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A New Homobifunctional *p*-Nitro Phenyl Ester Coupling Reagent for the Preparation of Neoglycoproteins

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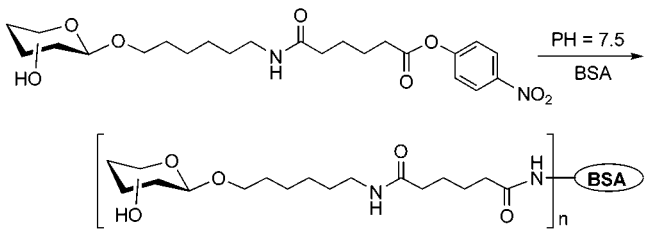
ABSTRACT



A new linker system has been designed and applied to neoglycoprotein synthesis. Reaction of oligosaccharide ω-aminoalkyl glycosides with homobifunctional adipic acid *p*-nitrophenyl diesters in dry DMF gave the corresponding amide half ester in good yields and of sufficient stability to permit chromatographic purification. Subsequent conjugation with bovine serum albumin under very mild conditions afforded the corresponding neoglycoproteins with good efficiency. The method is well suited for the coupling of very small amounts (mg) of oligosaccharide and protein.

Oligosaccharides covalently attached to proteins, insoluble matrixes, and surfaces find applications as vaccines,[1] antigens in solid-phase assays,[2] and immunoadsorption columns[3] and in biosensors.[4] A wide variety of conjugation reactions[5] have been employed to effect the covalent linkage of oligosaccharides to proteins and solid supports, and the majority of these methods rely upon the use of a large excess of derivatized oligosaccharide to achieve acceptable degrees of substitution.[6,7] Since most complex oligosaccharides are derived from challenging multistep syntheses, conjugation methods that achieve modest yields are wasteful of precious oligosaccharide sample. Lemieux introduced a very effective method based on acyl azide conjugation methodology, but this approach requires the construction of complex oligosaccharide on a nine-carbon linker arm that is most often carried through the entire synthetic scheme, thereby imposing orthogonal protection strategies that can become limiting.[8] Synthesis of complex oligosaccharides as simple allyl or pentenyl glycosides affords oligosaccharide final products that can be easily derivatized for different applications. Consequently, there remains a need for improved coupling reactions that would ideally proceed in near quantitative yield with oligosaccharide glycosides of this type.

One of the most efficient coupling methods involves the use of the homobifunctional reagent, diethyl squarate,[9−11]

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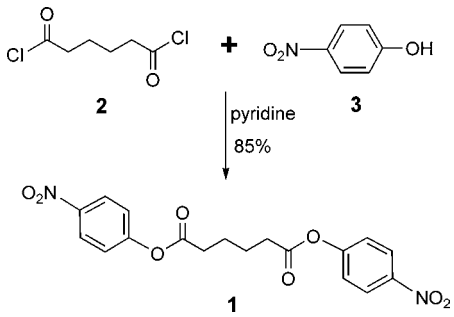
which affords reproducible conjugation in high yields under mild conditions with small amounts of oligosaccharide and protein at low concentrations. However, its use in conjugate vaccine applications has been correlated with a reduced immune response to the oligosaccharide epitope[12] and with potential immune response to the squarate residue itself.[13] Reductive amination is a favored method employed in the preparation of conjugate vaccines but suffers from the major drawback of the large excess of oligosaccharide that is required to achieve respectable degrees of conjugation.[14] Homobifunctional succinimide esters, while commercially available and very effective for amide bond formation, are too reactive to permit chromatographic workup of the oligosaccharide half ester intermediate. We conclude on the basis of our data[15] as well as recent reports[12,16] that the development of an aliphatic straight-chain coupling reagent is highly desirable for vaccine applications.

Here we apply a simple, efficient, bifunctional linker for the preparation of neoglycoproteins that fulfils the following demands: (i) chemoselective reaction of the linker with an oligosaccharide ω-aminoalkyl glycoside without affecting unprotected hydroxy groups; (ii) the activated intermediate (half ester) should be sufficiently stable to permit purification of the activated oligosaccharide; (iii) coupling of the purified, activated intermediate with proteins such as bovine serum albumin (BSA) should proceed with good to high efficiency.

To achieve this objective we selected the homobifunctional reagent, adipate 4-nitrophenyl diester **1**, which was readily synthesized from commercially available adipoyl chloride **2**. Reaction of **2** with 4-nitrophenol **3** in pyridine gave **1** in 85% yield, as shown in Scheme 1. The corresponding

Scheme 1. Synthesis of Homobifunctional Linker **1**



N-succiminide diester was found to be too reactive to allow purification of the activated oligosaccharide, but we reasoned that the electron-withdrawing effect of the 4-nitrophenyl ester would modulate this reactivity to acceptable levels, while preserving sufficient reactivity to permit efficient coupling with free amino groups present in the target proteins.[17] The literature describing the use of **1** was consistent with this expectation. Although it has not been used to create glycoconjugates, **1** has been used to effectively create bivalent ligands[18] and cyclic peptides[19] and as a bivalent affinity labeling reagent for antibody specific for the nitrophenyl hapten.[20]

To confirm the feasibility of this strategy, ω-aminoalkyl glycosides (**I**−**IV** in Figure 1) were employed. 6-Amino-

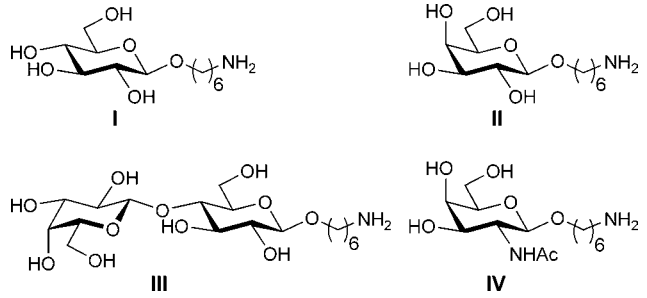


Figure 1. Oligosaccharide amines **I**−**IV**.

hexyl-β-D-glucopyranoside **I**,[21] 6-aminohexyl-β-D-galactopyranoside **II**,[21] and 6-aminohexyl-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside **III**[22] were synthesized according to published procedures.

6-Aminohexyl 2-acetamido-2-deoxy-β-D-galactopyranoside **IV** was synthesized from 6-azido alcohol **4**, prepared in 96% yield by substitution of 6-bromo-hexanol **5**. Glycosylation of **4** with the 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl trichloroacetimidate **6**[23] promoted by trimethylsilyl trifluoromethane sulfonate (TMSOTf) in dichloromethane afforded the intermediate glycoside **7** in excellent yield (93%). Treatment of **7** in methylamine (33% in ethanol) overnight, followed by selective acetylation with acetic anhydride in dry methanol, gave compound **8** in 97% yield, which was hydrogenated to afford free amine **IV** in 70% yield, as shown in Scheme 2.

The synthetic mono- or disaccharides used in this paper contained the 6-aminohexyl aglycone employed by Andersen

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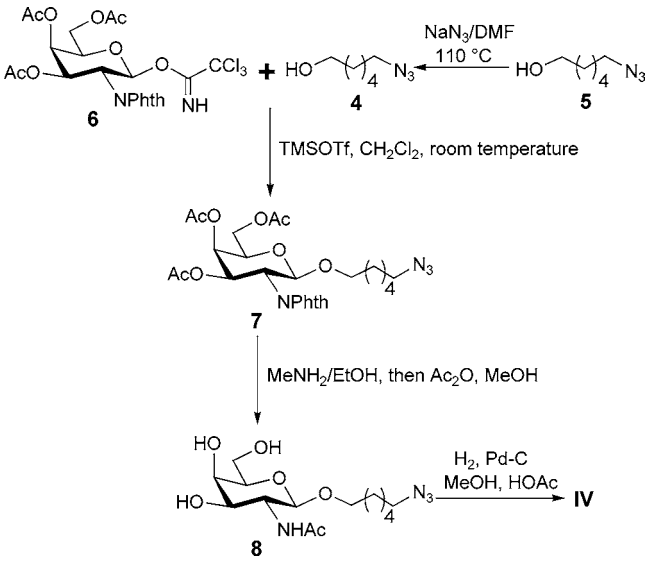
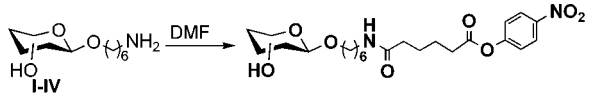
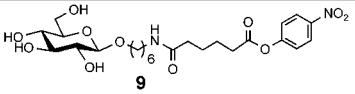
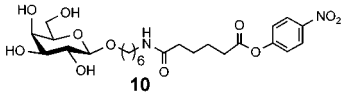
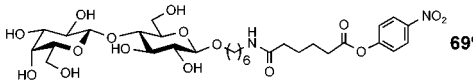
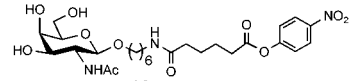
Scheme 2. Synthesis of Compound **IV**



et al. as a linking arm for the attachment to proteins and sepharose gel.[3c] Previously, coupling of such compounds to bovine serum albumin (BSA) protein was accomplished through a squarate linker.[7−9] Diethyl squarate, 3,4-diethoxy-3-cyclobutene-1,2-dione, conveniently permits reaction with the tether amino group in methanol or aqueous carbonate solution to afford the corresponding squarate half esters, which were subsequently coupled to BSA through the terminal amine group of exposed lysine residues of the protein. Under these conditions the percentage of the oligosaccharide attached to BSA varied in the range 50−80%. In the present method, the oligosaccharide amines were treated with 5 equiv of homobifunctional linker in dry DMF at room temperature for 5 h, affording the corresponding half esters **9**−**12** in good yields, as shown in Table 1. The reaction is readily monitored by TLC or by UV spectroscopy, and the half esters are stable to both silica gel chromatograph and reverse-phase isolation under acidic conditions. Excess linker could be removed by washing with dichloromethane, and the yields of this reaction were in the range of 65−82%. The half esters were then coupled to BSA by 18 h incubation in buffer (PH = 7.5) at ambient temperature. The BSA conjugates were obtained as white powders after dialysis against deionized water and then freeze-dried.

Table 1. Synthesis of Half Esters **9**−**12**



Entry	Substrate	Product	Yield
1	I	 **9**	76%
2	II	 **10**	82%
3	III	 **11**	69%
4	IV	 **12**	65%

The degree of incorporation of the oligosaccharides on BSA was established by MALDI-TOF MS using sinapinic acid as the matrix. The mass spectra (positive ion mode) obtained are shown in Figure 2. The spectrum obtained for

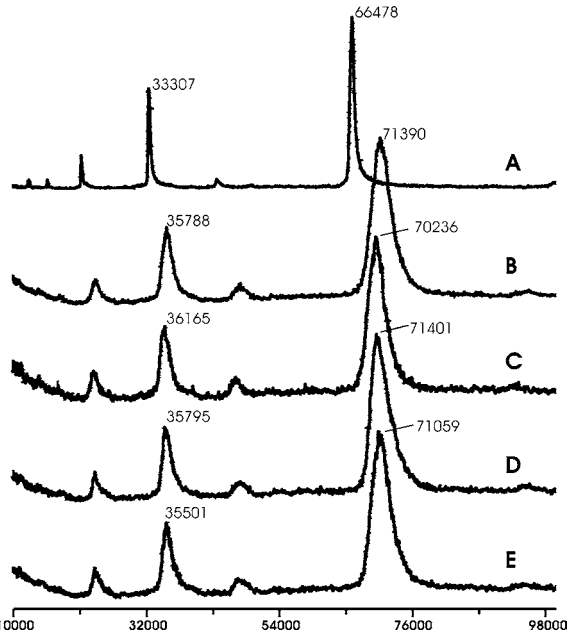
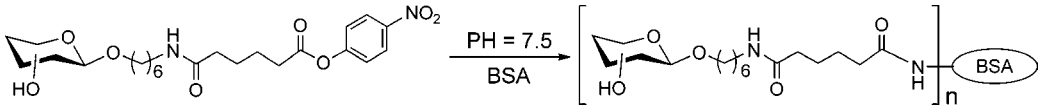


Figure 2. MALDI-TOF spectra of (A) BSA calibration standard, (B) lactose-BSA (n = 8.9), (C) glucose-BSA (n = 12.6), (D) galactose-BSA (n = 9.6), and (E) GalNAc-BSA (10.6).

BSA itself (panel A) shows a sharp peak for the singly charged protein (m/z 66478). Panels B−E (Figure 2) show the corresponding MALDI-TOF mass spectra obtained from the neoglycoprotein. The peaks become broader compared with that of BSA, because each neoglycoprotein sample is made up of a collection of oligosaccharide−protein conjugates with a range of different incorporation values centered around the average. The average molecular weight and this distribution are obtained from the spectra in Figure 2. This average molecular weight allows the calculation of the average number of oligosaccharide residues attached to BSA.

Table 2 summarizes the results obtained for the preparation of neoglycoproteins. When a ligand/protein ratio of 10 was employed, the average number of ligands per BSA ranged from 4.8 to 7.5 as determined by MALDI-TOF-MS. When this ratio was increased to 20/1, the average number of ligand per BSA increased to 8.9−12.6, and when this ratio was

Table 2. Summary of the Coupling Efficiency to BSA



product	saccharide (mg)	molar ratio of BSA:monoester	monoester concentration (μM)	BSA concentration (μM)	hapten incorporated	incorporation efficiency (%)
Lac-BSA	1	1:10	724	72.4	5	50
	2	1:20	1448	72.4	9	45
	3	1:30	2172	72.4	16	53
Gal-BSA	0.8	1:10	756	75.6	7	70
	1.6	1:20	1512	75.6	13	65
	2.4	1:30	2268	75.6	19	63
Glc-BSA	0.8	1:10	756	75.6	5	50
	1.6	1:20	1512	75.6	10	50
	2.4	1:30	2268	75.6	13	43
GalNAc-BSA	0.9	1:10	790	79.0	5	50
	1.8	1:20	1580	79.0	10	50
	1.7	1:30	2370	79.0	15	50

increased to 30/1, the average number of ligands per BSA increased to 14.8−18.4.

In summary, a novel, simple, linear homobifunctional linker has been developed for coupling 6-aminohexyl glycosides to BSA with high efficiency under very mild conditions. The method is also suitable for coupling of very small amounts of oligosaccharide. The strong UV absorption of the intermediates **I**−**IV** allows ready monitoring of the purification by chromatography. Preliminary data show that this linker is also suitable for coupling larger oligosaccharides to BSA.

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Supporting Information Available: Experimental procedures and ^{1}H NMR and other spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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