ORGANIC LETTERS

2002
Vol. 4, No. 19
3191−3194

A Method for Bioconjugation of Carbohydrates Using Diels–Alder Cycloaddition

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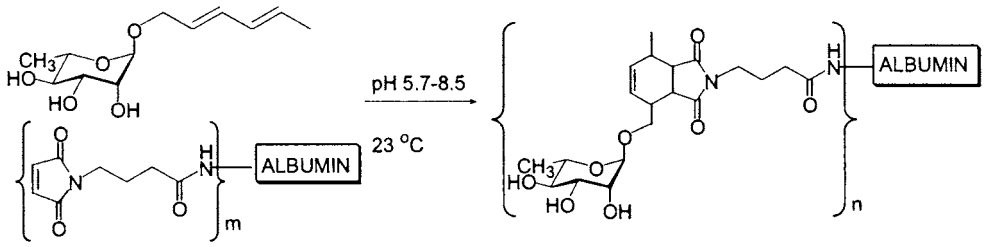
Received May 13, 2002

ABSTRACT



Diels–Alder-type cycloaddition of an electronically matched pair of saccharide-linked conjugated dienes and a dienophile-equipped protein gives neoglycoproteins at ambient temperature in pure water with a reaction half-life of approximately 2 h. Uncoupled saccharides can be recovered by diafiltration with complete conservation of the diene moiety, thus allowing their repeated use. The procedure described is the first for creating a carbon–carbon covalent bond in the bioconjugation step between a saccharide and a protein.

Covalent attachment of small molecules to proteins has been a target of numerous synthetic endeavors because this process converts nonimmunogenic molecules into immunogenic materials.[1] The application of this concept to carbohydrates provided the foundation to generate anti-oligosaccharide antibodies. In addition to being valuable reagents in glycobiology, protein conjugates of the capsular polysaccharides of several human pathogens became the most successful glyco-pharmaceuticals used as vaccines for infants and adults including licensed conjugate vaccines against *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis* group C.[2]

The choice of the methods for bioconjugation of carbohydrates and proteins is restricted by their limited solubility in organic solvents as well as by their pH and temperature sensitivity. Therefore, water at or near neutral pH is the only suitable solvent in almost all cases for this purpose.[3] In the protocols published so far,[1,4−6] the yields do not usually exceed 40% and are often in the 10−30% range. In most cases, the oligosaccharides cannot be recovered in their reactive form after the coupling procedure.[7] Recently, the

(1) For a recent review on bacterial oligosaccharide-protein conjugates as vaccine candidates, see: Pozsgay, V. *Adv. Carbohydr. Chem. Biochem.* **2001**, *56*, 153−199.

(2) Robbins, J. B.; Schneerson, R.; Anderson, P.; Smith, D. H. *J. Am. Med. Assoc.* **1996**, *276*, 1181−1185.

(3) Peeters, C. C. A. M.; Lagerman, P. R.; de Weers, O.; Oomen, L. A.; Hoogerhout, P.; Beurret, M.; Poolman, J. T. Polysaccharide-Conjugate Vaccines. In *Vaccine Protocols*; Robinson, A., Farrar, G., Wiblin, C., Eds.; Humana Press Inc.: Totowa, NJ, 1996; pp 111−133.

(4) For a comprehensive review of oligosaccharide conjugation methods up to 1994, see: Lee, Y. C.; Lee, R. T. *Methods in Enzymology*; Academic Press: San Diego, 1994; p Vol 242.

(5) For a review on conjugation methods of bacterial polysaccharides to proteins, see: Jennings, H. J.; Sood, R. K. Synthetic glycoconjugates as human vaccines. In *Neoglycoconjugates. Preparation and Applications*; Lee, Y. C., Lee, R. T., Eds.; Academic Press: New York, 1994; pp 325−371.

(6) For recent in-depth coverage of oligosaccharide conjugation methods up to 2002, see: Davis, B. G. *J. Chem. Soc., Perkin Trans. 1* **1999**, 3215−3237. Davis, B. G. *Chem. Rev.* **2002**, *102*, 579−601.

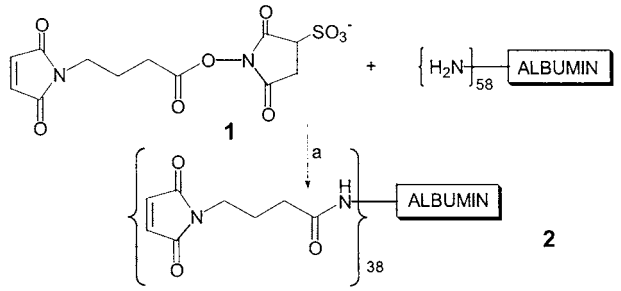
10.1021/ol026179v
Published on Web 08/23/2002

squarate method has appeared as a promising procedure.[8] Considering the expensive nature of oligosaccharide synthesis, development of an efficient bioconjugation method is critically important.

Here, we describe a new approach to bioconjugation utilizing the Diels−Alder reaction that may take place between an electronically matched pair of a double bond (dienophile) and a conjugated diene to form a six-membered unsaturated ring.[9] Although the Diels−Alder reaction has been studied in the carbohydrate field,[10] it has not previously been used for bioconjugation of saccharides. Crucial to our choice of the Diels−Alder cycloaddition is the recognition that water has an extraordinary rate-accelerating effect on these processes even at ambient temperatures.[11−13]

In the work reported herein, we have incorporated electron-deficient double bonds in the protein human serum albumin (HSA) by treating HSA with 3-sulfosuccinimidyl 4-maleimidobutyrate (**1**) in pH 7.5 phosphate buffer to yield the chemically modified protein **2** (Scheme 1). Depending on

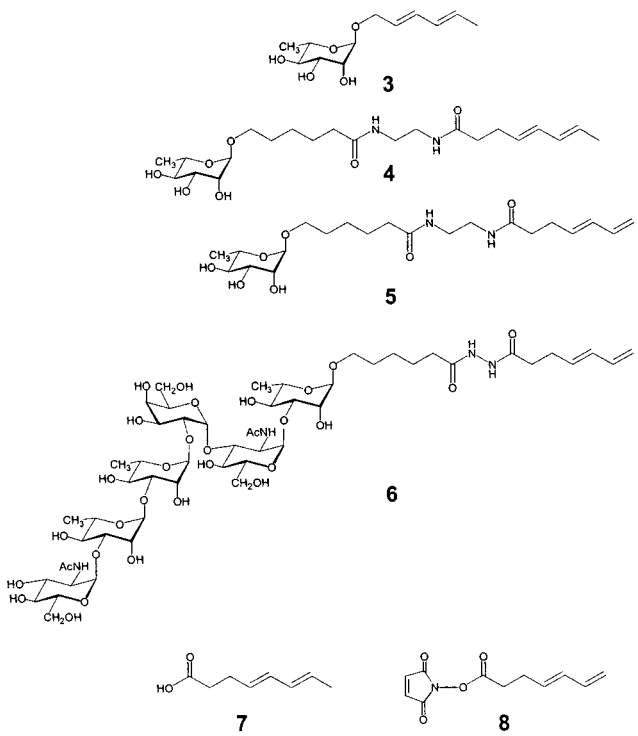
Scheme 1. Formation of the Dienophile-Functionalized HSA[a]



[a] Key: (a) 0.1 M phosphate buffer, pH 7.5 0 → 23 °C, 15 min.

the excess of the reagent, up to 38 (average) maleimido moieties per HSA were incorporated (MALDI-TOF) out of the 58 primary amino groups present.[14,15] Such high loading may, however, not be necessary for most applications.

We have prepared a panel of diene-derivatized saccharides **3**−**6**[16] to test the proposed approach. The selection of these reagents was motivated by our long-standing interest in L-rhamnose-containing oligosaccharides particularly in the O-specific polysaccharide of *Shigella dysenteriae* type 1.[17] Compound **3** was obtained from a per-*O*-acylated L-rhamnopyranosyl trichloroacetimidate and *trans*,*trans*-2,4-hexadien-1-ol in the usual manner[18] followed by deacylation. Compounds **4** and **5** were prepared from 5-methoxycarbonyl α-L-rhamnopyranoside by reaction with $(CH_2NH_2)_2$ followed by *N*-acylation with **7**[19] and **8**,[19] respectively. Compound **6** was obtained from the 5-hydrazinocarbonylpentyl glycoside precursor by *N*-acylation with **8**.



To demonstrate that cycloaddition between the diene and the dienophile components does, indeed, take place, the maleimide derivative **1** and the diene **5** were allowed to react in aqueous solution at 23 °C for 12 h (Scheme 2). As expected, the characteristic olefinic signals in the ^{1}H NMR spectrum of **5** at 6.38, 6.15, 5.74, 5.18, and 5.04 ppm almost completely disappeared and new signals appeared at 5.95 and 5.78 ppm as one-proton multiplets. The ^{1}H NMR also

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(8) Tietze, L. F.; Schröter, C.; Gabius, S.; Brinck, U.; Goerlach-Graw, A.; Gabius, H.-J. *Bioconjugate Chem.* **1991**, *2*, 148−153.

(9) Diels, O.; Alder, K. *Justus Liebigs Ann. Chem.* **1931**, *490*, 243−257.

(10) Lubineau, A.; Auge, J. *Top. Curr. Chem.* **1999**, *206*, 1−39.

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(12) Garner, P. P. Diels−Alder reactions in aqueous media. In *Organic synthesis in water*; Grieco, P. A., Ed.; Blackie Academic and Professional: London, 1998; pp 1−46.

(13) Kumar, A. *Chem. Rev.* **2001**, *101* (1), 1−19.

(14) Carter, D. C.; Ho, J. X. *Adv. Protein Chem.* **1994**, *45*, 153.

(15) The MALDI-TOF mass spectra were run on an Applied Biosystems Voyager DE-STR mass spectrometer (Framingham, MA); the mass accuracy is 0.05%.

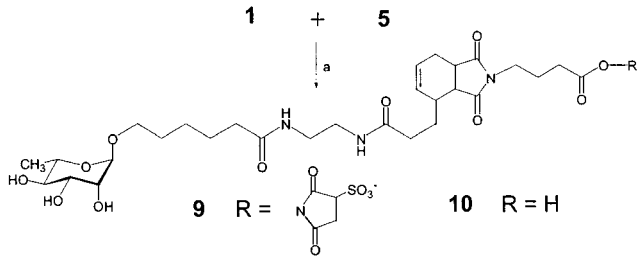
(16) **3**: ^{1}H NMR (300 MHz, D_2O) δ 6.33 (dd, 1 H), 6.18 (ddd, 1 H), 5.86 (m, 1 H), 5.69 (m, 1 H), 4.22 and 4.06 (2 dd, 2 × 1 H), 3.92 (dd, 1 H), 3.72 (dd, 1 H), 3.69 (m, 1 H), 3.44 (t, 1 H), 1.75 (d, 3 H, J = 6.8 Hz), 1.28 (d, 3 H, J = 6.3 Hz). **4**: ^{1}H NMR (500 MHz, CD_3OD) δ 6.09 (m, 2 H), 5.70 (m, 1 H), 5.57 (m, 1 H), 4.77 (br s, 1 H), 3.92 (dd, 1 H), 3.63−3.75 (m, 4 H), 3.53 (m, 1 H), 3.43 (t, 1 H), 3.32 (m, 4 H), 2.32 (m, 4 H), 2.22 (m, 2 H), 1.72 (d, 3 H), 1.53−1.67 (m, 4 H), 1.30−1.42 (m, 2 H), 1.29 (d, 3 H). **5**: ^{1}H NMR (500 MHz, D_2O) δ 6.39 (m, 1 H), 6.16 (m, 1 H), 5.76 (m, 1 H), 5.20 (d, 1 H, J = 17 Hz), 5.05 (d, 1 H, J = 9.7 Hz), 4.79 (br s, 1 H), 3.93 (ddd, 1 H), 3.73 (dd, 1 H, J = 3.3, 9.8 Hz), 3.70 (m, 2 H), 3.53 (dq, 1 H), 3.30 (m, 4 H), 2.36 (m, 4 H), 2.24 (t, 1 H), 1.61 (m, 4 H), 1.36 (m, 2 H), 1.29 (d, 3 H, J = 6.3 Hz). **6**: ^{1}H NMR (300 MHz, D_2O): δ 6.37 (m, 1 H), 6.17 (m, 1 H), 5.76 (m, 1 H), 5.58 (d, 1 H, J = 3.6 Hz), 5.72 (d, 1 H, J = 16 Hz), 5.08 (br s, 1 H), 5.03−5.00 (m, 3 H), 4.98 (d, 1 H, J = 3.4 Hz), 4.80 (br s, 1 H), 2.35 (m, 4 H), 2.22 (m, 1 H), 2.02 (br s, 6 H), 1.60 (m, 4 H), 1.33 (m, 2 H), 1.33−1.20 (m, 9 H).

(17) Pozsgay, V.; Chu, C.; Pannell, L.; Wolfe, J.; Robbins, J. B.; Schneerson, R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5194−5197.

(18) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21−123.

(19) For the methyl ester of **7** and the precursor acid of **8**, see: T. Hudlicky, F. J. Koszyk, T. M. Kutchan, J. P. Sheth, *J. Org. Chem.* **1980**, *45*, 5020−5027.

Scheme 2. Diels–Alder Cycloadditon of Maleimide Derivative **1** and the Diene-Functionalized Saccharide **5**[a]

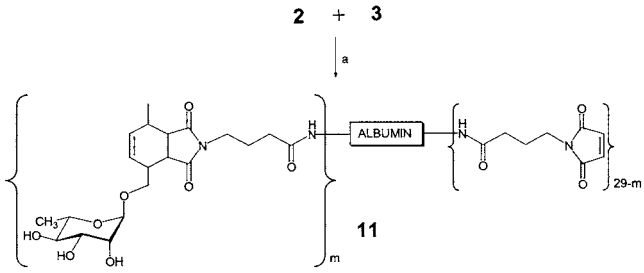


[a] Key: (a) H_2O, 23 °C, 12 h.

showed that ca. 40% of the active ester **9** was hydrolyzed (→ **10**). Structures **9** and **10** received further support from FAB mass spectral data.

Having established that cycloaddition between the selected diene and dienophile partners proceeds at an acceptable rate, our attention turned to bioconjugation of the dienes **3** and **4** with the derivatized protein **2** containing an average of 29 maleimido groups per molecule (initial range of derivatization: 25–33) in water at 23 °C using the diene component in about 3–4-fold molar excess relative to the protein's dienophile moieties (Scheme 3). The molecular weights of samples taken at intervals for up to 50 h were determined by MALDI-TOF mass spectrometry.

Scheme 3. Covalent Attachment of the Diene-Equipped Monosaccharide **3** and the Maleimide-Functionalized Human Serum Albumin **2** through Carbon–Carbon Bond Formation[a]



[a] Key: (a) H_2O, 23 °C, 53 h.

From the increase in molecular weight of the conjugates **11**, relative to the mass of the protein with the ene linkers attached, it appears that, over time, the molecular weight of each of the conjugates approaches a limiting value and that this value differs with the mass of the carbohydrate being attached. We have fit the observed increases in molecular weight as a function of time to a pseudo-first-order reaction kinetic equation of the form

$$\Delta MW = \Delta MW_{max}(1 - e^{-kt})$$

where ΔMW is the increase in molecular weight, ΔMW_{max} is the maximum increase that could be obtained if all the protein-linked starting material were to react, k is the rate constant, and t is time. The results are summarized in Table 1.

Table 1. Kinetic Parameters for Conjugation Reactions

	kinetic parameters		
diene	ΔMW_{max}	k (min^{-1})	$t_{1/2}$ (min)
construct **3**	4350	0.0045	154
construct **4**	9000	0.007	99

The faster incorporation of construct **4** relative to **3** is remarkable. The observed difference seems to correlate with the distance between the hydroxylated, and thus most hydrophilic, rhamnose moiety and the hydrophobic diene part of the molecule. It is noted that the conjugation reaction does not proceed to completion and some initially appended maleimide residues appear to be unreactive, even after long reaction times. The effect of the diene structure appears less pronounced: in separate experiments, the levels of incorporations of constructs **4** and **5** having interchain and terminal diene moieties, respectively, were found to be identical. On the other hand, the rate of the Diels–Alder cycloaddition is markedly affected by the pH. As shown by the data in Table 2. lower pH favors the addition reaction in phosphate–borate

Table 2. Effect of pH on the Cycloaddition between Diene **5** and the Derivatized Protein **2**

pH of phosphate–borate buffer	average incorporation[a,b] per HSA molecule[c,d]
5.7	20
7.0	13
8.5	8
ion-exchanged water	20

[a] Reaction temperature: 22 °C. [b] Reaction time: 30 h. [c] Containing 30 dienophile units. [d] Data indicate partial modification of the attached maleimide moieties.

buffers in the range studied. Interestingly, the coupling efficiency in pure water is as good as in the pH 5.7 buffer; therefore, all our conjugations are now routinely carried out in unbuffered aqueous solutions. For general experimental procedures, see ref 20.

The utility of the new bioconjugation method for linking a large oligosaccharide to HSA was demonstrated by coupling HSA containing an average of 22 maleimidoyl moieties with hexasaccharide **6** that represents one and a half repeating units of the O-specific polysaccharide of *Shigella* dysenteriae type 1.[21] Incorporation of **6** into the protein did, indeed, take place: after 2 h, the average incorporation was six hexasaccharide chains per HSA molecule. After 8 h, the incorporation reached an average of 13. Remarkably, the uncoupled saccharide could be recovered by diafiltration in pure form.

In summary, we have developed a new method for bioconjugation based on the Diels−Alder cycloaddition reaction. The method requires the introduction of an activated double bond and a conjugated diene sector into the components to be covalently linked together. All the steps of the new bioconjugation method including the attachment of the reacting moieties and the cycloaddition itself proceed under biocompatible conditions, at or below physiological temperatures allowing the conjugation of the most sensitive saccharides and proteins. The new method makes possible for the first time the complete recovery the uncoupled saccharide by diafiltration or by gel filtration in its original, bioconjugatable form. Since the conjugation is equally effective in unbuffered aqueous solutions, the amount of the recovered material can be directly quantitated without the need for prior desalting and purification.

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(20) **General Procedure for the Acylation of *ω*-Aminoalkyl Glycosides with *N*-Hydroxysuccinimidyl Hexa-4,6-dienoate.** To a stirred solution of an *ω*-alkyl glycoside (10 mg) in methanol (1 mL) at 0 °C was added *N*-hydroxysuccinimidyl hexa-4,6-dienoate (10 mg) in methanol (1 mL). After 10 min, the solution was concentrated under reduced pressure. The residue was stirred in water (2 mL) for 5 min followed by filtration. The solids were discarded. The filtrate was applied to a column (3 cm × 0.5 cm) of C-18 reversed-phase silica gel that was eluted with a gradient of methanol in water. The fractions containing saccharide (Dubois-assay) were pooled and concentrated under reduced pressure. After removal of MeOH, the aqueous solution was freeze-dried to yield an amorphous white solid. **General Procedure for Derivatization of Albumin with Maleimidobutyryl Groups.** To a stirred solution of human serum albumin (10 mg) in 0.1 M pH 7.5 phosphate buffer at 0 °C was added 8.5 mg of sulfosuccinimidyl 4-maleimidobutyrate (Pierce). After 5 min, the ice bath was removed. After a further period of 10 min, the solution was transferred to an Amicon diafiltration apparatus equipped with a YM-10 (10 kDa cutoff) membrane. The solution was diafiltered using five changes of water (5 mL each). The final volume was approx 0.3 mL. **General Procedure for the Diels−Alder-Type Bioconjugation of the Diene-Spacer-Equipped Saccharides to Maleimido Group Functionalized Albumin.** To the solution of the derivatized protein described above was added the diene-equipped saccharide (12 mg) at 22 °C. The mixture was stirred for a period of 24 h followed by diafiltration through a YM-10 (10 kDa cutoff) membrane using five changes of water. After the final filtration, the solution containing the conjugate was freeze-dried. The composition of the conjugate so obtained was determined by MALDI-TOF mass spectrometry. The filtrate was pooled and freeze-dried. The ^{1}H NMR spectrum of the residue was identical to the spectrum of the starting saccharide−diene construct.

(21) For the synthesis of oligosaccharides related to **6**, see: (a) Pozsgay, V. *J. Am. Chem. Soc.* **1995**, *117*, 6673−6681. (b) Pozsgay, V. *J. Org. Chem.* **1998**, *63*, 5983−5999. (c) Pozsgay, V. *Tetrahedron*: *Asymmetry* **2000**, *11*, 151−172.