Bioconjugate Chemistry

Communication

pubs.acs.org/bc

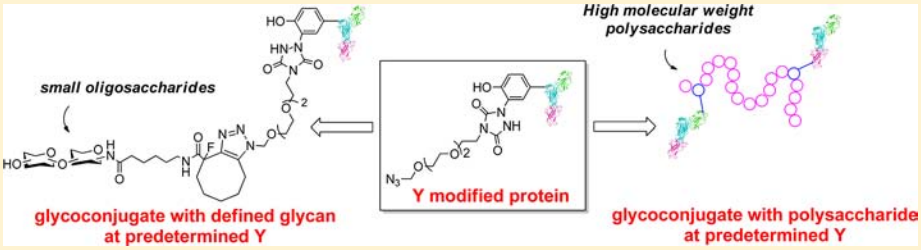
Tyrosine-Directed Conjugation of Large Glycans to Proteins via Copper-Free Click Chemistry

Alberto Nilo,[†] Martin Allan,[‡] Barbara Brogioni,[†] Daniela Proietti,[†] Vittorio Cattaneo,[†] Stefano Crotti,[†] Samantha Sokup,[‡] Huili Zhai,[‡] Immaculada Margarit,[†] Francesco Berti,[†] Qi-Ying Hu,[‡,*] and Roberto Adamo*[,†]

[†]Novartis Vaccines & Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

[‡]Novartis Institutes for BioMedical Research, 100 Technology Square, Cambridge, Massachusetts 02139, United States

Supporting Information



ABSTRACT: We have demonstrated that the insertion of alkyne-containing bifunctional linkers into the tyrosine residues of the carrier protein, followed by the copper mediated azide−alkyne [3 + 2] cycloaddition of carbohydrates, is a robust approach for the preparation of glycoconjugates with defined glycans, carrier, and connectivity. Conjugation of Group B *Streptococcus* (GBS) capsular polysaccharides to streptococcal pilus protein could extend the vaccine coverage to a variety of strains. Application of our protocol to these large charged polysaccharides occurred at low yields. Herein we developed a tyrosine-directed conjugation approach based on the copper-free click chemistry of sugars modified with cyclooctynes, which enables efficient condensation of synthetic carbohydrates. Most importantly, this strategy was demonstrated to be more effective than the corresponding copper catalyzed reaction for the insertion of GBS onto the tyrosine residues of GBS pilus proteins, previously selected as vaccine antigens through the so-called *reverse vaccinology*. Integrity of protein epitopes in the modified proteins was ascertained by competitive ELISA, and conjugation of polysaccharide to protein was confirmed by SDS page electrophoresis and immunoblot assays. The amount of conjugated polysaccharide was estimated by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The described technology is particularly suitable for proteins used with the dual role of vaccine antigen and carrier for the carbohydrate haptens.

INTRODUCTION

Site-selective bioconjugation methods represent powerful tools to generate novel therapeutic proteins, molecular probes, targeted delivery systems, and bionanomaterials.[1,2] Glycosylation of proteins can profoundly affect the pharmacokinetics and activity of protein-based medicines.[3] Glycans are poorly immunogenic; however, conjugation to protein carriers ensures elicitation of a carbohydrate specific T-cell-dependent memory response.[4] Carbohydrate-based vaccines are often produced by coupling the saccharides to the surface abundant lysine residues of the protein carrier, but regioselectivity of the conjugation is hard to achieve. Thus, batch-to-batch variability in the immunological properties can be introduced. In contrast, tyrosine residues are usually far less present and accessible than lysine residues, and hence regioselective conjugation can be expected. We have recently developed a robust tyrosine-selective amination via a triazolidinone-ene reaction in Tris buffer.[5] The method enabled the creation of an anti-candidiasis vaccine through Cu(I) catalyzed azide−alkyne [3 + 2] cycloaddition (CuAAC)[6,7] of a synthetic β-glucan onto specific tyrosine residues of the genetically detoxified diphtheria toxin CRM_{197}. By this strategy we obtained a detailed structure−immunogenicity relationship of glycoconjugates with defined sugars at predetermined sites, and deciphered the contribution of the glycan, the linker, and the attachment site on their immune properties.[8]

A few carrier proteins, such as CRM_{197}, diphtheria (DT), and tetanus toxoid (TT) are generally employed for licensed vaccines or candidates under clinical development.[9] The use of the same carriers for multivalent vaccines or subsequent vaccinations can in some cases interfere with the antipolysaccharide immune response.[10−12] Therefore, the identification of novel carrier proteins is a relevant topic for the development of the vaccines.

Group B *Streptococcus* (*Streptococcus agalactiae* - GBS) is a Gram positive pathogen that causes severe invasive neonatal

Received: September 22, 2014
Revised: October 31, 2014
Published: November 19, 2014

   dx.doi.org/10.1021/bc500438h | *Bioconjugate Chem.* 2014, 25, 2105−2111

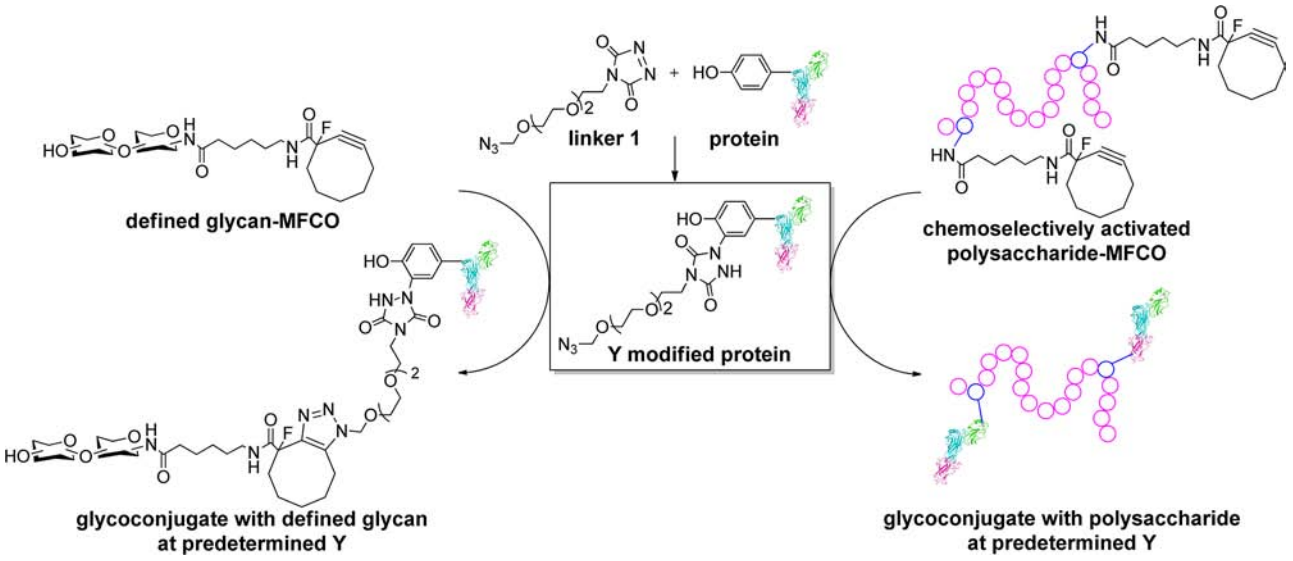


Figure 1. Synthesis of glycoconjugates by tyrosine-ligation copper-free azide−alkyne [3 + 2] cycloaddition.

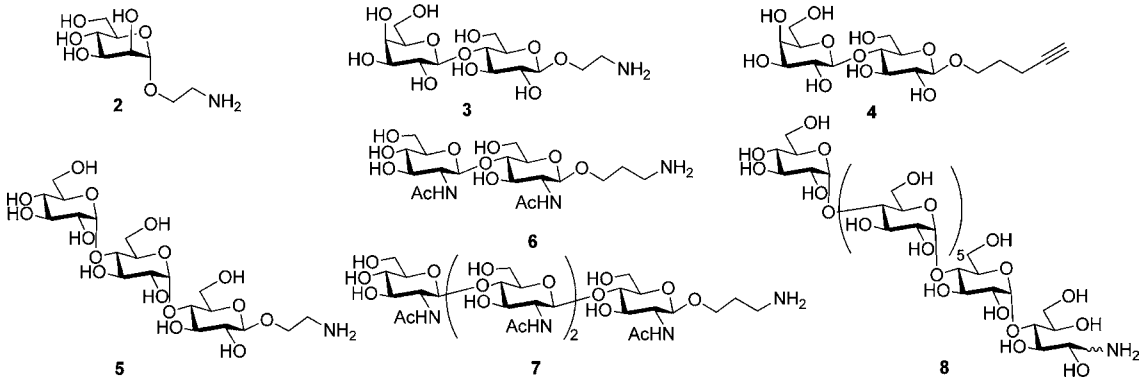
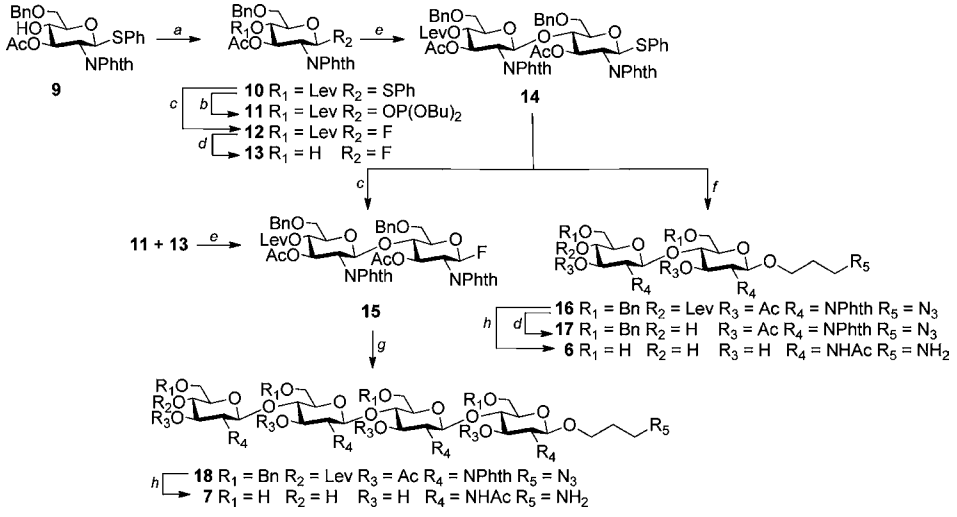


Figure 2. Structures of the defined glycans used for the tyrosine ligation via copper-free azide−alkyne [3 + 2] cycloaddition.

Scheme 1. Reactions Leading to Chitin Dimer 6 and Tetramer 7[a]

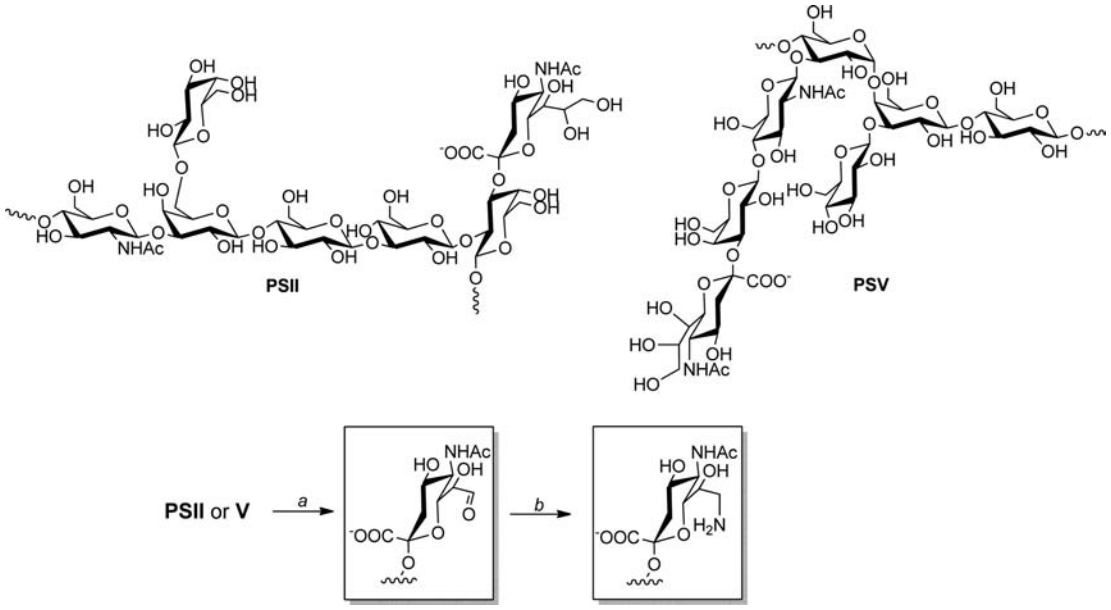


[a]Reagents and conditions: a. Levulinic acid, DMAP, DCC, CH_2Cl_2, 91%; b. $HOPO(OBu)_2$, NIS-TfOH, CH_2Cl_2, −40 °C to rt, 87%; c. DAST-NBS, CH_2Cl_2, −10 °C to rt, 80% from **10**, 65% from **14**; d. H_2NNH_2.AcOH, CH_2Cl_2, 85% from **12**, 94% from **16**; e. TMSOTf, **9**, CH_2Cl_2, −30 °C to rt, 93%; f. $HO(CH_2)_2N_3$, CH_2Cl_2, −40 °C to rt, 69%; g. Cp_2HfCl_2−AgOTf, CH_2Cl_2, −10 °C to rt, 55%; h. $NH_2CH_2CH_2NH_2$, EtOH, 60 °C; Ac_2O, MeOH; NH_3, MeOH; H_2, 5% Pd−C, MeOH, 64% from **16**, 53% from **18**.

infections, such as pneumonia, septicemia, and meningitis.[13] Of the 10 serotypes identified on the basis of the capsular polysaccharide (PS) structure,[14,15] five (Ia, Ib, II, III, and V) are commonly associated with human diseases. A glycoconjugate

dx.doi.org/10.1021/bc500438h | Bioconjugate Chem. 2014, 25, 2105−2111

Scheme 2. Modification of PSII or V[a]



[a]Reagents and conditions: a. $NaIO_4$, 10 mM NaPi; b. NH_4OAc, $NaCNHB_3$, 5 mM NaOAc, 60−65%.

vaccine comprising conjugates of PS Ia, Ib, and III, which covers the majority of the infections, is currently under phase II clinical trial.[16,17] After genome sequencing of GBS strains, and high-throughput expression and testing of a number of surface proteins by the *reverse vaccinology* approach,[18] pilus proteins have been found to protect immunized mice against a lethal challenge with GBS.[19,20] The conjugation of the capsular polysaccharides to proteins of the same pathogen could be a general strategy to broaden the strain coverage of glycoconjugate vaccines.

In the newly developed tyrosine-ligation we envisaged a powerful method to direct polysaccharide conjugation at predetermined sites of the protein, and ensure higher batch-to-batch consistency in comparison to classic nonspecific conjugation procedures, particularly when the protein is used with the dual role of antigen and carrier.

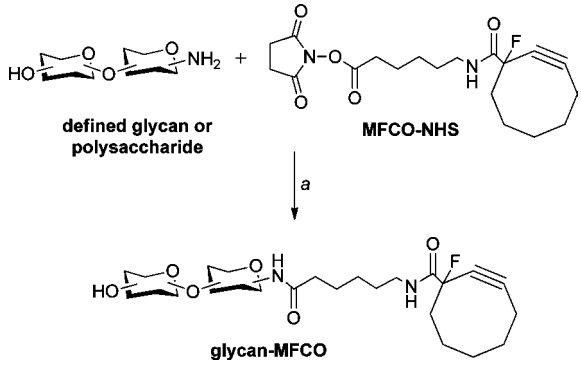
To our surprise, preliminary attempts to conjugate high molecular weight negatively charged GBS polysaccharides to the tyrosine residues of CRM_{197} by CuAAC provided the desired glycoconjugates at poor yield. Conversely, condensation of 4-pentinyl lactose **4**[21] (Figure 2) was successful, indicating that our method, which was proven very efficient with small−medium-sized oligosaccharides,[5] was not suitable for larger polysaccharides, such as the ones from GBS. We reasoned that the CuAAC could be hampered by the reduced accessibility of the catalyst to these large coupling partners.[22]

We, therefore, hypothesized that strain-promoted azide−alkyne [3 + 2] cycloaddition (SPAAC),[23−25] which benefits of high rate of the cycloaddition bypassing the need for a catalyst,[26] would represent an optimal approach for the conjugation of this type of polysaccharides. Herein, we report a two-step strategy for copper-free conjugation of defined glycans equipped with a cyclooctyne arm to predetermined tyrosine residues of CRM_{197} and GBS pilus proteins modified with a novel azide-linker, and its utilization for the coupling of streptococcal polysaccharides (Figure 1). Integrity of protein epitopes in the modified proteins was ascertained by competitive ELISA, and covalent attachment of protein and polysaccharide in the glycoconjugates was confirmed by immunoblot assays. The conjugated polysaccharide was quantified by HPAEC-PAD.

RESULTS AND DISCUSSION

To develop a general strategy suitable for tyrosine directed conjugation of both well-defined glycans and complex polysaccharides through SPAAC (Figure 1), we initially designed and synthesized a set of different length sugars bearing an amine function for coupling to *N*-hydroxysuccinimyl ester of the monofluoro-cyclooctyne (MFCO)[23] linker (Figure 2).

Scheme 3. Insertion of the MFCO Linker on the Carbohydrates[a]



[a]Reagents and conditions: MFCO-NHS, DMSO or 9:1 DMSO-H_2O, TEA, 80−90%.

Compounds **2**−**5** and **8** were prepared by standard manipulations of commercially available sugars (as described in Supporting Information).[27] To further test our approach in the context of a convergent total synthesis of glycoconjugates, the β-(1 → 4)-chitin dimer **6** and tetramer **7** were assembled as depicted in Scheme 1. These structures are biologically relevant, since chitins have been found in the inner core of

dx.doi.org/10.1021/bc500438h | *Bioconjugate Chem.* 2014, 25, 2105−2111

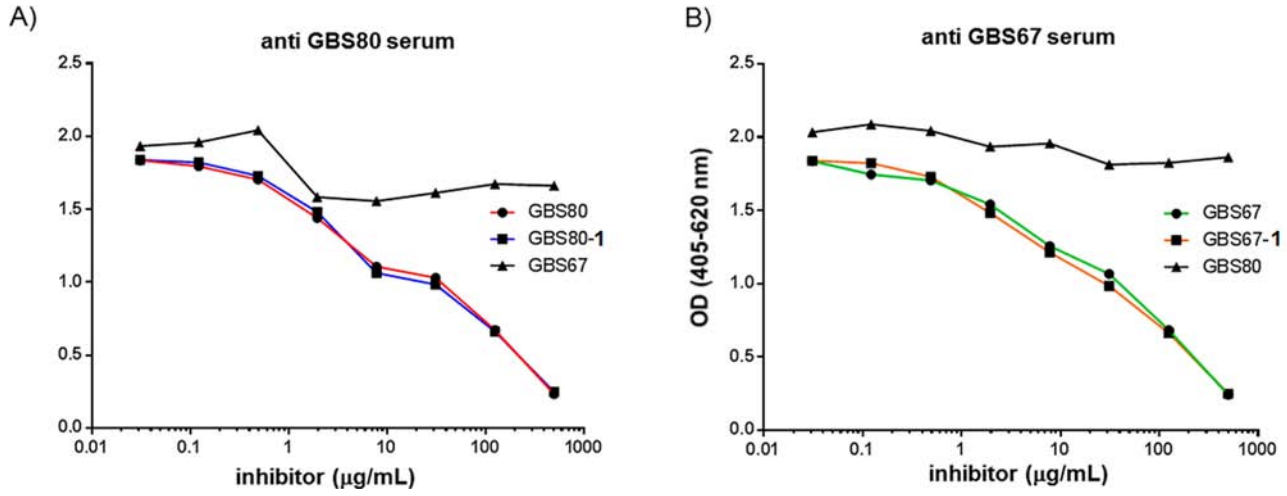


Figure 3. Competitive ELISA of the binding between specific antiprotein polyclonal sera and the protein as coating reagent, using unmodified and labeled proteins as inhibitors. (A) GBS80 and GBS80-**1** are equipotent inhibitors of the binding (IC_{50} = 15 and 10 μg/mL, respectively); GBS67 is the negative control. (B) GBS67 and GBS67-**1** are equipotent inhibitors of the binding (IC_{50} = 33 and 18 μg/mL, respectively); GBS80 is the negative control.

Table 1. Conjugation of Different Length Oligosaccharides to the Proteins

	glycan loading (coupling efficiency %)		
glycan[a]	CRM_{197}	GBS80	GBS67
2	3.5 (>95%)	2.0 (90%)	3.3 (94%)
3	3.5 (>95%)	2.0 (>95%)	3.5 (>95%)
4	nd[b]	2.0 (>95%)	nd[b]
5	3.5 (>95%)	2.0 (>95%)	3.5 (>95%)
6	3.5 (>95%)	2.0 (95%)	3.5 (>95%)
7	3.5 (>95%)	2.0 (>95%)	3.1 (89%)
8	3.5 (>95%)	2.0 (>95%)	2.8 (85%)

[a]All glycans were coupled to MFCO-NHS prior to conjugation as described in Supporting Information , with the exception of **4**, which was directly coupled via CuAAC. [b]not determined.

the fungal cell wall[28] and as part of bacterial peptidoglycan.[29] The assembly of the β-(1 → 4)-oligoglucosamines is particularly challenging due to the presence of the deactivating acetamido group at the C-2 position of each repeating unit.[30,31] Our synthesis commenced from the known thioglycoside **9**,[31] which was used as acceptor for trimethylsilyl trifluoromethanesulfonate (TMSOTf) promoted glycosylation with **11**, obtained by levulinoylation at the C-3 hydroxyl group and following conversion to phosphate donor, to give disaccharide **14**.[32]

The dimer **6**, derivatized at the end terminus with an arm for conjugation, was prepared by reaction of **14** with 3-azidepropanol in the presence of N-iodosuccinimide (NIS)/Trifluoromethanesulfonic acid (TfOH) and a subsequent series of deprotecting steps that include removal of N-phthalamido groups, reacetylation, methanolysis of the acetyl esters, and final debenzylation. The assembly of the tetramer **6** was achieved from the disaccharide acceptor **17**, readily available by 3-*O*-develvulinoylation of **16**, through a [2 + 2] convergent approach. For this purpose, the disaccharide fluoride donor **15** was prepared from thioglycoside **14** by treatment with diethylaminosulfur trifluoride (DAST)/*N*-bromosuccinimide (NBS)[33] or, alternatively, by chemoselective TMSOTf promoted glycosylation with phosphate donor **11** of fluoride acceptor **13**, obtained from **10** by reaction with DAST/NBS succeeded by orthogonal removal of the 3-*O*-levulinoyl ester. To the best of our knowledge, this represents the first example of selective glycosylation using phosphate and fluoride partners. Bis(cyclopentandienyl) hafnium(IV) dichloride (Cp_2HfCl_2)/silver trifluoromethanesulfonate (AgOTf) promoted glycosylation of **16** with **15** yielded the protected tetramer **18**. Deprotection of **18**, similarly to dimer **6**, afforded the target tetrasaccharide **7**.

Insertion of the amine groups in GBS PSII and V for coupling with MFCO-NHS was accomplished by targeting 20% chemoselective periodate oxidation of the NeuAc 9,8-diol, and

Table 2. Characteristics of the Synthesized GBS Glycoconjugates

entry	glycoconjugate	carbohydrate:protein stoichiometry[a] (w/w)	glycosylation ratio[b,c] (w/w)	free saccharide[b] (%)	yield[d] (%)
1	PSII-Y-CRM_{197}	3:1	1.8	<2.8	41
2	PSII-Y-GBS80	6:1	2.7	<1.8	36
3	PSII-Y-GBS80[e]	6:1	1.9	13.5	9
4	PSII-Y-GBS80	1:1	1.1	<4.5	32
5	PSII-Y-GBS67	4:1	1.3	<3.8	32
6	PSV-Y-GBS80	4:1	2.2	<5.5	37
7	PSV-Y-GBS67	4:1	2.3	<5.1	24

[a]Ratio of reagents used in the conjugation reaction. [b]Carbohydrate:protein ratio in the purified glycoconjugate. [c]Conjugated and unconjugated PS in the purified products were estimated by HPAEC-PAD quantification of Gal for PSII adducts or GlcNAc for PSV adducts. [d]Determined by micro-BCA quantification of purified glycoconjugate vs the starting protein. [e]This conjugate was prepared by CuAAC.

dx.doi.org/10.1021/bc500438h | *Bioconjugate Chem.* 2014, 25, 2105−2111

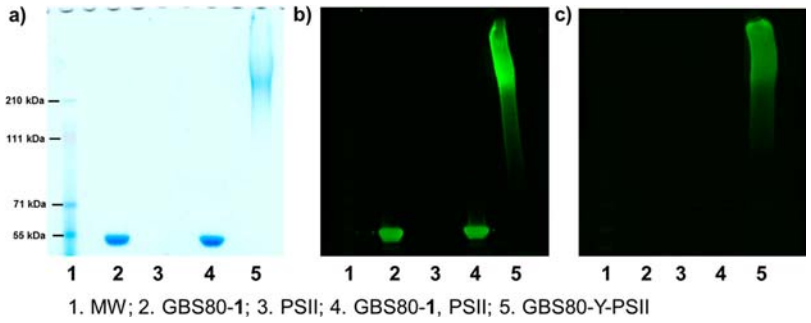


Figure 4. Example of immunoblot characterization of GBS glycoconjugates. (a) SDS page of GBS80-Y-PSII (entry 4; Table 1), using the labeled protein, PS, and their mixture as controls; (b) WB analysis with antiprotein murine sera shows staining for the starting protein, its mixture with the sugar and the glycoconjugate; (c) WB analysis with anti-PS murine sera highlights staining only for the glycoconjugate, clearly confirming the covalent linkage of PS with the protein.

subsequent reductive amination of the generated aldehyde intermediate (Scheme 2).[34]

Both defined glycans and polysaccharides were then coupled to MFCO active ester to deliver the molecules ready for strain-promoted coupling to the labeled proteins (Scheme 3).

For the tyrosine-directed modification of the proteins, linker **1** was designed without the phenyl group present in our first generation of spacers, which we have shown to induce unwanted antibodies.[8] Incubation of CRM_{197} with linker **1** (prepared as described in Supporting Information), under the conditions previously reported,[5] gave the modified protein with an average incorporation of 3.5 azides, as determined by LC-MS analysis. As expected, MS/MS analysis of proteolytic digests showed, in agreement with our previous findings, that Y27, Y46, Y358, and Y380 were modified among the 18 tyrosine residues totally available in CRM_{197}.[5,8]

Having demonstrated that insertion of linker **1** proceeded uneventfully as reported for similar tyrosine modifications, we directed our attention to the GBS pilus proteins, GBS80 and GBS67. GBS assembles two types of pili on its surface that mediate bacterial adherence to host cells.[35] Two pilus island (PI) variants, PI-1 and PI-2, have been described, with the latter differentiated into components 2a and 2b.[36] GBS80 is a three-domain protein, which constitutes the major component of GBS PI-1 pilus,[37] while GBS67 is an ancillary highly conserved four-domain protein of 2a pilus.[38]

GBS80 and GB67 were modified with **1** in a fashion similar to CRM_{197}, and an average labeling of 2 and 3.5 tyrosine residues, respectively, was ascertained by LC-MS. Semi-quantitative MS analysis of peptide digests enabled coverage of 17 of the 18 tyrosine residues of GBS80 and identification of Y16, Y23, Y44, and Y135 as the modified sites (Table S1, Supporting Information). Interestingly, in the case of GBS67 36 of the 39 tyrosine residues were covered, and Y744 resulted in a higher level of modification, while Y282/283, Y336/337, and Y403 were modified at a lower extent (Table S2, Supporting Information).

By competitive ELISA we ascertained that the modification of the tyrosine residues did not impair the exposition of peptide epitopes onto GBS80 or GBS67, as unmodified and modified proteins were comparably strong as inhibitors of the binding of polyclonal antiprotein murine sera to the immobilized proteins (Figure 3).

Following our main purpose, we first developed a procedure for conjugation of the defined glycans **2**–**8** with the MFCO linker installed. In spite of the moderate constant rate reported for the MFCO in SPAAC,[23] a good progression of the reaction was observed already in 1 h by mixing the labeled protein at 300 μM azide concentration (~5 mg/mL protein concentration) with a 10-fold excess of MFCO-glycan (Supporting Information Figure S1). In general, overnight incubation of the labeled proteins at 200–300 μM azide concentration with a 2-fold excess of MFCO-glycan was sufficient to achieve complete reaction regardless of the sugar length (Table 1; Supporting Information Figures S2–S6 for SDS page electrophoresis and MALDI TOF MS).[39] Although cycloalkynes enabling more expeditious click reactions have been recently described,[25] we deemed these conditions satisfying for the preparation of the target GBS PS conjugates.

Next, we tested the optimized protocol in the conjugation of GBS PSII and PSV to the different proteins. Modified proteins (~5 mg/mL) and MFCO-activated polysaccharides were gently shaken overnight at different w/w ratios, as summarized in Table 2. After chromatography of the produced glycoconjugates on the hydroxyapatite column, the amount of conjugated and unconjugated sugar in the final products was estimated by HPAEC-PAD (Table 2).[40] Notably, when the same stoichiometry of polysaccharide and protein was applied, conjugation by SPAAC gave the GBS-PSII construct at higher yield in comparison to CuACC (36% vs 9%; Table 2, entries 2 and 3). The amount of condensed polysaccharide was proportional to that used in the conjugation step (Table 2, entries 3 and 4).

For instance, a 1:1 carbohydrate–protein ratio (w/w) during the coupling step (~4:1 mol MFCO/mol azide) was sufficient to attain the glycoconjugate PSII-GBS80 with a final glycosylation degree of 1.1 (w/w). Incubation of polysaccharide and protein in 3–4:1 ratio (w/w) (12–16:1 mol MFCO/mol azide) guaranteed a higher glycosylation degree (1.8–2.3:1, w/w).

The covalent linkage between the protein and polysaccharide was confirmed by Western blot (WB) analysis (Figure 4 and Figure S7–S9 of Supporting Information), demonstrating that only glycoconjugates were stained concomitantly by specific antiprotein and antipolysaccharide murine sera, when compared to the protein, the polysaccharide, and their physical mixture as controls.

■ CONCLUSIONS

We described the development of a two-step conjugation strategy based on copper-free [3 + 2] cycloaddition of sugars modified with a cyclooctyne and proteins derivatized at the tyrosine residues with the novel azido-linker **1**. This method was first proven efficient in the preparation of glycoconjugates

dx.doi.org/10.1021/bc500438h | Bioconjugate Chem. 2014, 25, 2105–2111

with defined attachment point from minimal amounts of synthetic carbohydrates, whose preparation generally requires multistep syntheses and substantial effort. Next, the novel approach was successfully applied to the preparation of glycoconjugates from GBS PSII and PSV and the pilus proteins GBS80 and GBS67, previously selected as vaccine antigens through the *reverse vaccinology* approach. By competitive ELISA we verified that the proteins are not affected by the selective ligation of the linker onto the tyrosine residues. The covalent linkage of the saccharides and the protein was ascertained by immunoblot analysis. This technology appears very appealing for the conjugation of carbohydrate haptens to protein antigens,[41] as it ensures high consistency in the conjugation step and easily processed MS analysis of the modified protein whose functionality needs to be preserved. This approach will be useful to extend the coverage of glycoconjugate vaccines. Ongoing immunological evaluation of the prepared candidates will be presented in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Protocols for the synthesis of the defined glycans **2**−**8** and linker **1**, protocols for modification of the polysaccharides and proteins, ESI MS spectra of modified proteins and determination of labeled sites, procedures for conjugation of defined glycans and polysaccharides, SDS page and MALDI TOF MS spectra of glycoconjugates, protocols for HPAEC-PAD, competitive ELISA and immunoblot analysis, NMR spectra. This material is available free of charge via Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: qiying.hu@novartis.com.

*E-mail: roberto.adamo@novartis.com.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Spicer, C. D., and Davis, B. G. (2014) Selective chemical protein modification. *Nat. Commun. 5*, doi:10.1038/ncomms5740.

(2) Shimanovich, U., Bernardes, G. J. L., Knowlesa, T. P. J., and Cavaco-Paulo, A. (2014) Protein micro- and nano-capsules for biomedical applications. *Chem. Soc. Rev. 43*, 1361−1371.

(3) Wang, L.-X., and Amin, M. N. (2014) Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem. Biol. 21*, 51−66.

(4) Avci, F. Y., and Kasper, D. L. (2010) How bacterial carbohydrates influence the adaptive immune system. *Annu. Rev. Immunol. 28*, 107−110.

(5) (a) Hu, Q.-Y., Allan, M., Adamo, R., Quinn, D., Zhai, H., Wu, G., Clark, K., Zhou, J., Ortiz, S., Wang, B., Danieli, E., Crotti, S., Tontini, M., Brogioni, G., and Berti, F. (2013) Synthesis of a well-defined glycoconjugate vaccine by a tyrosine-selective conjugation strategy. *Chem. Sci. 4*, 3827−3832. (b) Adamo, R., Martin, A., Berti, B., Danieli, E., and Hu, Q.-Y. (2013) Tyrosine ligation process and crosslinking agents used to form tyrosine-contg. polypeptide conjugates for raising an immune response in a mammal, From PCT Int. Appl., WO 2013009564 A1 20130117.

(6) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective ligation of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596−2599.

(7) Tørnoe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-Triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057−3064.

(8) Adamo, R., Hu, Q.-Y., Torosantucci, A., Crotti, S., Brogioni, G., Allan, M., Chiani, P., Bromuro, C., Quinn, D., Tontini, M., and Berti, B. (2014) Deciphering the structure−immunogenicity relationship of anti-Candida glycoconjugate vaccines. *Chem. Sci. 5*, 4302−4311.

(9) Costantino, P., Rappuoli, R., and Berti, F. (2011) The design of semi-synthetic and synthetic glycoconjugate vaccines. *Expert Opin. Drug Discovery 6*, 1045−1067.

(10) Pollabauer, E. M., Petermann, R., and Ehrlich, H. J. (2009) The influence of carrier protein on the immunogenicity of simultaneously administered conjugate vaccines in infants. *Vaccine 27*, 1674−1679.

(11) Barington, T., Skettrup, M., Juul, L., and Heilmann, C. (1991) Non-epitope specific suppression of the antibody response to Haemophilus influenzae type b conjugate vaccine by preimmunization with vaccine components. *Infect. Immun. 61*, 432−438.

(12) Granoff, D. M., Holmes, S. J., Belshe, R. B., Osterholm, M. T., McHugh, J. E., and Anderson, E. L. (1994) Effect of carrier protein priming on antibody responses to Haemophilus influenzae Type b conjugate vaccines in infants. *J. Am. Med. Assoc. 272*, 1116−1121.

(13) Gibbs, R. S., Schrag, S., and Schuchat, A. (2004) Perinatal infections due to Group B streptococci. *Obstet. Gynecol. 104*, 1062−1076.

(14) Madoff, L. C., Paoletti, L. C., and Kasper, D. L. (2006) Surface structures of Group B streptococci important in human immunity. Gram-Positive Pathogens (Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A., and Rood, J. I., Eds) pp 169−185, American Society of Microbiology, Washington, DC.

(15) Berti, F., Campisi, E., Toniolo, C., Morelli, L., Crotti, S., Rosini, R., Romano, M. R., Pinto, V., Brogioni, B., Torricelli, G., Janulczyk, R., Grandi, G., and Margarit, I. (2014) Structure of the type IX Group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. *J. Biol. Chem. 289*, 23437−2348.

(16) Heath, P. T., and Feldman, R. G. (2005) Vaccination against Group B Streptococcus. *Expert Rev. Vaccines 4*, 207−218.

(17) Adamo, R., Nilo, A., Castagner, B., Boutureira, O., Berti, F., and Bernardes, G. J. L. (2013) Synthetically defined glycoprotein vaccines: Current status & future directions. *Chem. Sci. 4*, 2995−3008.

(18) Rappuoli, R. (2000) Reverse vaccinology. *Curr. Opin. Microbiol. 3*, 445−450.

(19) Maione, D., Margarit, I., Rinaudo, C. R., Masignani, V., Mora, M., Scarselli, M., Tettelin, H., Brettoni, C., Iacobini, E. T., Rosini, R., D'Agostino, N., Miorin, L., Buccato, S., Mariani, M., Galli, G., Nogarotto, R., Nardi Dei, V., Vegni, F., Fraser, C., Mancuso, G., Teti, G., Madoff, L. C., Paoletti, L. C., Rappuoli, R., Kasper, D. L., Telford, J. L., and Grandi, G. (2005) Identification of a universal Group B Streptococcus vaccine by multiple genome screen. *Science 109*, 148−150.

(20) Margarit, I., Rinaudo, C. R., Galeotti, C. L., Maione, D., Ghezzo, C., Buttazzoni, E., Rosini, R., Runci, Y., Mora, M., Buccato, S., Pagani, M., Tresoldi, E., Berardi, A., Creti, R., Baker, C. J., Telford, J. L., and Grandi, G. (2009) Preventing bacterial infections with pilus-based vaccines: the Group B Streptococcus paradigm. *J. Infect. Dis. 199*, 108−115.

(21) Crotti, S., Zhai, H., Zhou, J., Allan, M., Proietti, D., Pansegrau, W., Hu, Q.-Y., Berti, F., and Adamo, R. (2014) Defined conjugation of glycans to the lysines of CRM_{197} guided by their reactivity mapping. *ChemBioChem 14*, 836−843.

(22) Meldal, M., and Tornøe, C. W. (2008) Cu-catalyzed azide-alkyne cycloaddition. *Chem. Rev. 108*, 2952−3015.

(23) Agard, N. J., Baskin, J. M., Prescher, J. A., Lo, A., and Bertozzi, C. R. (2006) A comparative study of bioorthogonal reactions with azides. *ACS Chem. Biol. 1*, 644−648.

(24) Hudak, J. E., Barfield, R. M., de Hart, G. W., Grob, P., Nogales, E., Bertozzi, C. R., and Rabuka, D. (2012) Synthesis of heterobifunctional protein fusions using copper-free click chemistry and the aldehyde tag. *Angew. Chem., Int. Ed. 51*, 4161−4165.

(25) Jewett, J. C., and Bertozzi, C. R. (2010) Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev. 39*, 1272−1279.
(26) Kurra, Y., Odoi, K. A., Lee, Y.-J., Yang, Y., Lu, T., Wheeler, S. E., Torres-Kolbus, J., Deiters, A., and Liu, W. R. (2014) Two rapid catalyst-free click reactions for in vivo protein labeling of genetically encoded strained alkene/alkyne functionalities. *Bioconjugate Chem. 25*, 1730−1738.
(27) Šardzík, R., Noble, G. T., Weissenborn, M. J., Martin, A., Webb, S. J., and Flitsch, S. L. (2010) Preparation of aminoethyl glycosides for glycoconjugation. *Beilstein J. Org. Chem. 6*, 699−703.
(28) Mora-Montes, H. M., Netea, M. G., Ferwerda, G., Lenardon, M. D., Brown, G. D., Mistry, A. R., Kullberg, B. J., O'Callaghan, C. A., Sheth, C. C., Odds, F. C., Brown, A. J. P., Munro, C. A., and Gow, N. A. R. (2011) Recognition and blocking of innate immunity cells by Candida albicans chitin. *Infect. Immun.*, 1961−1970.
(29) Vollmer, W., Blanot, D., and de Pedro, M. A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiol. Rev. 32*, 149−167.
(30) Kanie, O., Ito, Y., and Ogawa, T. (1994) Orthogonal glycosylation strategy in oligosaccharide synthesis. *J. Am. Chem. Soc. 116*, 12073−12074.
(31) Yamago, S., Yamada, T., Maruyama, T., and Yoshida, J. (2004) Iterative glycosylation of 2-deoxy-2-aminothioglycosides and its application to the combinatorial synthesis of linear oligoglucosamines. *Angew. Chem., Int. Ed. 43*, 2145−2148.
(32) Plante, O. J., Palmacci, E. R., Andrade, R. B., and Seeberger, P. H. (2001) Oligosaccharide synthesis with glycosyl phosphate and dithiophosphate triesters as glycosylating agents. *J. Am. Chem. Soc. 123*, 9545−9554.
(33) Toshima, K. (2000) Glycosyl fluorides in glycosidations. *Carbohydr. Res. 327*, 15−26.
(34) Wessels, M. R., Paoletti, L. C., Kasper, D. L., Di Fabio, J. L., Michon, F., Holme, K., and Jennings, H. J. (1990) Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III Group B Streptococcus. *J. Clin. Invest. 86*, 1428−1433.
(35) Lauer, P., Rinaudo, C. D., Soriani, M., Margarit, I., Maione, D., Rosini, R., Taddei, A. R., Mora, M., Rappuoli, R., Grandi, G., and Telford, J. L. (2005) Genome analysis reveals pili in Group B Streptococcus. *Science 309*, 105.
(36) Rosini, R., Rinaudo, C. D., Soriani, M., Lauer, P., Mora, M., Maione, D., Taddei, A., Santi, I., Ghezzo, C., Brettoni, C., Buccato, S., Margarit, I., Grandi, G., and Telford, J. L. (2006) Identification of novel genomic islands coding for antigenic pilus-like structures in Streptococcus agalactiae. *Mol. Microbiol. 61*, 126−141.
(37) Vengadesan, K., Ma, X., Dwivedi, P., Ton-That, H., and Narayanan, S. V. L (2010) Purification, crystallization and halide phasing of a Streptococcus agalactiae backbone pilin GBS80 fragment. *Acta Crystallogr. F66*, 1666−1669.
(38) Hendrickx, A. P. A., Budzik, J. M., Oh, S.-Y., and Schneewind, O. (2011) Architects at the bacterial surface - sortases and the assembly of pili with isopeptide bonds. *Nat. Rev. 9*, 167−176.
(39) Dougherty, C. A., Furgal, J. C., van Dongen, M. A., Goodson, T., III, Banaszak Holl, M. M., Manono, J., and DiMaggio, S. (2014) Isolation and characterization of precise dye/dendrimer ratios. *Chem.—Eur. J. 20*, 4638−4645.
(40) van der Put, R. M., de Haan, A., van den IJssel, J. G., Hamidi, A., and Beurret, M. HPAEC-PAD quantification of Haemophilus influenzae type b polysaccharide in upstream and downstream samples. *Vaccine*, DOI: 10.1016/j.vaccine.2014.07.028.
(41) Adamo, R., Berti, F., and Hu, Q.-Y. (2014) Cycloalkyne derivatized saccharides. From PCT Int. Appl., WO 2014111344 A1 20140724.