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On the Preparation of Carbohydrate−Protein Conjugates Using the Traceless Staudinger Ligation

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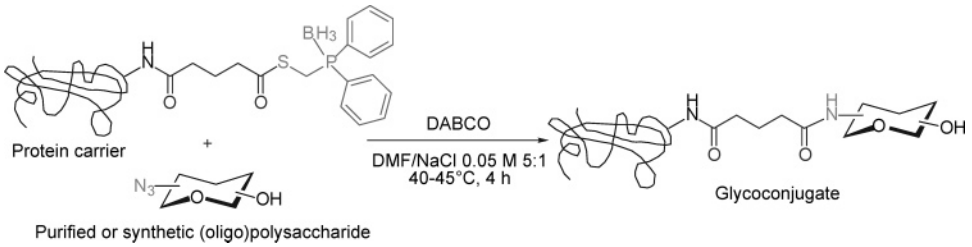

The nature of a linker used for preparing glycoconjugate vaccines is of utmost importance as it may lead to immunogenic biomolecules. We report the conjugation of carbohydrate haptens to protein carriers leading to potential vaccines using the traceless Staudinger ligation. The ligation relies on the selective transfer of a phosphane substituent to an azide to form a native amide bond in the final product upon release of an oxidized phosphane byproduct. We designed new phosphino-functionalized cross-linkers suitable for protein carrier derivatization. We evaluated their utility in preparing conjugates using both synthetic and purified bacterial carbohydrates. The use of a borane-protected phosphane which is deprotected at the time of the ligation reaction led to the best results observed thus far in terms of stability toward oxidation and reactivity.

Introduction

It has long been known that conjugating carbohydrate haptens to appropriate protein carriers often results in biomolecules capable of inducing anti-carbohydrate antibodies.[1] This has stimulated the development of glycoconjugates as potential anti-infectious and anti-cancer vaccines. Some of these are currently licensed as pharmaceuticals.[2]

Numerous methods have been developed to link oligosaccharides covalently to proteins.[3] Ideally, the coupling reaction should be site-selective and efficient. This avoids forming ill-defined glycoconjugates and the resultant loss of costly starting materials. The choice of a method is often restricted by the solubility of starting materials and products in organic solvents as well as by the pH and temperature stability of these materials. The availability of functional groups and the stability and biocompatibility, in particular the absence of immunogenicity of the resulting covalent bond, are also important. Attachment via an amide bond is attractive since this type of bond is ubiquitous in nature. However, the basic amine/carboxylic acid condensation reaction is not often convenient. Indeed, yields are usually low as the activated species are hydrolyzed in a competing side-reaction.[4]

These limitations may be circumvented if the amide link is created by a chemoselective ligation reaction. This is the coupling of two mutually and uniquely reactive

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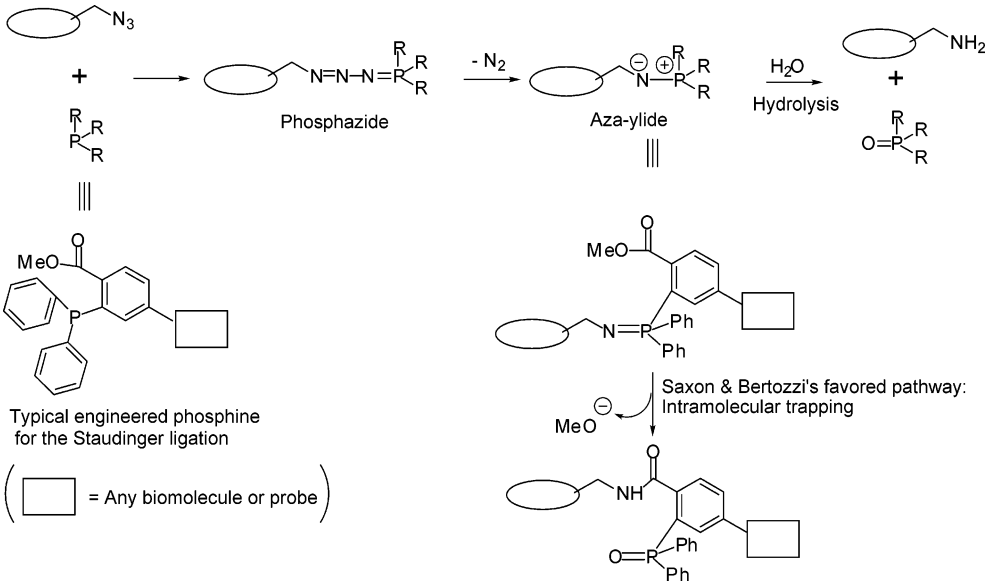
SCHEME 1. Principle of the Staudinger Reaction and of Its Chemoselective Ligation Variant



functional groups on two biomolecules in an aqueous environment.[5] The direct coupling of an azide with a thio acid to give the corresponding amide, in the absence of protecting groups, has been recently reported.[6] However, transition metals, trace amounts of which are difficult to remove from the product, are sometimes required to promote the reaction. Also, the utility of this azide/thio acid condensation for the preparation of biomolecules has not yet been demonstrated.

The Staudinger ligation, which couples two biomolecules bearing an azide and an engineered trivalent phosphine, respectively, has been used successfully for cell surface engineering, for probing post-translational modification, for coating microarrays, and for the blockwise synthesis of peptides and proteins.[7−9] The Staudinger reaction[10] consists of the nucleophilic attack of a phosphine on an azide to give a phosphazide, which through the loss of nitrogen gives a reactive intermediate aza-ylide, which is further hydrolyzed upon addition of water to give a phosphine oxide and an amine.[11] This reaction tolerates a wide range of reactants. It is mainly limited by the sensitivity of the phosphine to oxidation. Careful design of a triarylphosphine allowed Saxon and Bertozzi to trap the aza-ylide intermediate intramolecularly. This avoids shortcut hydrolysis of the aza-ylide in a truly aqueous medium and results in amide-linked products (Scheme 1). One of the three aryl groups on the phosphorus atom is functionalized by an ester, such as a methyl ester, at the position ortho to the phosphorus. The aza-ylide now reacts preferentially via a nucleophilic attack of the nitrogen atom on the ester, eliminating a methoxide, rather than being hydrolyzed. This transformation was so efficient that it was executed at the surface of living cells.[7a]

However, any bioconjugate produced by using this method contains a triarylphosphine oxide residue. The presence of this phosphine oxide may not be suitable for preparing glycoconjugate vaccines, and it is highly desirable to remove it from the actual glycoconjugates. Indeed, this side product possesses all characteristics of a hapten,[1] although it has not yet been tested. Therefore, the desired immune response may be partly diverted or even absent, preventing extensive use of this method in the development of immunogens. For instance, partial diversion of the immune response has been occasionally documented for maleimide spacers:[12] when observed in the series, their immunogenicity was shown to increase from the aliphatic members to the alicyclic ones, with the aromatic linkers being the most immunogenic.

Saxon and Bertozzi[13] and Nilsson et al.[8a] have independently prepared a new generation of phosphines which are released from the conjugated product, in a so-called traceless Staudinger ligation, to give a true amide link, thus allowing broader use of the reaction. In this new generation, the phosphine is incorporated in the leaving group and not in the transferred acyl group (Chart 1).

Previous studies have shown the difficulty in tuning the structure of the phosphine to avoid the two main limitations: premature oxidation of the phosphorus atom,

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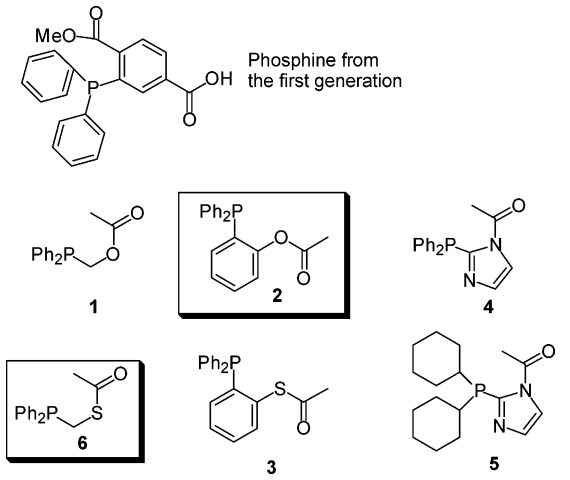
CHART 1. Known Phosphines Designed To Test the Traceless Staudinger Ligation



as in dicyclohexylphosphine **5**, and competitive hydrolysis of the iminophosphorane intermediate, as for **1** and **3**. The use of compound **4**[13] overcame these problems but needed prolonged reaction times, whereas phosphines **2**[13] and **6**[8a] did not need such long reaction times. Therefore, we designed new phosphino-functionalized cross-linkers derived from these two reagents but adapted them to allow construction of potential glycoconjugate vaccines. In this paper, we report the synthesis of such cross-linkers. We assessed their stability toward oxidation when stored either neat or in aqueous solution We evaluated their reactivity in model traceless Staudinger ligations. The most promising reagents were activated as a succinimidyl ester and coupled to tetanus toxoid (TT) or bovine serum albumin (BSA). The resulting phosphine-derivatized carriers were further conjugated to either synthetic bacterial carbohydrates or purified carbohydrates functionalized with a pendent azido group. Finally, we assessed the efficiency of the ligation by determining the carbohydrate/protein ratio and evaluating the antigenicity of the glycoconjugates.

Results and Discussion

Synthesis and Evaluation of Phosphino-Functionalized Cross-Linkers. The synthesis of phosphane cross-linkers bearing a carboxylic function was easily devised as the derivatization of proteins using activated ester reagents is an extremely convenient and well-known technique.[14] Thus, *o*-(diphenylphosphino)phenol **7**, previously used by Saxon et al. as a precursor of compound **2**,[13] was reacted gently with glutaric anhydride to give the carboxy-functionalized phosphine **8** in about 74% yield (Scheme 2). Next, we attempted to prepare the phosphino-thioester **10a** from the known thioacetate **9**.[8b] According to a one-pot, two-step procedure, thioacetate **9** was treated with sodium methoxide and glutaric anhydride, yielding several products. Since a side product resulting from the premature oxidation of the phosphorus atom was isolated, we turned to the borane protected phosphine **11**,[8] a synthetic precursor of **9**. The reaction of **11** under the previously mentioned conditions gave the expected acid **12** in an acceptable 60% yield. Subsequent deprotection with DABCO gave **10a** in a quantitative yield. However, although the phosphorus atom was stabilized by two phenyl groups, this compound was highly susceptible to deleterious oxidation. Isolation of the phosphino-thioester was extremely difficult, with the purified product being partially oxidized to **10b** during analysis.[15]

To avoid a premature oxidation, we used the inductive effect of *p*-chloro substituents on the phenyl groups to stabilize the phosphine. The halogen atoms could also favor intramolecular acylation by increasing the nucleophilic character of the nitrogen atom of the aza-ylide intermediate. Thus, chloromethylphosphonic dichloride was reacted with commercially available (4-chlorophenyl)magnesium bromide as described by Herd et al.[16] to give intermediate **13**, which was then treated with potassium thioacetate to give **14** in 54% overall yield. Potassium thioacetate proved better for the nucleophilic substitution than the previously used thioacetic acid/triethylamine mixture.[8a] Thioester **14** was then deacetylated with sodium methoxide to give an intermediate thiol, which was trapped with glutaric anhydride to afford acid **15** in 53% yield. Reduction of **15** with excess trichlorosilane gave air-stable phosphino-thioester **16**.

We then investigated whether these phosphino-functionalized cross-linkers were suitable for a Staudinger ligation process. In preliminary experiments, we used the known 2-azidoethyl 2-acetamido-2-deoxy-β-D-glucopyranoside **17**[17] as a model azide (Scheme 3).

We obtained the required amide **18** in 30% yield on reaction of phosphine **8**, which was shown to be inert toward oxidation, with azide **17** in CH_3CN/H_2O. As for compound **2**,[13] the reaction proceeded very slowly and reached completion after 96 h. However, unlike for compound **2**, there was predominant hydrolysis of the aza-ylide intermediate resulting in amine **19** being formed in 70% yield (Table 1, entry a). This may be due to increased steric hindrance in the intermediates when replacing the acetyl in **2** by the longer acyl chain in **8**. Merkx et al.[18a] and Bianchi et al.[18b] have recently reported similar results when attempting to synthesize peptides or glycopeptides using closely related C-terminal peptide or amino acid *o*-(diphenylphosphine)phenyl esters.

When the condensation was carried out with the air-stable phosphino-thioester **16**, amide **18** was formed as the major product with only traces of the hydrolyzed product **19**. However, the reaction stopped at 40% conversion, using a 2:1 molar ratio of **16** versus **17**, with the remaining phosphane **16** being completely oxidized (Table 1, entry c). TLC of pure phosphine **16**, in acetonitrile, confirmed its susceptibility toward oxidation. Therefore, we developed a one-pot deprotection and

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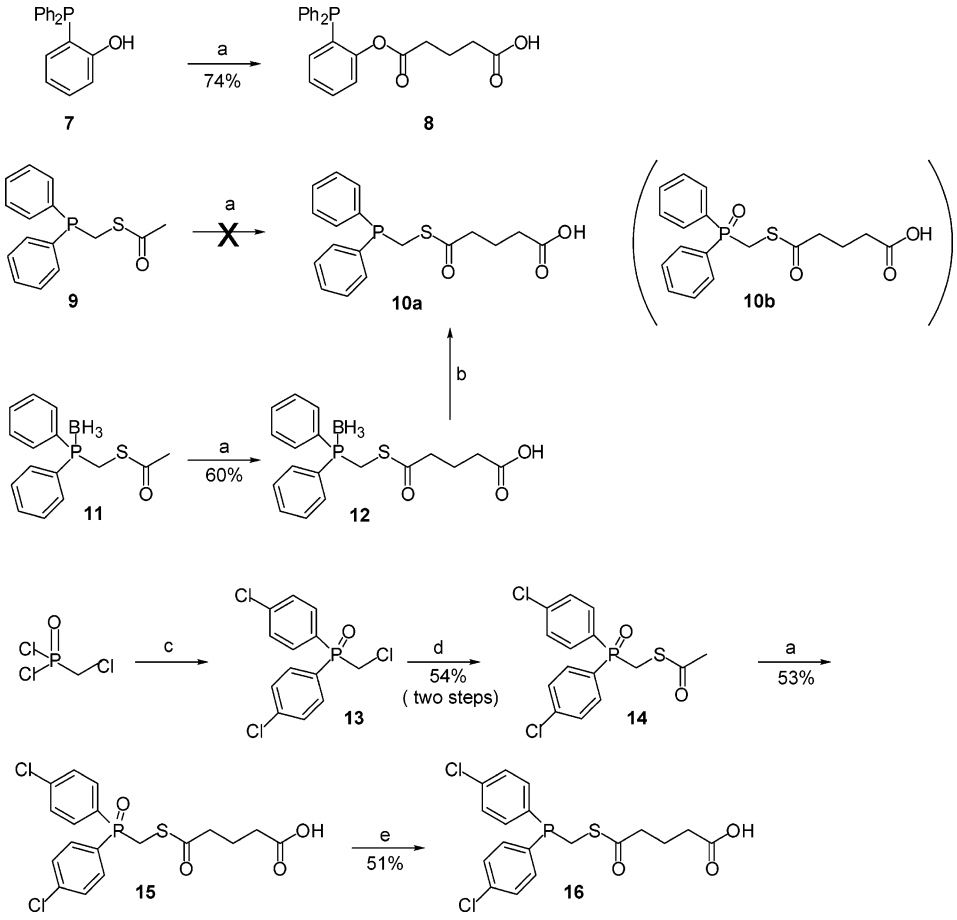
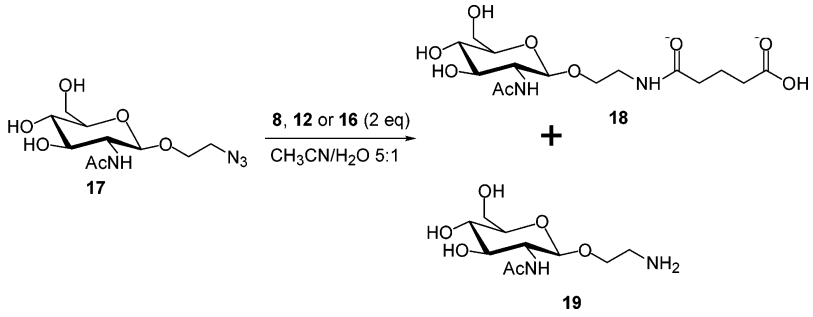
SCHEME 2. Synthesis of Phosphino-Functionalized Crosslinkers[a]



[a] Reagents and conditions: (a) NaOMe (1.1 equiv), then glutaric anhydride (0.9 equiv), DMF, 0 °C to rt, 30 min; (b) DABCO (1 equiv), toluene, 40 °C, 4 h, quantitative; (c) ClPhMgBr (2 equiv), THF, reflux, 24 h; (d) KSAc (1.1 equiv), THF, reflux, overnight; (e) $HSiCl_3$ (15 equiv), $ClCH_2CH_2Cl$, reflux, 72 h.

SCHEME 3. Model Staudinger Ligation



Staudinger ligation procedure using synthetic intermediate **12**, whose phosphorus atom is protected against oxidation. This method has recently been used to prepare lactams. In the lactam synthesis, the phosphine was kept protected as the borane complex until the cyclization step to avoid a spontaneous, premature, Staudinger ligation.[19] As expected, when **17** and **12** were heated in the presence of a slight excess of a strong base, such as DABCO, amide **18** was formed almost quantitatively (Table 1, entry d). Moreover, RP-HPLC showed that phosphine **12** was not significantly degraded over a 1 month period when kept at 4 °C in various aqueous solutions ranging from pH 5.5 to 8.3. Stability in aqueous solution is important as common protein carriers such as TT are kept in aqueous buffers once derivatized. Finally, unreacted phosphane was removed from the carrier by hydrolysis of **12** and **8** with an excess of hydroxylamine (Table 1, last column), to yield the corresponding glutaryl-modified protein.

We selected compounds **12** and **8** for the preparation of glycoconjugates. They were activated into their corresponding succinimide ester **20** and **21** in 90% and 63% yield, respectively (Scheme 4).

Preparation of Carbohydrate−Protein Conjugates. A large excess of heterobifunctional cross-linker **20** was used to derivatize ϵ-amino groups of TT lysines

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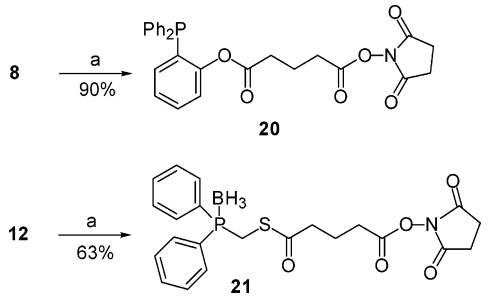
TABLE 1. Properties of the Phosphino-functionalized Crosslinkers

entry	phosphine	stability	yields of products[a] (%) azide **17**	amide **18**	amine **19**	experimental conditions	hydrolysis[b]
a	**8**	stable upon storage neat or in solution		30	70	rt, 96 h	200 equiv of NH_2OH 52% after 1 h 95% after 4 h
b	**10a**	rapidly oxidized[c]					
c	**16**	stable (stored neat) rapidly oxidized in solution	61	37	2	rt, 96 h	
d	**12**	stable (stored neat or in solution)		>95	<5	DABCO (4 equiv), 40 °C, 4 h	50 equiv of NH_2OH, >95% after 1 h

[a] Yield determined by RP-HPLC. [b] Reaction performed in potassium phosphate buffer 0.2 M, pH 8.3. [c] Too unstable to be determined.

SCHEME 4. Activation of Phosphino-functionalized Crosslinkers[a]



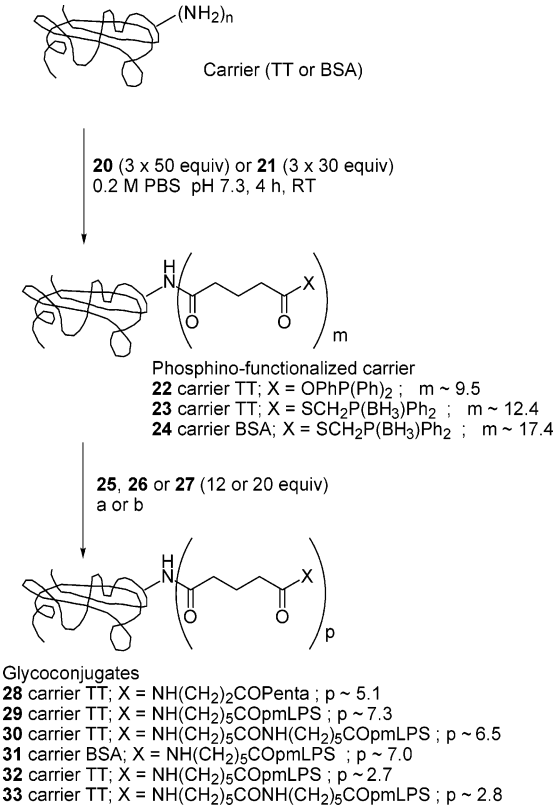
[a] Reagents and conditions: (a) HOSu (1 equiv), EDC (1 equiv), CH_2Cl_2, overnight.

to give the phosphino-functionalized carrier **22**. Analogously, reaction of cross-linker **21** with TT or BSA gave the corresponding protein carriers **23** and **24**, respectively (Scheme 5). Due to its low solubility, we used a somewhat smaller but still large excess of borane−phosphine complex **21**.

We compared the SELDI-TOF (surface enhance laser desorption ionization-time of flight) mass spectra of these different preparations with those of the parent protein to determine the extent of derivatization (Figure 1). The degrees of derivatization were in the range of 10−20 mol of phosphane per mole of protein depending on the assay and on the protein, with a higher substitution being observed for BSA. These rates remained inferior to those we obtained when using either highly hydrophilic or small cross-linkers, such as maleimide-functionalized or bromoacetylated succinimidyl esters. For these cross-linkers, 30−40 linker-to-protein molar ratios were typically observed.[20] After derivatization, **24** was reacted with an excess of hydroxylamine at pH 8.3 and the resulting protein analyzed by SELDI-TOF mass spectroscopy (Figure 1e). This confirmed that the phosphane residues could be completely removed from the protein, resulting in a modified protein bearing only glutaryl chains.

We next prepared carbohydrate haptens bearing a complementary azide group. As a representative example, we selected a synthetic pentasaccharide **34**, corresponding to the biological repeating unit of the O-SP of *Shigella flexneri* 2a LPS (Chart 2).[21]

SCHEME 5. Derivatization and Conjugation of the Protein Carriers[a]



[a] Reagent and conditions: (a) Potassium phosphate buffer 0.1 M, pH 7.4, 47 °C, 6 h; (b) DABCO (40 + 40 equiv), DMF/NaCl 0.05 M 5:1, 40−45 °C, 4 h. *m* and *p* are average values. For further details, see Table 2.

We also used the polysaccharide moiety (pmLPS) of the lipopolysaccharide (LPS) of *Vibrio cholerae* O1 serotype Inaba, a causative agent of cholera. This surface polysaccharide is the major target of the human's protective immune response against this disease.[22] Thus, LPS, purified from bacterial culture, was treated with mild acid treatment to remove the lipid A, giving the corresponding pmLPS **35** in about 25% yield (Chart 2).[20] Both compounds **34** and **35** possess a unique amine functionality which can be used for derivatization. Amines can be

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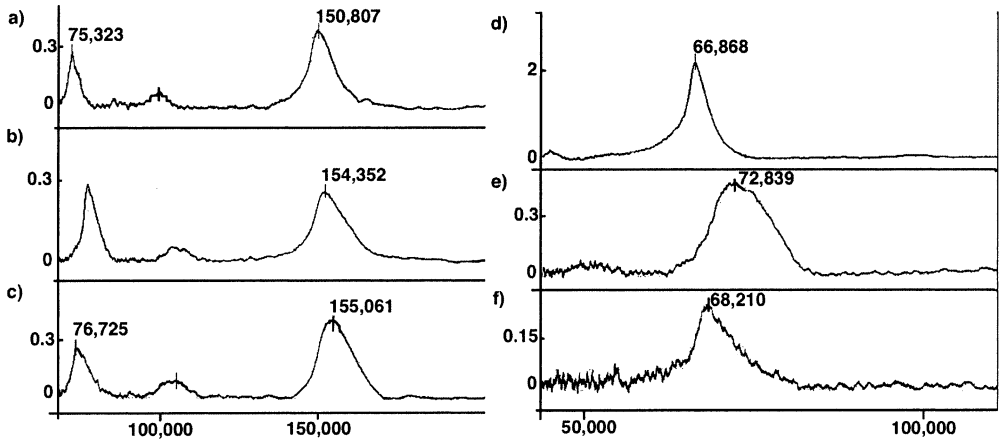
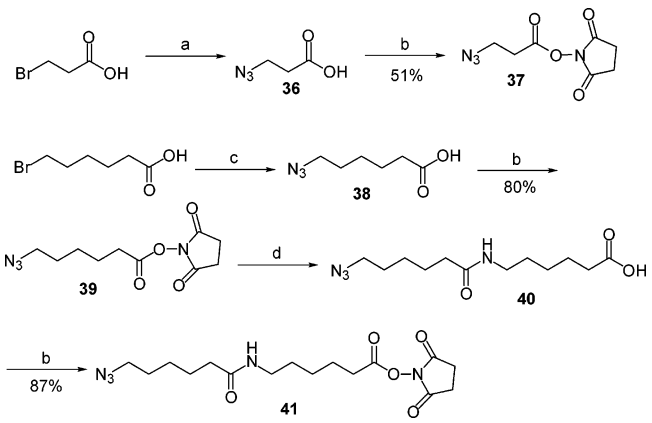


FIGURE 1. SELDI-TOF-MS analysis of TT, BSA, and their phosphino-functionalized derivatives. The average content of phosphine residues was determined on the basis of a 374 or 342 Da mass increment per incorporated molecule of **20** or **21**, respectively: (a) TT; (b) **22**, average ratio phosphine/protein: 9.5; (c) **23**, average ratio phosphine/protein: 12.4; (d) BSA; (e) **24**, average ratio phosphine/protein: 17.4; (f) **24** after NH_2OH treatment for 1 h, theoretical average molecular mass: 68,562 Da. For further details, see the Supporting Information.

SCHEME 6. Synthesis of Azido-Functionalized Linkers[a]



[a] Reagents and conditions: (a) NaN_3 (1.5 equiv), CH_3CN, reflux, 3 h, 51%; (b) HOSu (1 equiv), EDC (1 equiv), CH_2Cl_2, rt, overnight; (c) NaN_3 (2 equiv), DMF, 85 °C, 3 h, 82%; (d) 6-aminocaproic acid (1 equiv), Et_3N (2 equiv), DMF, rt, 4 h, quantitative.

selectively converted into azides by a metal-catalyzed diazo transfer reaction[24] or by coupling to azido-functionalized linkers.[25] Using the latter approach, we reacted 3-bromopropionic acid or 6-bromocaproic acid with sodium azide to give intermediates **36** and **38** in 51% and 82% yield, respectively (Scheme 6). Treatment of these derivatives with *N*-hydroxysuccinimide gave the corresponding activated esters **37** and **39** in 51% and 80% yield, respectively. Partial water solubility of compounds **36** and **37** resulted in a loss of product during workup, explaining the observed modest yields. We then reacted compound **39** with 6-aminocaproic acid to afford derivative **40**, which was transformed into the long *N*-hydroxysuccinimidyl ester spacer **41** in 87% overall yield.

We then coupled **34** with **37** to give the azido-functionalized pentasaccharide (penta-C_3N_3, **25**) in 70% yield. Similarly, we coupled **39** and **41** with pmLPS **35** in PBS (pH 7.3) to give the azido-containing derivatives [pmLPSC_6N_3, **26** and pmLPS$C_{12}N_3$, **27**] in 38 and 58% yield, respectively. We recovered 20−25% of the starting polysaccharide after RP-HPLC purification. We characterized **26** and **27** by MALDI-TOF-MS analysis. Comparison of their MS spectra with that of the parent compound **35** showed an increase of 140 or 253 Da, respectively, for all signals from the polymeric mixture. These were consistent with the introduction of a single azido-linker on **35**.

For the traceless Staudinger ligation we found we could not use a strong base such as DABCO under the previously mentioned conditions to couple **26** or **27** with the derivatized proteins **23** or **24**. We found predominant, premature cleavage of the thioester bond under these conditions. In aqueous buffers, the pH was either too acidic or too basic for the decomplexation to occur. Under basic conditions, hydrolysis was strongly favored compared to the decomplexation−iminophosphorane formation/acyl transfer sequence. However, we found acceptable levels of Staudinger products in the organic medium where the ion pairs are only slightly dissociated and the pH effect is minimized. Activated carriers **23** and **24** tended to precipitate in acetonitrile, which had been used in our initial assays. Therefore, 5/1 (v/v) DMF/aq NaCl 0.05 M was typically used for the conjugation. Thus, haptens **25**, **26** and **27** were successfully coupled to TT derivative **23** under these conditions to give conjugates **28**, **29** and **30**, referred to as TT-C_3Penta, TT-C_6pmLPS$_I$ and TT-C_{12}pmLPS$_I$, respectively. We also conjugated **26** to BSA-derivative **24** to give **31**, referred to as BSA-C_6-pmLPS (Scheme 5 and Table 2). Similarly, phosphino-functionalized TT **22** was reacted with **26** or **27** according to the optimized procedure developed by Kiick et al.[7c] to give conjugates **32** and **33**, referred as TT-C_6pmLPS$_{II}$ and TT-C_{12}pmLPS$_{II}$, respectively (Scheme 5 and Table 2).

All conjugates were isolated between 45% and 80% yields (Table 2). The carbohydrate contents were estimated using a colorimetric method, based on the an-

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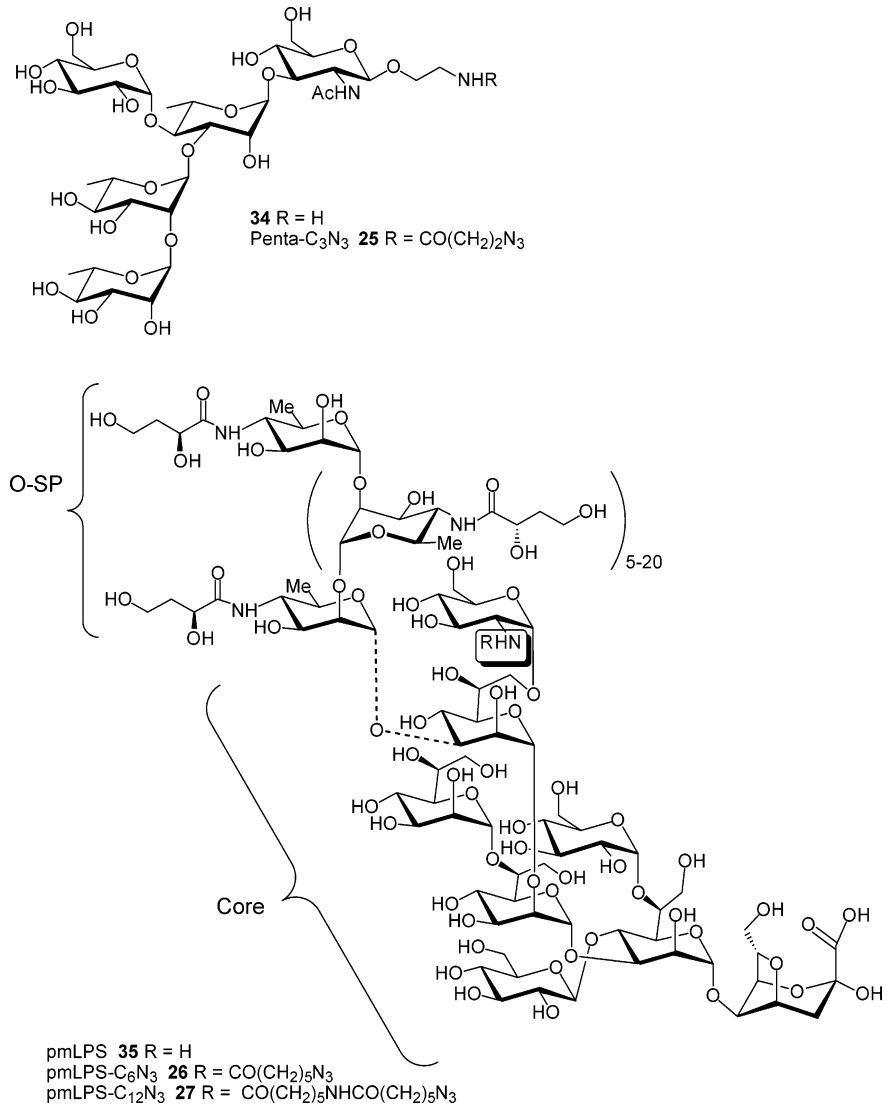
CHART 2. Structures of the Model Carbohydrate Haptens and of Their Azido-Functionalized Derivatives[a]



[a] The pmLPS structure is according to Vinogradov et al.[23] The point of attachment (dotted line) between the specific polysaccharide (O−SP) and the core has not yet been clearly established.

throne reaction.[27] Carbohydrate/protein molar ratios were in the range of 2.7−7.0. These values can be easily explained by the low degree of derivatization of the protein carriers and the small excess of carbohydrate haptens used for the ligation reactions (12−20 equiv/protein). Competitive hydrolysis of the aza-ylide intermediates when using carrier **22** or of the thioester bonds when using carriers **23** or **24** may also account for the overall moderate sugar/protein ratios.

We assessed the antigenicity of derivatives **29**−**33**, obtained from Inaba pmLPS, by ELISA inhibition assays, to test whether the functionality of the carbohydrate haptens was altered upon conjugation (Table 3). All conjugates inhibited the interaction between *V. cholerae* O1 serotype Inaba LPS and the monoclonal antibody I-24-2. This is an IgG3 known to possess a protective activity against *V. cholerae* O1 oral challenge in the suckling mouse model.[28] We could not strictly compare the different conjugates because of the differences in their extent of derivatization. Antigenicity of glycoconjugates **29** and **30** was somewhat lower than antigenicity of Inaba LPS used as a reference compound. This apparent loss may be caused by the use of an organic solvent, namely DMF, and/or heating to perform the conjugation. However, we found that **31**, obtained using the same protocol from BSA instead of TT, as well as **32** and **33** were almost as potent as Inaba LPS. These latter results suggested that, for these glycoconjugates, neither pmLPS derivatization nor the ligation reaction conditions strongly altered the unique antigenic determinant recognized by mIgG I-24-2. This determinant is known to involve residues from both the core and the O-SP.[29]

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TABLE 2. Characterization of the Conjugates

entry	conjugate	carbohydrate hapten	derivatized carrier[a]	method[b]	yield[c] (%)	sugar/protein ratio (mol/mol)
a	**28**	**25**	**23**	A	50	5.1
		pentaC$_3$N$_3$	12.4			
b	**29**	**26**	**23**	A	70	7.3
		pmLPSC$_6$N$_3$	12.4			
c	**30**	**27**	**23**	A	60	6.5
		pmLPSC$_{12}$N$_3$	12.4			
d	**31**	**26**	**24**	A	52	7.0
		pmLPSC$_6$N$_3$	**17**			
e	**32**	**26**	**22**	B	59	2.7
		pmLPSC$_6$N$_3$	9.5			
f	**33**	**27**	**22**	B	83	3
		pmLPSC$_{12}$N$_3$	9.5			

[a] Compound number followed by the extent of derivatization. [b] Method A: DABCO (40 + 40 equiv), DMF/aq NaCl 0.05 M 5:1, 40−45 °C, 4 h. Method B: Potassium phosphate buffer 0.1 M, pH 7.4, 47 °C, 6 h. [c] Yields were calculated on the basis of the weight of protein in the conjugates compared with the amount of starting protein estimated by the Lowry method using BSA as the standard.[26]

TABLE 3. Inhibition of the Interaction between Inaba LPS and MAb I-24-2 by LPS or Conjugates 29−33

entry	inhibitor		IC_{50} (ng·mL^{-1})[a]
a	*V. cholerae* O1 Inaba LPS		45
b	TT-C$_6$pmLPS$_I$	**29**	820
c	TT-C$_{12}$pmLPS$_I$	**30**	410
d	BSA-C$_6$pmLPS	**31**	100
e	TT-C$_6$pmLPS$_{II}$	**32**	110
f	TT-C$_{12}$pmLPS$_{II}$	**33**	100

[a] The amount of inhibitor is calculated from its estimated sugar content.

Conclusion

The traceless Staudinger ligation reaction has been successfully applied to the conjugation of carbohydrate haptens to immunogenic protein carriers, giving a fully stable and biocompatible amide link between the haptens and protein carriers. Further work should aim at increasing the water solubility of the phosphino-functionalized linker. This should give greater derivatization of the carrier and, consequently, contribute to an improved sugar/protein ratio. A phosphine that is totally stable toward oxidation, which would allow intramolecular acylation in the absence of an organic cosolvent, is still in need.

Experimental Section

2-(Diphenylphosphanyl)phenyl Glutarate 8. Diphenyl-(2-hydroxyphenyl)phosphine **7** (758 mg, 2.72 mmol) was dissolved in dry DMF (3 mL) under argon at 0 °C, and sodium hydride (60% dispersion in oil, 120 mg, 3 mmol, 1.1 equiv) was added in several portions. The reaction mixture was stirred at 0 °C for 20 min, and then solid glutaric anhydride (311 mg, 2.73 mmol) was added. After 30 min at 0 °C, the reaction mixture was poured over 0.1 N HCl and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc 8:2) to give acid **8** (786 mg, 74% for the two steps) as a colorless oil: R_f = 0.46 (EtOAc/cyclohexane 3:7); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.81 (br s, 1 H), 7.83−7.74, 7.68−7.58, 7.55−7.49, 7.48−7.36, 7.27−7.13 and 7.94−7.90 (m, 14 H), 2.48−2.40 (m, 4 H), 1.92 (q, 2 H, J = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.4, 171.3, 153.1 (d, J = 17.1 Hz), 136.0−129.1 (12 C), 126.7, 123.1, 33.3 (2 C), 19.8; ^{31}P NMR (162 MHz, $CDCl_3$) δ 14.8; positive CI-MS m/z 393 [M + NH_4]$^+$; HR-MS (FAB) m/z 393.1276 (calcd for [M + H]$^+$ $C_{23}H_{22}BO_4P$ 393.1256).

Succinimidyl 2-(Diphenylphosphanyl)phenyl Glutarate 20. To a stirred solution of **8** (418 mg, 1.07 mmol) and *N*-hydroxysuccinimide (122 mg, 1.07 mmol) in CH_2Cl_2 (4 mL) was added EDC (205 mg, 1.07 mmol), and the mixture was stirred at rt overnight. The crude reaction mixture was diluted with CH_2Cl_2, and this solution washed with 0.1 N aq HCl and then brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by chromatography on silica gel (eluent: CH_2Cl_2/EtOAc 96:4) to give the succinimidyl ester **20** (470 mg, 90%) as a colorless oil: R_f = 0.71 (EtOAc/CH_2Cl_2 4:96); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41−7.32 (m, 11 H), 7.17 (t, 1 H, J = 3.8 Hz), 7.15 (t, 1 H, J = 7.1 Hz), 6.88−6.85 (m, 1 H), 2.74 (br s, 4 H), 2.59 (t, 2 H, J = 7.3 Hz), 2.43 (t, 2 H, J = 7.2 Hz), 1.93 (q, 2 H, J = 7.2 Hz; ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 169.6 (2 C), 168.5, 153.0 (d, 1 C, J = 17.3 Hz), 135.8 (d, 2 C, J = 10.0 Hz), 134.4 (d, 4 C, J = 20.6 Hz), 134.1 (d, 1 C, J = 0.9 Hz), 130.7 (d, 1 C, J = 14.7 Hz), 130.4, 129.6 (2 C), 129.1 (d, 4 C, J = 7.3 Hz), 126.7, 123.0, 32.8, 30.3, 26.0 (2 C), 19.8; ^{31}P NMR (162 MHz, $CDCl_3$) δ 15.0; positive FAB-MS m/z 522.2 [M + Na]$^+$, 490.2 [M + H]$^+$. Anal. Calcd. for $C_{27}H_{24}NO_6P\cdot{}^1/_3H_2O$: C, 65.45; H, 5.02; N, 2.83; O, 20.45. Found: C, 65.54; H, 4.87; N, 2.73; O, 20.19.

Borane−{4-Carboxythiobutyric acid *S*-[(diphenylphosphanyl)methyl] ester} Complex 12. Compound **11** (520 mg, 1.80 mmol) was dissolved in DMF (2.8 mL) and degassed under vacuum. Argon was then bubbled through the solution cooled at 0 °C for 45 min. Sodium methoxide (108 mg, 2.0 mmol) was added in several portions at 0 °C. The mixture was stirred at 0 °C for 30 min, and then glutaric anhydride (186 mg, 1.62 mmol) was added. The reaction mixture was stirred for another 20 min at 0 °C, poured over an ice−water mixture, and extracted with CH_2Cl_2. The organic layer was washed with 0.1 M aq citrate buffer at pH 3, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by flash chromatography on silica gel (eluent: cyclohexane/EtOAc 6:4) to give the acid **12** (390 mg, 60%) as an oil: R_f = 0.18 (EtOAc/cyclohexane 3:7); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.18 (br s, 1 H), 7.68−7.54 (m, 4 H), 7.48−7.30 (m, 6 H), 3.73 (d, 2 H, J = 6.7 Hz), 2.55 (t, 2 H, J = 7.3 Hz), 2.25 (t, 2 H, J = 7.3 Hz), 1.84 (q, 2 H, J = 7.3 Hz), 1.51−0.52 (br m, 3 H; ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.5, 178.7, 133.0 (d, 4 C, J = 9.4 Hz), 132.1 (d, 2 C, J = 2.3 Hz), 129.3 (d, 4 C, J = 10.1 Hz), 127.9 (d, J = 55.5 Hz), 42.7, 32.9, 23.8 (d, J = 35.2 Hz), 20; ^{31}P NMR (162 MHz, $CDCl_3$) δ 20.6 (d, J = 41.5 Hz); positive CI-MS m/z 378 [M + NH_4]$^+$, 362 [M + H]$^+$, 347 [M + BH_3]$^+$; HRMS (FAB) calcd for [M + H − H_2]$^+$ 359.1046, found 359.1042.

Preparation of Succinimidyl Derivative 21. To a solution of acid **12** (500 mg, 1.39 mmol) and *N*-hydroxysuccinimide (160 mg, 1.39 mmol) in CH_2Cl_2 (4 mL) was added EDC (267 mg, 1.39 mmol), and the mixture stirred at rt overnight. The

crude reaction mixture was diluted with CH_2Cl_2 and washed with 0.1 N aq HCl and then brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by chromatography on silica gel (cyclohexane/EtOAc 7:3) to give the succinimidyl ester **21** (400 mg, 63%) as an oil: R_f = 0.32 (EtOAc/cyclohexane 4:6); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76−7.70 (m, 4 H), 7.58−7.45 (m, 6 H), 3.74 (d, 2 H, J = 6.6 Hz), 2.86 (br s, 4 H, 2 CH_2), 2.62 (t, 2 H, J = 7.2 Hz), 2.50 (t, 2 H, J = 7.4 Hz), 1.95 (q, 2 H, J = 7.3 Hz), 1.51−0.52 (br m, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.0, 169.3 (2 C), 168.1, 133.0 (d, 4 C, J = 9.4 Hz), 132.2 (d, 2 C, J = 2.3 Hz), 129.2 (d, 4 C, J = 10.1 Hz), 127.8 (d, J = 55.4 Hz), 42.1, 30.0, 26.0 (2 C), 23.8 (d, J = 35.2 Hz), 20.7; ^{31}P NMR (162 MHz, $CDCl_3$) δ 20.73 (d, J = 64.3 Hz); positive CI-MS m/z 475 $[M + NH_4]^+$, 444 $[M + BH_2]^+$; HRMS (FAB) calcd for $[M + H - H_2]^+$ 456.1210, found 456.1200.

6-Azidohexanoic Acid 38. To a stirred solution of 6-bromohexanoic acid (1.5 g, 7.7 mmol) in DMF (5 mL) was added sodium azide (1.0 g, 15.4 mmol), and the mixture was heated at 85 °C for 3 h. The crude reaction mixture was diluted in CH_2Cl_2, and this solution washed with 0.1 N aq HCl. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give **38** (1.0 g, 82%) as an oil, which was used without further purification: R_f = 0.63 (EtOAc/CH_2Cl_2 3:7); ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.00 (br s, 1 H), 3.29 (t, 2 H, J = 6.9 Hz), 2.39 (t, 2 H, J = 7.4 Hz), 1.73−1.58 (m, 4 H), 1.49−1.40 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.3, 51.6, 34.2, 28.9, 26.5, 24.5; CI-MS m/z 175 $[M + NH_4]^+$.

6-Azidohexanoic Acid Succinimidyl Ester 39. To a stirred solution of 6-azidohexanoic acid **38** (590 mg, 3.76 mmol, 1 equiv) and *N*-hydroxysuccinimide (432 mg, 3.76 mmol) in $CHCl_3$/DMF 9:1 (1 mL) was added EDC (720 mg, 3.76 mmol), and the mixture stirred at rt overnight. The crude reaction mixture was diluted with CH_2Cl_2 and washed with 1 N aq HCl, 5% aq $NaHCO_3$, and then brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give the succinimidyl ester **39** (760 mg, 80%) as an oil which was used without further purification: R_f = 0.76 (EtOAc/CH_2Cl_2 3:7); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.29 (t, 2 H, J = 6.8 Hz), 2.82 (br s, 4 H), 2.62 (t, 2 H, J = 7.4 Hz), 1.77 (q, 2 H, J = 7.4 Hz), 1.68−1.56 (m, 2 H), 1.54−1.44 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.6 (2 C), 168.8, 51.5, 31.1, 28.8, 26.2, 26.0 (2 C), 24.5; CI-MS m/z 272 $[M + NH_4]^+$. Anal. Calcd for $C_{10}H_{14}N_4O_4$: C, 47.24; H, 5.55; N, 22.04. Found: C, 47.11; H, 5.55; N, 22.05.

Derivatization of the Carbohydrate Haptens: Preparation of 25 and Derivatization of Pentasaccharide 34[21] with *N*-Hydroxysuccinimidyl Ester 37. To a solution of **34** (10.5 mg, 13 μmol) in 0.2 M potassium phosphate buffer saline, pH 7.3 (1 mL), was added **37** dissolved in CH_3CN (50 μL), (3 × 26.9 mg, 3 × 130 μmol) in three portions at rt every 2 h. After an additional period of 2 h, the crude reaction mixture was concentrated under reduced pressure and the residue purified by RP-HPLC (gradient: 0−5% B over 15 min). The collected fractions were diluted with H_2O, frozen, and freeze-dried to give the corresponding activated pentasaccharide **25** as a white foam (8.5 mg, 70%): ^{1}H NMR (400 MHz, D_2O) (selected data) δ 5.13 (d, 1 H, J = 2.7 Hz), 5.00 (br s, 1 H), 4.90 (br s, 1 H), 4.73 (br s, 1 H), 4.48 (d, 1 H, J = 8.6 Hz), 2.46 (t, 2 H, J = 6.0 Hz), 1.92 (s, 3 H), 1.27 (d, 3 H, J = 6.2 Hz), 1.24 (d, 3 H, J = 6.0 Hz), 1.22 (d, 3 H, J = 5.9 Hz); ^{13}C NMR (100 MHz, D_2O) (selected data) δ 102.9, 101.5, 101.4, 101.0, 97.9, 81.8, 79.7, 79.4, 76.3, 72.9, 72.4, 72.3, 72.2, 71.7, 71.1, 70.4, 70.4, 70.1, 70.0, 69.7, 69.6, 69.3, 68.8, 68.5, 61.1, 61.0, 55.6, 47.6, 39.7, 35.4, 22.6, 18.2, 17.2, 17.0; positive FAB-MS m/z 984 $[M + Na]^+$; HRMS (FAB) calcd for $[M + Na]^+$ 984.3761, found 984.3777.

Preparation of cOmpounds 26 and 27: Derivatization of pmLPS 35 with Succinimidyl Esters 39 and 41. To a solution of **35** (1.5 to 3.6 μmol) in 0.2 M potassium phosphate buffer saline, pH 7.3 (0.9−1.5 μL), was added the succinimidyl ester **39**, or **41** [3 × 10 equiv dissolved in CH_3CN (50 μL)], in three portions every 2 h. Following an additional reaction period of 2 h, the crude reaction mixture was purified by RP-HPLC (gradient: 0% B for 5 min then 0−30% B over 60 min). The collected fractions were diluted with H_2O, frozen, and freeze-dried to give the corresponding activated-pmLPS **26**, or **27**, as a white foam. pmLPSC_6N_3 **26** (8.5 mg, 38%) was obtained as well as recovered starting material **35** (5.2 mg, 24%): (negative MALDI-TOF-MS) m/z 7197, 6951, 6702, 6455, 6207, 5960, 5713, 5466, 5219, 4970 $(M - H)^-$, 7169, 6922, 6675, 6429, 6181, 5933, 5686, 5438, 5192 $(M - N_2 - H)^-$ (22-mer to 14-mer). pmLPS$C_{12}N_3$ **27** (7.3 mg, 58%) was obtained as well as recovered starting material **35** (2.0 mg, 20%): (negative MALDI-TOF-MS) m/z 7312, 7066, 6814, 6572, 6321, 6070 $(M - H)^-$ (22-mer to 17-mer).

Derivatization of the Protein Carriers. In a typical experiment, a stock solution of TT (18 mg, 820 μL, 0.12 μmol) was diluted with 0.2 M potassium phosphate buffer saline, pH 7.3 (340 μL). To this solution was added succinimidyl 2-(diphenylphosphanyl)phenyl glutarate **20** (3 × 2.61 mg dissolved in 50 μL of CH_3CN) or the borane−phosphine complex **21** (3 × 1.63 mg dissolved in 100 μL of CH_3CN) in three portions every 2 h. Following an additional reaction period of 4 h, the crude reaction mixtures were dialyzed against 0.1 M potassium phosphate buffer, pH 6 (3 × 2 L over 2 days), at 4 °C to remove excess reagent. Phosphino-functionalized TT **22** and **23** were used without further purification.

Similarly, BSA (5 mg, 0.08 μmol) was diluted with 0.2 M potassium phosphate buffer saline, pH 7.3 (340 μL). To this solution was added the borane−phosphine complex **21** (3 × 1.02 mg dissolved in 100 μL of CH_3CN, 3 × 30 equiv), in three portions every 2 h. Following an additional reaction period of 4 h, the crude reaction mixture was dialyzed against 0.1 M potassium phosphate buffer, pH 6 (3 × 2 L over 2 days) at 4 °C to remove excess reagent. Phosphino-functionalized BSA **24** was used without further purification. The extent of derivatization of these protein carriers was determined by comparison of the SELDI-TOF mass spectra of compounds **22**−**24** with those of their parent protein.

Staudinger Ligation Procedures. Method A. Azido-activated carbohydrate hapten **25**, **26**, or **27** was added in one portion to the corresponding modified TT or BSA, **23** or **24**, respectively, in solution in DMF/aq NaCl 0.05 M 5/1, at a 20:1 molar ratio. DABCO (40 equiv) (at a 60 mg·mL^{-1} solution in H_2O) was added to the reaction mixture and further heated at 45 °C. Another 40 equiv of DABCO was added 1 h later. The reaction mixture was further heated for another 3 h. Then, 2 M NH_2OH,HCl in 0.1 M phosphate buffer, pH 6 (20−40 μL), was added to the mixture, and the reaction was kept at room temperature for a further 2 h. Purification and analyses of the conjugates **28**−**31** were performed as for the derivatization procedure.

Method B. Azide-containing oligosaccharides **26** and **27** were mixed with the phosphino-functionalized TT **22** in a 0.2 M phosphate buffer saline solution at a 12:1 molar ratio. Reaction mixtures were heated at 47 °C for 6 h. The crude reaction mixtures were then dialyzed against 0.1 M potassium phosphate buffer saline, pH 7.4 (3 × 2 L over 2 days) at 4 °C, and further purified by gel permeation chromatography on a Sepharose CL-6B column (1 m × 160 mm) (Pharmacia Biotech), using 0.1 M potassium phosphate buffer saline, pH 7.4 as eluent, at a flow rate of 0.2 mL.min^{-1}, with optical density at 280 nm and refractive index detection. The fractions containing the conjugates were pooled and concentrated using Vivaspin 15R centrifugal concentrators (Vivascience), having a membrane cutoff of 10 000 Da, and at a centrifugal force of 5000*g*. The conjugates **32** and **33** were stored at 4 °C in the presence of thimerosal (0.1 mg.mL^{-1}) and assessed for total carbohydrate[27] and protein content.[26]

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Supporting Information Available: General procedures, experimental procedures and analyses for compounds **10a**,**b**, **13**−**16**, **18**, **25**−**27**, and **33**−**41**. This material is available free of charge via the Internet at http://pubs.acs.org.

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