ORGANIC LETTERS

2006
Vol. 8, No. 14
3081−3084

A Highly Efficient Chemoenzymatic Approach toward Glycoprotein Synthesis

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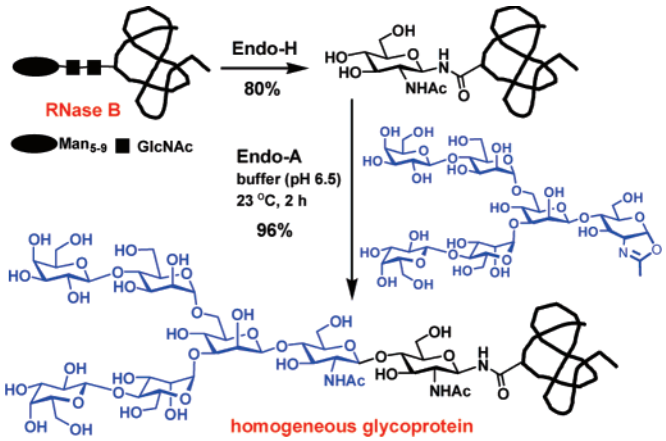
Received May 1, 2006

ABSTRACT



A highly efficient endoglycosidase-catalyzed synthesis of homogeneous glycoproteins is described. By using ribonuclease B as a model system, it was demonstrated that Endo-A could efficiently attach a preassembled oligosaccharide to a GlcNAc-containing protein in a regio- and stereospecific manner, when the corresponding sugar oxazoline was used as the donor substrate. The method allows the synthesis of both natural and tailor-made N-linked glycoproteins in excellent yield.

Asparagine-linked glycosylation is a predominant form of posttranslational modifications of eukaryotic proteins. The covalently attached oligosaccharides of glycoproteins can profoundly affect a protein's structure and in vivo activity, and are directly involved in a number of important biological recognition processes such as cell adhesion and immune response.[1] Since natural and recombinant glycoproteins are typically produced as a mixture of heterogeneous glycoforms, synthesis of homogeneous glycoproteins carrying structurally defined oligosaccharides has become essential both for detailed structure−function relationship studies and for developing glycoprotein-based therapeutics.[2] To address the need, a variety of synthetic strategies have been explored for constructing large glycopeptides and glycoproteins.[3,4]

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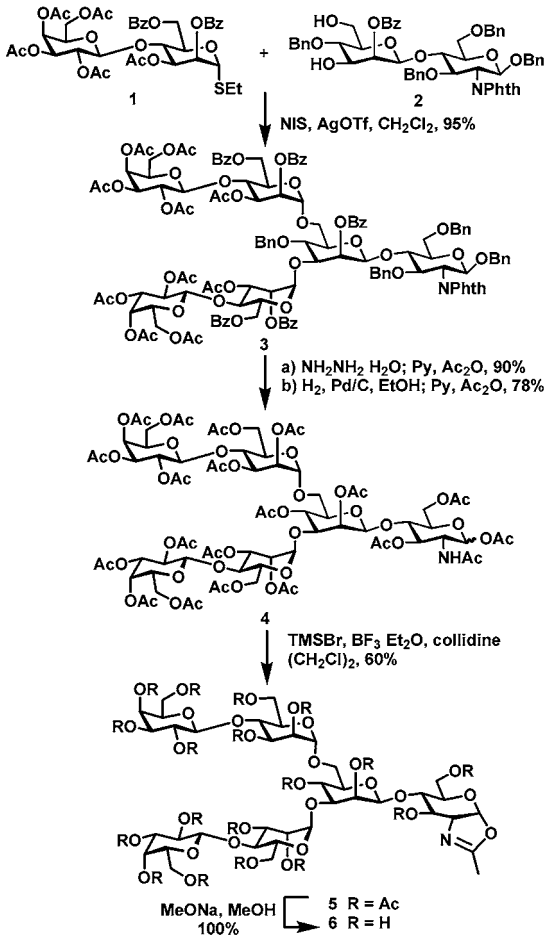
10.1021/ol061056m CCC: $33.50
Published on Web 06/06/2006

The chemoenzymatic approach involving the *endo*-β-*N*-acetylglucosaminidase (ENGase)-catalyzed trans-glycosylation is particularly appealing, as it has the unique advantage to attach an intact oligosaccharide to a preassembled GlcNAc-containing peptide or protein in a single step without the need of any protecting groups.[4−6] However, the enzymatic trans-glycosylation has suffered with low efficiency of trans-glycosylation and has hitherto been limited to the use of only natural *N*-glycopeptides as the donor substrates that themselves are difficult to synthesize. To overcome these problems, we have recently explored sugar oxazolines, the mimics of the presumed transition state, as donor substrates for glycopeptide synthesis.[7] There was a precedent that a disaccharide oxazoline could act as substrate for ENGases.[8] The use of the transition state mimics not only expended the substrate availability, but also led to a very high-yield synthesis of large *N*-glycopeptides.[7,9] We describe in this paper the first extension of this methodology to glycoprotein synthesis and remodeling. Using ribonuclease B as a model system, we have found that Endo-A (from *Arthrobacter protophormiae*) can efficiently transfer a pre-assembled oligosaccharide from the corresponding synthetic sugar oxazoline to the GlcNAc-containing protein under mild conditions to form a tailor-made glycoprotein in excellent yield.

Our preliminary studies have shown that Endo-A can tolerate certain modifications on the mannose moiety of the Manβ1,4GlcNAc-oxazoline, the minimal substrate structure recognized by Endo-A.[9] This prompted us to examine whether the endoglycosidase could also tolerate selective modifications on the outer mannose moieties of the Manα1,3-(Manα1,6)Manβ1,4GlcNAc core of *N*-glycans. Thus, a non-natural hexasaccharide ($Gal_2Man_3GlcNAc$) oxazoline was designed and synthesized, which has two galactose residues β-1,4-linked to the terminal mannose residues in the Man_3-GlcNAc core (Scheme 1). This hexasaccharide derivative can be regarded as a mimic of a bi-antennary complex type *N*-glycan without the interlinked GlcNAc moieties. Glycosidation of the disaccharide acceptor **2** with two units of the disaccharide donor **1** under the catalysis of NIS and AgOTf gave the hexasaccharide intermediate **3** in excellent yield. NMR analysis confirmed that the newly formed glycosidic bonds were in the desired α-glycosidic linkage. Compound **3** was then converted to the per-*O*-acetylated hexasaccharide **4** through a series of protecting group manipulations. The formation of the oxazoline ring was achieved by the treatment of compound **4** with Lewis acid (TMSBr, BF_3, Et_2O) and collidine to provide compound **5** in 60% yield. Finally, de-*O*-acetylation of **5** with a catalytic amount of MeONa in dry MeOH afforded the free hexasaccharide oxazoline **6** in quantitative yield.

Scheme 1. Synthesis of an Unnatural Hexasaccharide Oxazoline



To examine whether the endoglycosidase Endo-A is able to recognize the synthetic nonnatural sugar oxazoline for trans-glycosylation, a model reaction was carried out with a small GlcNAc-peptide, Ac-Asn(GlcNAc)-Ile-Thr (**7**),[9] as the acceptor. The enzymatic reaction was monitored by reverse-phase HPLC. It was observed that the reaction between **6** and **7** (molar ratio 2:1) occurred quickly in the presence of

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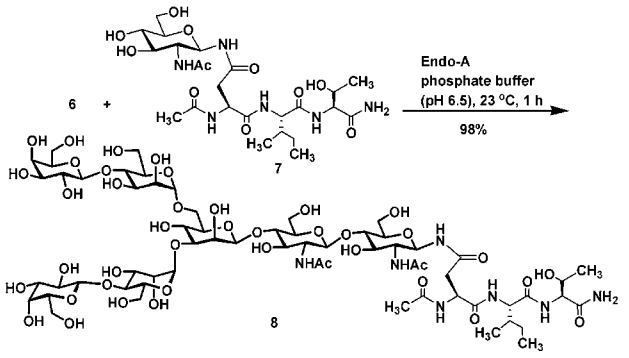
Endo-A, while no ligation would occur in the absence of the enzyme. The glycosylation of the acceptor **7** with oxazoline **6** under the catalysis of Endo-A was essentially complete within 30 min to form the glycopeptide **8** (Scheme 2), which was isolated in 98% yield. The identity of the

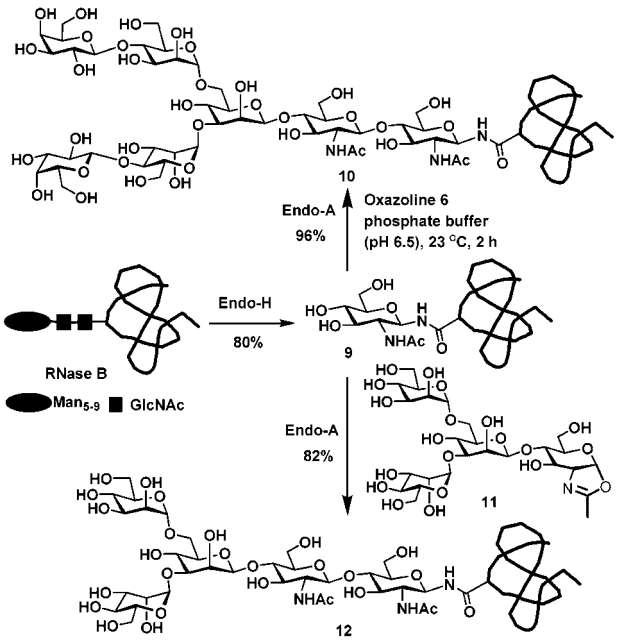
Scheme 2. Synthesis of the Glycopeptide **8**



glycopeptide was characterized by ESI-MS and NMR (see the Supporting Information). The observed MS (1604.1 Da) as revealed by ESI-MS matched well with the calculated MS (exact mass, 1603.63 Da) of glycopeptide **8**, indicating that it is the adduct of the hexasaccharide oxazoline and the acceptor Ac-Asn(GlcNAc)-Ile-Thr. On the other hand, a doublet at δ 4.56 with a relatively large coupling constant (J = 7.5 Hz) assigned for the H-1 of the second GlcNAc suggested that the hexasaccharide was attached to the GlcNAc in the peptide moiety via the expected β1,4-glycosidic linkage. The results clearly indicate that the synthetic hexasaccharide oxazoline can serve as an excellent substrate for the Endo-A catalyzed trans-glycosylation, making it possible to incorporate nonnatural sugar chains into peptides.

To examine the feasibility of the chemoenzymatic method for glycoprotein synthesis and remodeling, bovine ribonuclease B was chosen as a model system, which has been used previously as a system for demonstrating glycoprotein remodeling.[5,10] Ribonuclease B is a small glycoprotein that consists of 124 amino acids and contains a single glycosylation site at Asn-34. Natural ribonuclease B is a mixture of several glycoforms carrying a range of high-mannose type *N*-glycans ($Man_{5-9}GlcNAc_2$) at Asn-34. Treatment of ribonuclease B with Endo-H (an endoglycosidase that cleaves high-mannose type *N*-glycans at the chitobiose core) removed the *N*-glycans, which leaves only the innermost *N*-acetylglucosamine (GlcNAc) at the Asn-34 site, giving the homogeneous GlcNAc-RB (**9**). It was found that when the hexasaccharide oxazoline **6** and GlcNAc-RB (molar ratio, 2:1) were incubated in a phosphate buffer (pH 6.5) at 23 °C in the presence of Endo-A, the GlcNAc-RB was smoothly glycosylated to give the trans-glycosylation product **10**, which was eluted earlier than GlcNAc-RB under reverse-phase HPLC. The transformation was essentially quantitative after 2 h reaction and the glycoprotein product was isolated in 96% yield (Scheme 3). Deconvolution of the ESI-MS of

Scheme 3. Synthesis of the Glycoproteins **10** and **12**



10 gave a molecular mass of 14901 Da, which is in good agreement with the calculated MS (14900 Da) of the glycoprotein **10**. These results clearly indicated that the chemoenzymatic approach was equally efficient for the synthesis of homogeneous glycoproteins carrying structurally defined oligosaccharides.

Interestingly, the glycoprotein **10**, once formed, was found to be resistant to Endo-A catalyzed hydrolysis. This is understandable because glycoprotein **10** carries a nonnatural *N*-glycan and Endo-A is known to hydrolyze only high-mannose type natural *N*-glycans. Since the corresponding hexasaccharide oxazoline **6** could serve as an excellent donor substrate for the trans-glycosylation, these results suggest that the sugar oxazolines as transition state mimics are kinetically much more active for the enzymatic reaction than the "ground state" glycoprotein products thus formed. We also found that the Endo-A catalyzed trans-glycosylation of the sugar oxazoline **6** was much faster in the presence of the acceptor (GlcNAc-containing peptide or protein) than its enzymatic hydrolysis (data not shown). All these factors contribute to the formation of the trans-glycosylation product. Similarly, Endo-A catalyzed reaction of GlcNAc-RB with the tetrasaccharide oxazoline **11**[7] gave the glycoprotein **12** carrying the core N-linked pentasaccharide $Man_3GlcNAc_2$ in 82% yield (Scheme 3). Again, the observed MS (14574.5 Da, from the deconvolution of the ESI-MS data) of the isolated product matches well with the calculated MS (14575.6 Da) of glycoprotein **12**. It should be noted that the efficient attachment of the core N-linked pentasaccharide ($Man_3GlcNAc_2$) to a protein would provide a key starting structure for a quick assembly of a variety of glycoforms via sequential glycosylations of the core with various glycosyltransferases.[11] The identity and homogeneity of the synthetic glycoproteins **10** ($Gal_2Man_3GlcNAc_2$-RB) and **12**

($Man_3GlcNAc_2$-RB) were clearly demonstrated by their ESI-MS (Figure 1).

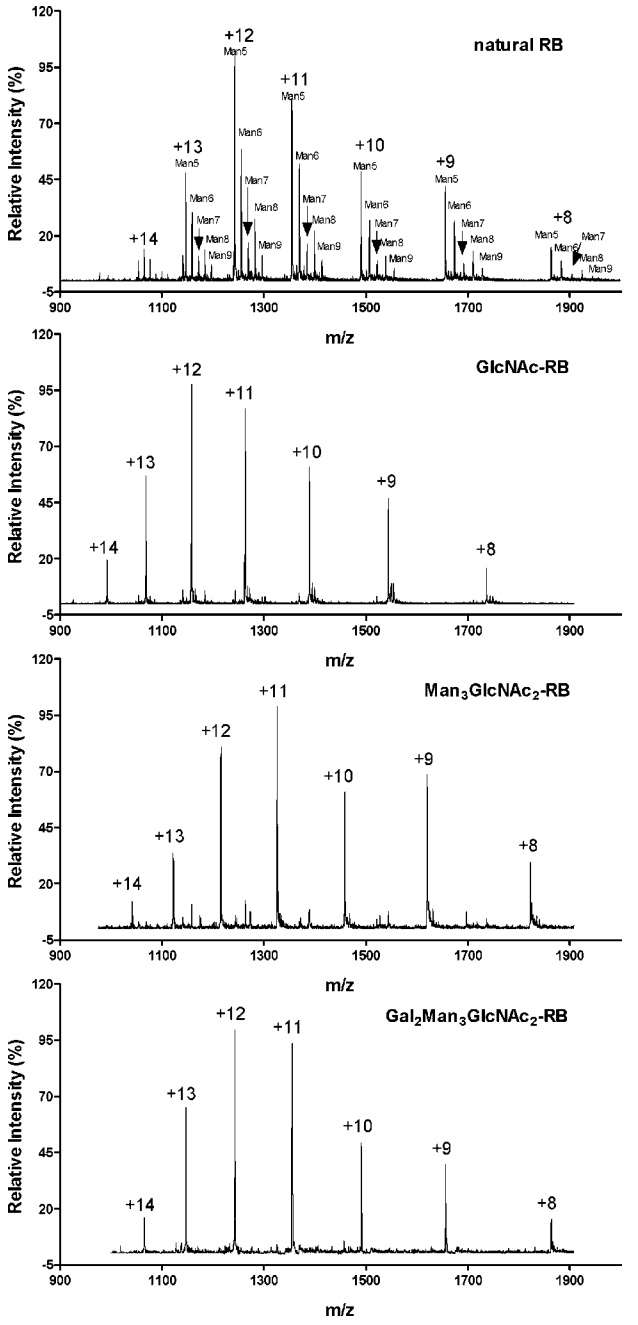


Figure 1. The ESI-MS spectra of the synthetic glycoproteins.

The high-yield enzymatic trans-glycosylation with the use of synthetic sugar oxazoline as the donor substrate opens a new avenue toward glycoprotein synthesis and remodeling. The endoglycosidase-catalyzed ligation with GlcNAc-containing proteins proceeds in a regio- and stereospecific manner and in an excellent yield under mild conditions, without the need of any protection groups. The chemoenzymatic approach is highly convergent and allows totally independent preparation of the oligosaccharide and protein portions, thus avoiding the long-standing problem of "incompatibility" of protecting group manipulations in glycopeptide synthesis. In addition, this study has demonstrated that nonnatural oligosaccharide could also serve as efficient donor substrate, making it possible to construct both natural and tailor-made glycoproteins. Since a given GlcNAc-protein could be efficiently prepared by several established approaches, including (a) overproduction of the protein in a high-yield yeast expression system with subsequent removal of the high-mannose type *N*-glycan by Endo-H or Endo-A, (b) total protein synthesis via native chemical ligation or expressed protein ligation with the incorporation of a GlcNAc-tag at a predetermined site during the synthesis,[12] and (c) overproduction of a GlcNAc-containing protein in *E. coli* through the novel in vivo suppressor tRNA technology,[13] the chemoenzymatic method described here may become generally applicable for constructing various natural as well as tailor-made homogeneous glycoproteins.

Acknowledgment. We thank Prof. K. Takegawa (Kagawa University) for kindly providing the pGEX-2T/Endo-A plasmid that was used for overproducing the enzyme Endo-A.

Supporting Information Available: Detailed experimental procedures; the ^1H and ^{13}C NMR spectra of key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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