen pressure to afford partially de-O-acetylated CPs1 (67.6% O-acetyl content, 95%). To an aqueous solution of de-O-acetylated CPs1 (1 mg/mL) was added H_2O_2 (30%) to the final molarity of 150 mmol/L. The reaction mixture was stirred at 60 °C for 2 h and the product purified by diafiltration through a series of regenerated cellulose membranes (10–30 kDa) with 10 water volumes each time and 4 bar of nitrogen pressure to afford CPs1 (10–30 kDa, $K_d = 0.35$, 63% O-acetyl content, 93%).

Procedure for the Fragmentation and Periodic Oxidation of CPs6B from *S. pneumoniae*. To an aqueous solution of natural CPs6B ($K_d = 0.08$) (2 mg/mL), trifluoroacetic acid (100 mM) was added. The reaction mixture was stirred at 70 °C for 1 h and the product purified by diafiltration through a series of regenerated cellulose membranes (10–30 kDa) with 10 water volumes each time and 4 bar of nitrogen pressure to afford CPs6B (10–30 kDa, $K_d = 0.512$, 69%). Fragmented CPs6B (5 mg/mL) was mixed with NaIO_4 (2.5 mM) and the reaction mixture was protected from light and stirred at 10 °C for 3 h. An excess of ethylene glycol was added to the reaction mixture, and then it was diafiltrated through a regenerated cellulose membrane (10 kDa) with 10 water volumes and 4 bar of nitrogen pressure to afford oxCPs6B (1 C=O every 6 repetitive units (RU), $K_d = 0.65$, 76%).

Procedure for the Fragmentation and Periodic Oxidation of CPs18C from *S. pneumoniae*. To an aqueous solution of natural CPs18C ($K_d = 0.17$) (2 mg/mL) was added trifluoroacetic acid (25 mmol/L). The reaction mixture was stirred at 70 °C for 2.5 h and then diafiltrated through a series of regenerated cellulose membranes (10–30 kDa) with 10 water volumes each time and 4 bar of nitrogen pressure to afford CPs18C (10–30 kDa, $K_d = 0.49$, 88%). Fragmented CPs18C (5 mg/mL) was mixed with NaIO_4 (2 mmol/L) and the reaction mixture was protected from light and stirred at room temperature for 3 h. An excess of ethylene glycol was added to the reaction mixture that was then diafiltrated through a regenerated cellulose membrane (10 kDa) with 10 water volumes and 4 bar of nitrogen pressure to afford oxCPs18C (1 C=O every 6 repetitive units (RU), $K_d = 0.49$, 92%).

Procedure for the Fragmentation and Oxidation of CPs14 from *S. pneumoniae* with TEMPO. Trifluoroacetic acid (200 mmol/L) was added to an aqueous solution of natural CPs14

($K_d = 0.18$) (2 mg/mL). The reaction mixture was stirred at 70 °C for 0.5 h and then diafiltrated through a series of regenerated cellulose membranes (10–30 kDa) with 10 water volumes each time and 4 bar of nitrogen pressure to afford CPs14 (10–30 kDa, $K_d = 0.57$, 88%). To a solution of fragmented CPs14 [10–30 kDa, (10 mg, 14.5 mmol relative to the repetitive unit mass)] in 20 mL of carbonate buffer (2 mol/L, pH 10.5) were added TEMPO (0.042 equiv, 0.53 mmol), NaBr (0.28 equiv, 8 mmol), and NaClO (11%). The reaction mixture was protected from light and stirred during 1 h at 0 °C and then quenched with ethanol (10 equiv). The product was purified by diafiltration through a regenerated cellulose membrane (1 kDa) with 10 water volumes and 4 bar of nitrogen pressure to afford TEMPO-oxidized CPs14 (oxidation degree: 1 –COOH every 4 RU, 1 –CO every 5.3 RU, $K_d = 0.43$, 78.5%). Fragmented and TEMPO-oxidized CPs14 [10–30 kDa (10 mg, 14.5 mmol)] was dissolved in NaCl solution (0.9%) and mixed with NaBH_4 (2 mg, 0.533 mmol). The reaction mixture was stirred at 37 °C for 2 h and the product was purified by diafiltration through a regenerated cellulose membrane (1 kDa) with 10 water volumes and 4 bar of nitrogen pressure to afford TEMPO-oxidized CPs14 (oxidation degree: 1 carboxylic acid every 4.7 RU, no carbonyl groups detected, $K_d = 0.4$, 74.8%).

Modification of TT with Hydrazine. To a solution containing the detoxified protein TT with a concentration in the range from 3 to 5 mg/mL (determined by Lowry's method) in phosphate buffer (pH 6.0) were added hydrazine hydrochloride and *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide hydrochloride (20 mM). The reaction mixture was stirred for 4 h at room temperature in the darkness. After completion, the pH was adjusted to a value between 10.4 and 10.6. The product was purified by diafiltration through a regenerated cellulose membrane (30 kDa) with 10 volumes of a carbonate buffer (10 mmol/L, pH 10.4) and 4 bar of nitrogen pressure to afford hydrazide-activated TT (50 amino/hydrazide groups per mole of protein, 97%).

General Synthetic Procedure for the Conjugation of Carbonyl- and Carboxyl-Containing CPs by Ugi-4CR. To a solution containing the detoxified/hydrazide-activated protein in PBS (10 mmol/L, pH 7.4) was added the fragmented and periodate-oxidized polysaccharide in a 1.5:1 ratio related to the mass of the protein to reach a concentration of 10 mg/mL, and the mixture was stirred overnight at room temperature. Then, the carboxypolysaccharide (1:1 to 1.5:1 ratio related to the first polysaccharide) was added, the reaction mixture was stirred for 15 min, and then *tert*-butyl isocyanide (~10 equiv excess) was added. The reaction mixture was stirred for 24 h (a time of 48 h was also set for comparison by SE-HPLC monitoring). The conjugate was purified by diafiltration through a regenerated cellulose membrane (100 kDa) with 10 water volumes and 2 bar of nitrogen pressure. More information about the synthesis of each conjugate can be found in the [Supporting Information](#).

Immunological Evaluation. Immunization Schemes with Mice. Female Balb/c mice within 5–6 weeks old and between 18 and 20 g of weight [National Center for the Production of Laboratory Animals (CENPALAB)] were used. The mice were adapted to the laboratory conditions in Macrolon boxes (Panlab) during 7 days, under controlled humidity and temperature (22–24 °C), with 12 h of light and 12 h of darkness. Groups of eight Balb/c mice were immunized at 0, 14, and 28 days by the subcutaneous route and the blood samples were collected by the retro-orbital plexus 3 days before

starting the assay and 7 days after each immunization. Conjugate 18C–14–TT (2 $\mu\text{g}/\text{dose}$ of CPs18C/3.4 $\mu\text{g}/\text{dose}$ of CPs14) and a mixture containing conjugates 18C–TT (2 $\mu\text{g}/\text{dose}$ of CPs18C) and 14–TT (3.4 $\mu\text{g}/\text{dose}$ of CPs14) were administered in each immunization, while the control group was immunized with a mixture containing CPs 18C and 14 (10 $\mu\text{g}/\text{doses}$ of CPs). All immunogens were adsorbed on AlPO_4 at 125 $\mu\text{g}/\text{dose}$ of elemental aluminum. The extracted blood samples were centrifuged at 1500 rpm during 5 min and the sera were conserved at -20°C until the moment of the immunologic evaluation. The experimental protocols were approved by the Committee of Ethics for the Experimentation with Lab Animals and the Quality Department of the Finlay Institute of Vaccines.

Immunization Schemes with Rabbits. Female NZW rabbits within 10–12 weeks old and between 1.5 and 1.8 kg of weight (CENPALAB) were used. The rabbits were adapted to the laboratory conditions for 7 days, under controlled humidity ($60 \pm 5\%$) and temperature ($23\text{--}27^\circ\text{C}$), with 12 h of light and 12 h of darkness. Groups of three NZW rabbits were immunized at 0, 14, and 28 days by the subcutaneous route and the blood samples were collected from the marginal ear vein 3 days before starting the assay and 7 days after each immunization. Conjugate 6B–1–TT (2.4 $\mu\text{g}/\text{dose}$ of CPs1/4 $\mu\text{g}/\text{dose}$ of CPs6B) and the mixture of the correspondent monovalent conjugates 6B–TT (4 $\mu\text{g}/\text{dose}$ of CPs6B) and 1–TT (2.4 $\mu\text{g}/\text{dose}$ of CPs1) and conjugate 18C–1–TT (2 $\mu\text{g}/\text{dose}$ of CPs18C/8 $\mu\text{g}/\text{dose}$ of CPs1) and the mixture of the correspondent monovalent conjugates 18C–TT (2 $\mu\text{g}/\text{dose}$ of CPs18C) and 1–TT (8 $\mu\text{g}/\text{dose}$ of CPs1) were administered in each immunization, while the control group, immunized with the corresponding mixtures of CPs 1, 6B, and 18C, received 10 $\mu\text{g}/\text{doses}$ of CPs. The extracted blood samples were incubated at 37°C for 30 min, cooled to -20°C , and centrifuged at 1500 rpm for 5 min and the sera were conserved at -20°C until the moment of the immunologic evaluation. The experimental protocols were approved by the Committee of Ethics for the Experimentation with Lab Animals and the Quality Department of the Finlay Institute of Vaccines.

ELISA Analysis. Determination of the Recognition of the CPs and Carrier Protein on the Glycoconjugates by ELISA. Maxisorp 96-well microtiter plates were coated with the glycoconjugates containing CPs 1, 6B, 14, and 18C (1 $\mu\text{g}/\text{mL}$ of protein), the CPs of their respective serotypes (10 $\mu\text{g}/\text{mL}$), and the protein TT (1 $\mu\text{g}/\text{mL}$) in a coating buffer containing NaHCO_3 (pH 9.6) (50 $\mu\text{L}/\text{well}$) and were incubated overnight at 4°C . Every sample was applied to the plate in duplicate. Then, a blocking solution of BSA (1% in PBS pH 7.2) (150 $\mu\text{L}/\text{well}$) was applied and maintained for 30 min at 37°C . Specific serotype sera from Statens Serum Institute obtained in rabbits, type serum 1 (lot K111A1), type serum 14 (lot M1412A114), factor serum 6c (6B) (lot V6c11A1), and group serum 18 (18F, 18A, 18B, 18C) (lot M1812A3) were used. For the recognition of the protein TT, a polyclonal antibody obtained in rabbit and produced by the Finlay Institute of Vaccines was employed. All specific sera were diluted [1:10 000 (v/v) for anti-CPs 1, 6B, 18C sera; 1:20 000 (v/v) for anti-CPs 14 serum; and 1 $\mu\text{g}/\text{mL}$ for anti-TT serum] in a buffer (PBS pH 7.2, Tween₂₀ 0.3%, EDTA 0.01 mol/L, BSA 1%) (100 $\mu\text{L}/\text{well}$), and the plates were incubated for 90 min at room temperature. Anti-rabbit IgG (whole molecule) serum conjugated to peroxidase (lot 040M-6150) (Sigma-Aldrich) (100 $\mu\text{L}/\text{well}$) in a dilution of 1:20 000 (v/v) was

then added, and the plates were incubated for 90 min at room temperature. At the end, 100 $\mu\text{L}/\text{well}$ of a solution containing the buffer Na_2HPO_4 (52 mmol/L)/citric acid (25 mmol/L, pH 5.6), 3,3',5,5'-tetramethylbenzidine (1 mL), and H_2O_2 (2 μL) was added. The reaction was protected from light, carried out for 20 min, and stopped by adding 50 $\mu\text{L}/\text{well}$ of a solution of H_2SO_4 (3 mol/L). The optical density was determined at 405 nm using an ELISA reader (SUNRISE, Tecan) and the software Magellan 7.0. After each step, the plates were washed with a washing solution (PBS pH 7.2, Tween₂₀ 0.05%) using an automatic washer (MW-2001 ProWash, TecnoSUMA International) and then dried.

Determination of the Antibodies (IgG) toward the CPs and Carrier Protein. The determination of IgG titers induced by the conjugates against CPs serotypes 1, 6B, 14, and 18C from *S. pneumoniae* and TT was made in polystyrene 96-well microtiter plates (Maxisorp, NUNC). The plates were coated with natural CPs (10 $\mu\text{g}/\text{mL}$) or TT (1 $\mu\text{g}/\text{mL}$) dissolved in PBS pH 7.2 (50 $\mu\text{L}/\text{well}$) and incubated overnight at 4°C . All plates were washed with a solution of PBS (pH 7.2, Tween₂₀ 0.05%). Then, 150 $\mu\text{L}/\text{well}$ of a blocking solution of BSA (2%, in PBS pH 7.2) was added, and the plates were incubated at 37°C for 30 min. After washing, sera from the immunized mice were applied (100 $\mu\text{L}/\text{well}$) in serial dilutions (1/2) starting from 1:100 (v/v) in a buffer (PBS pH 7.2, Tween₂₀ 0.05%, EDTA 0.01 mol/L, BSA 1%), and the plates were incubated for 90 min at room temperature. Depending on the sample from mice or rabbit, 100 $\mu\text{L}/\text{well}$ of anti-mouse IgG (whole molecule) conjugated to peroxidase (lot RG7201 V) (Sigma-Aldrich) or anti-rabbit IgG conjugated to peroxidase (lot 040M-6150) (Sigma-Aldrich) in the corresponding dilution was added, and then the plates were incubated for 90 min at room temperature. After washing, 100 $\mu\text{L}/\text{well}$ of a solution containing buffer Na_2HPO_4 (52 mmol/L)/citric acid (25 mmol/L, pH 5.6), 3,3',5,5'-tetramethylbenzidine (1 mL), and H_2O_2 (2 μL) was added. The reactions were carried out during 20 min in the darkness and were stopped by adding 50 $\mu\text{L}/\text{well}$ of a solution of H_2SO_4 (3 mol/L). The optical density at 405 nm was determined using an ELISA reader (SUNRISE, Tecan) and the software Magellan 7.0. After each step, the plates were washed with a washing solution (PBS pH 7.2/Tween₂₀ 0.05%) using an automatic washer (MW-2001 ProWash, TecnoSUMA International) and then dried. The IgG titers against CPs were defined as the logarithm of the highest dilution giving 2- or 5-fold the absorbance value of preimmune sera, from rabbits or mice immunization, respectively.

Statistical analyses were performed using Graph Pad Prism 7.4. First was applied the Kruskal–Wallis nonparametric test and then the uncorrected Dunn's multiple comparison test.

Opsonophagocytic Killing Assays (OPKAs). The OPKAs were designed on the basis of the protocol described by Romero-Steiner and co-workers in 1997,³⁰ using human polymorphonuclear leukocytes as effector cells. The selected samples for the assay were the individual sera from the three rabbits of each group (groups immunized with 6B–1–TT, 6B–TT + 1–TT, 18C–1–TT, and 1–TT + 18C–TT) and the sera from the eight mice pooled in four pairs of each group (groups immunized with 18C–14–TT and 14–TT + 18C–TT). All the selected sera were those of the last immunization, in which all the correspondent animals were responsive. Heat-inactivated mouse and rabbit sera were serially diluted in 2-fold steps starting from 1:4 (v/v) in a 96-well microtiter plate (round-bottom, COSTAR) with opsono buffer [Hank's buffer

(Sigma) containing 0.1% gelatin] and were incubated with cells of *S. pneumoniae* serotypes (~1000 CFU per well) during 15 min at 37 °C in a 5% CO₂ atmosphere. Complement from baby rabbit serum (Pel-Freez Biological) and peripheral polymorphonuclear leukocytes purified from human blood was added at 4×10^5 cell per well. The mixture was incubated at 37 °C for 45 min with shaking (90 rpm). The reaction was stopped with ice-cooling for 5 min. After the phagocytic step, an aliquot of 5 μ L was extracted and applied onto an agar plate covered with a tilted Todd Hewitt yeast extract, and the plate was incubated at 37 °C with 5% CO₂ atmosphere for 18 h. OPKA titers were calculated as the reciprocal of the sera dilution that caused a 50% reduction of the CFU (killing) compared with the CFU from the control wells containing all reagents except for the mouse sera, and the dilution value of 1:8 was selected as a threshold.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00423>.

HPLC chromatograms, NMR spectra, and bioconjugation protocols for each specific glycoconjugate (PDF)

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Notes

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