Glycoproteins

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Chemoenzymatic Synthesis of a Phosphorylated Glycoprotein

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Abstract: *The majority of lysosomal enzymes are targeted to the lysosome by post-translational tagging with N-glycans terminating in mannose-6-phosphate (M6P) residues. Some current enzyme replacement therapies (ERTs) for lysosomal storage disorders are limited in their efficacy by the extent to which the recombinant enzymes bear the M6P-terminated glycans required for effective trafficking. Chemical synthesis was combined with endo-β-N-acetylglucosaminidase (ENGase) catalysis to allow the convergent synthesis of glycosyl amino acids bearing M6P residues. This approach can be extended to the remodeling of proteins, as exemplified by RNase. The powerful synergy of chemical synthesis and ENGase-mediated biocatalysis enabled the first synthesis of a glycoprotein bearing M6P-terminated N-glycans in which the glycans are attached to the peptide backbone by entirely natural linkages.*

Phosphorylation of the terminal mannose residues of N-glycans is a post-translational protein modification that occurs through a two-step process involving the sequential action of two enzymes.[1] The primary purpose of the installation of mannose-6-phosphate (M6P) at the non-reducing termini of high-mannose oligosaccharides is to facilitate the trafficking of proteins carrying such "tagged" glycans to the lysosome through interaction with the mannose-6-phosphate receptors (M6PRs).[2]

Targeting of enzymes to the lysosome through the M6PRs is of critical interest for the development and enhancement of enzyme replacement therapies (ERTs) for the treatment of lysosomal storage disorders (LSDs). For example, a variety of avenues have been pursued to try and enhance the delivery of recombinant human acid α-glucosidase (rhGAA, commercially marketed as Myozyme) to affected muscles by targeting through the M6PRs in order to increase the efficacy of ERT for the treatment of Pompe disease. Approaches have included the chemical conjugation of naturally derived or synthetic oligosaccharides containing M6P,[3,4] as well the enzymatic addition of M6P residues.[5] The total synthesis of a variety of N-glycan structures containing M6P units has also been reported, and these have been attached to a protein via a non-native linker.[6]

Amongst various synthetic approaches developed to access glycoproteins,[7] the use of *endo*-β-*N*-acetylglucosaminidases (ENGases) has allowed the synthesis of a variety of homogenous glycopeptides, proteins, and antibody fragments containing N-glycan structures.[8] Recent advances, such as the use of oxazolines as activated donors,[9] methods that allow the synthesis of these oxazolines in water from the corresponding reducing sugars,[10] and the production of efficient glycosynthase mutant enzymes,[11] have all greatly increased the utility of this approach. However whether this strategy can be applied to access glycoconjugates that contain phosphorylated saccharides has not been investigated. As part of ongoing exploration of the utility of ENGases, the synthesis of N-glycan structures containing M6P residues was undertaken, and the ability of ENGases to catalyze their transfer to peptides and proteins was investigated.

Studies of the interaction of glycans with the M6PRs have shown that multivalent presentation of terminal M6P residues significantly increases receptor binding.[12] An N-glycan pentasaccharide structure in which the two terminal mannose residues contained phosphate at the 6 position was identified as a preliminary synthetic target; with this material it would be possible to assay whether ENGases could process glycans containing M6P residues before undertaking more complicated and arduous syntheses of the fully extended structures required for optimal binding to the M6PRs. The synthetic strategy adopted was based upon sequences previously used to access N-glycan oxazolines.[9j] However, in this case, the installation of phosphate groups at position 6 of the mannose residues at the non-reducing terminus required a selective protection strategy. OH-6 of the known tetrol **1**[13] was selectively protected by reaction with TIPS chloride to give triol **2**; this was then benzylated to give thioglycoside **3**, which was to serve as a common donor for the introduction of both the 3- and 6-branches of the core N-glycan oligosaccharide. The acceptor disaccharide **4** was accessed as previously described,[9k] and glycosylation of **4** with **3** produced trisaccharide **5**. Regioselective reductive cleavage of the 4,6-benzylidene gave the primary alcohol **6**, followed by a second glycosylation with donor **3** to yield tetrasaccharide **7** (Scheme 1). Conversion of the N-phthalimide into the N-acetamide gave **8**, followed by removal of the TIPS groups to produce diol **9**. Phosphitylation of **9** with dibenzyl diisopropylphosphoramidate and immediate oxidation with MCBPA

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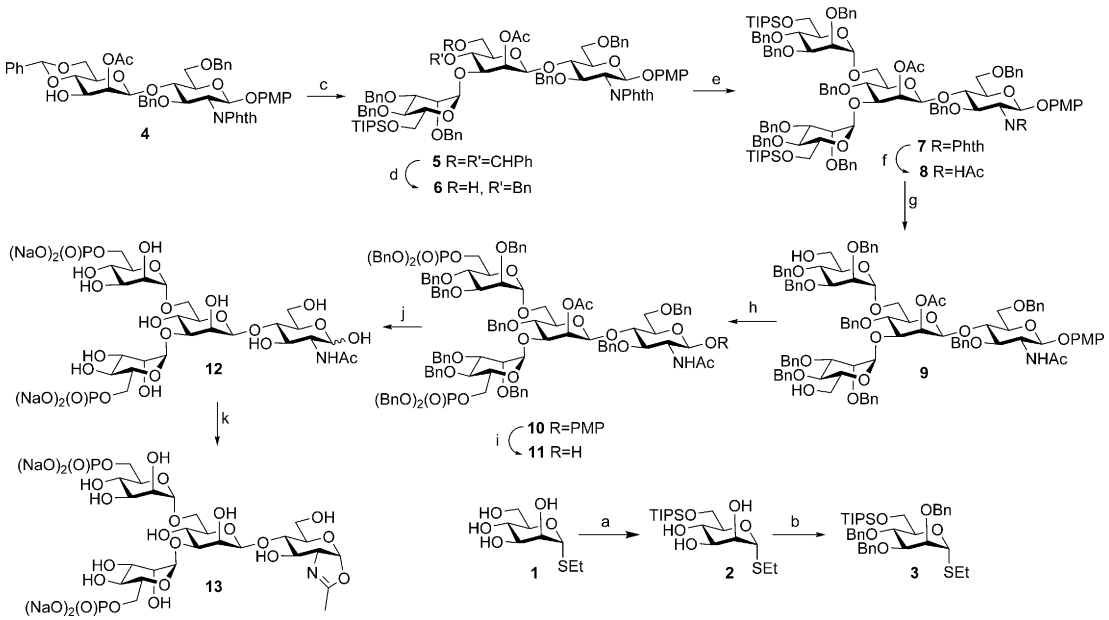


Scheme 1. Synthesis of oxazoline **13**. a) TIPSCl, imidazole, THF, 0°C to RT, 24 h, 89%; b) NaH, BnBr, THF, 60°C, 24 h, 78%; c) **3**, MeOTf, 3 Å mol. sieves, CH_2Cl_2, *t*-TBP, 0°C to RT, 18 h, 91%; d) Et_3SiH, $PhBCl_2$, 3 Å mol. sieves, CH_2Cl_2, −78°C, 50 min, 94%; e) **3**, MeOTf, 3 Å mol. sieves, CH_2Cl_2, *t*-TBP, 0°C to RT, 18 h, 79%; f) first $NH_2CH_2CH_2NH_2$, MeOH, reflux, 16 h; then Ac_2O, py, RT, 24 h, 91% over 2 steps; g) $BF_3{\cdot}OEt_2$, CH_2Cl_2, 0°C, 1 h, 76%; h) $(BnO)_2PNiPr_2$, tetrazole, CH_2Cl_2, RT, 18 h, then add MCPBA, −78°C to RT, 2 h, 58% over two steps; i) CAN, MeCN, H_2O, RT, 1 h, 78%; j) Na, $NH_{3(l)}$, −33°C, 1 h, 98%; k) DMC, Et_3N, H_2O, RT, 95%. TBP = 2,4,6-tri-*tert*-butylpyrimidine, CAN = ceric ammonium nitrate, DMC = 2-chloro-1,3-dimethylimidazolinium chloride, PMP = *p*-methoxyphenyl, TIPS = triisopropylsilyl, Bn = benzyl, Npth = naphthyl.

gave the bis(phosphate ester) **10**. Oxidative cleavage of the anomeric PMP protecting group then gave hemiacetal **11**, which was globally deprotected by Birch reduction to yield free sugar **12** in very high yield. Finally, **12** was converted into the corresponding oxazoline **13** in 95% yield by following the procedure of Shoda and co-workers[10] and using 2-chloro-1,3-dimethylimidazolinium chloride (DMC) in water.

Oxazoline **13** was investigated as a substrate for several of the family GH85 ENGase enzymes by using the glycosyl amino acid **14** as an acceptor (Scheme 2). Wild-type (WT) Endo A[14] was able to catalyze the glycosylation of **14**, and with three equivalents of donor **13**, product **15** was formed in 40% yield after 2 hours. Increasing the number of equivalents of **13** from three to six correspondingly increased the yield of **15** to a very respectable 73%. A time-course study of the reaction revealed that although **15** was a hydrolytic substrate for WT Endo A, hydrolysis occurred only slowly, thereby allowing effective kinetic control and isolation in high yield (Figure 1).

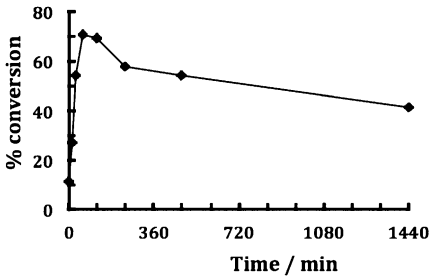
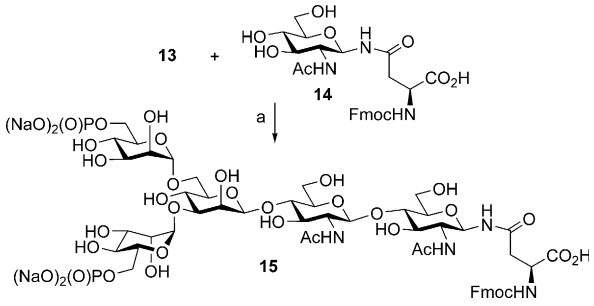


Figure 1. Time-course study for the conversion of **14** into **15** with WT Endo A.

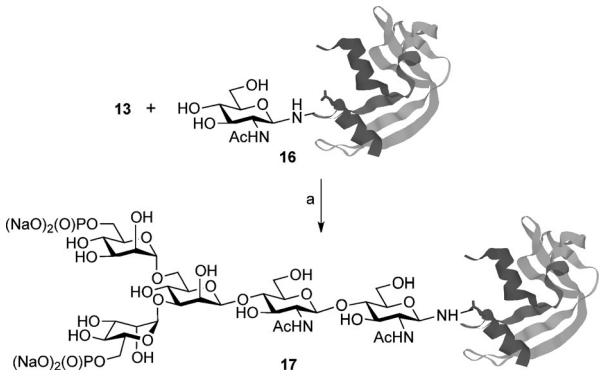


Scheme 2. ENGase-catalyzed glycosylation of **14**. a) ENGase, phosphate buffer pH 6.5, H_2O, 37°C, 2 h.

Other family GH85 enzymes assayed were found to be less effective. Interestingly WT Endo M[15] was unable to catalyze the formation of **15**, a result that was unexpected since Endo M has a broader hydrolytic capability than Endo A, being able to hydrolyze complex biantennary N-glycans as well as high-mannose structures. The fact that complex N-glycans are terminated by negatively charged sialic acid residues had led us to speculate that Endo M may perhaps be more tolerant of high-mannose glycans terminated in negatively charged phosphates. However, with the tetrasaccharide oxazoline **13**, this was not the case. Similarly the commercially available N175Q glycosynthase mutant of

Endo M[11d] was also unable to effect the glycosylation of **14** with oxazoline **13**. On the other hand, Endo D, which has considerably more specific structural requirements with respect to the N-glycans it will hydrolyze, was able to catalyze the production of **15**, albeit with very low yield (ca. 3%).

Attention was turned to the application of WT Endo A catalyzed glycosylation with **13** to the production of a phosphorylated glycoprotein. RNase B was chosen as the model protein substrate. Commercially available RNase B, which is a mixture of high-mannose glycoforms, was trimmed back to a single GlcNAc residue at the sole glycosylation site by treatment with Endo H, as previously described,[11b] to give dRNase B **16**. Treatment of **16** with 3 equivalents of oxazoline **13** and WT Endo A gave the phosphorylated glycoprotein $(M6P)_2$RNase **17** (Scheme 3), as demonstrated by SDS PAGE and high-resolution mass spectrometry (HRMS; Figures S2 and S3 in the Supporting Information).



Scheme 3. Enzymatic synthesis of remodeled RNase. a) WT Endo A, phosphate buffer pH 6.5, H_2O, 37 °C, 2 h.

The successful glycosylation of dRNase B **16** with oxazoline **13** represents the first report of the synthesis of an N-linked glycoprotein that contains M6P-terminated glycans in which the oligosaccharides are linked to the peptide by native linkages. Such a strategy should allow the production of recombinant lysosomal hydrolases decorated with M6P-terminated glycans, and so may augment the trafficking of administered enzymes to the lysosome through the M6PR; a development that could significantly improve the efficacy of ERT treatment of LSDs. The synthesis of full-length N-glycan structures bearing M6P residues of optimal structure for lysosomal targeting through the M6PR, and their biocatalytic attachment to glycopeptides and glycoproteins is currently in progress.

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