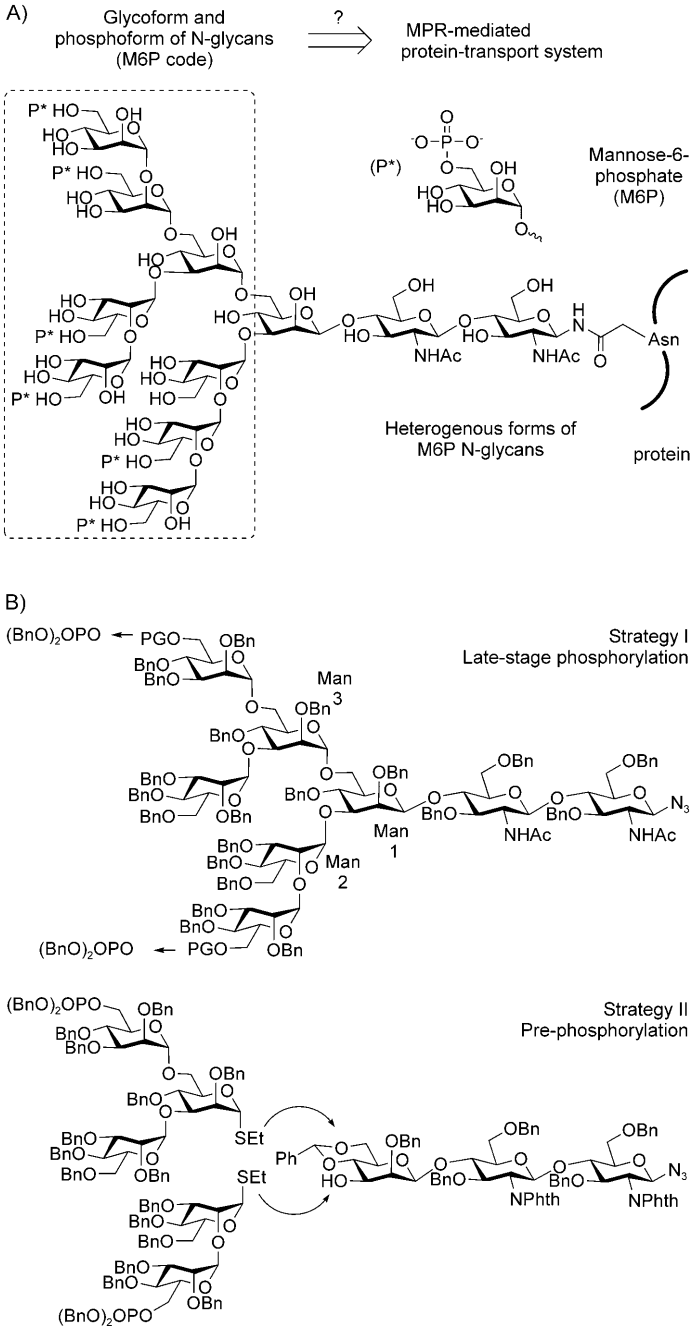
DOI: 10.1002/cbic.201000785

Chemical Synthesis of a Bisphosphorylated Mannose-6-Phosphate N-Glycan and its Facile Monoconjugation with Human Carbonic Anhydrase II for in vivo Fluorescence Imaging

Yunpeng Liu,[a] Yan Mei Chan,[a] Jianhui Wu,[a, c] Chen Chen,[a] Alan Benesi,[a] Jing Hu,[b] Yanming Wang,[b] and Gong Chen*[a]

Dedicated to Professor Raymond Funk on the occasion of his 60th birthday.

Mannose-6-phosphate (M6P) containing N-linked glycans (N-glycans) belongs to a class of high-mannose-type oligosaccharides with phosphorylation on the C6-OH groups of peripheral mannoses.[1] These M6P N-glcans function as a universal organelle-targeting signal that directs the transport of some 60 newly synthesized hydrolases and some non-hydrolase proteins from Golgi to endosome/lysosome compartments through specific interaction with two *p*-type lectins, cation-dependent and cation-independent M6P receptors (CD- and CI-MPRs) in eukaryotic cells.[2] On the cell surface, CI-MPR controls the level of a number of extracellular hydrolases and insulin-like growth factor II through receptor-mediated endocytosis. Defects in this carbohydrate–lectin recognition system are known to cause severe physiological conditions such as lysosomal storage diseases, and are closely related to the progress of cancers and liver fibrosis.[3] The current understanding of this complex protein-transport system is incomplete, particularly with regard to the detailed structure–activity relationship of the M6P sugar-coding sytem at the molecular level. Only limited knowledge is available on how the sugar structure, the location of the phosphate group(s), and the valency of M6P in N-glycans affect their binding to MPRs (Scheme 1 A).[4] Efforts to carry out detailed glycan receptor binding studies have been hampered by the inherent heterogeneity of M6P N-glycans and the formidable difficulties in obtaining adequate amounts of different, structurally well defined M6P N-glycans in good purity from natural resources. Powered by the recent advance of chemical synthesis of complex carbohydrates, total synthesis of M6P N-glycans with defined glycoform and phosphoform would provide an attractive solution to this long-standing problem.[5,6]



Scheme 1. Structure and synthetic strategies for M6P N-glycans.

Herein, we report the first chemical synthesis of a fully elaborated bis-phosphorylated triantennary M6P N-glycan

[a] *Dr. Y. Liu, Y. M. Chan, Dr. J. Wu, Dr. C. Chen, Prof. Dr. A. Benesi, Prof. Dr. G. Chen*
Department of Chemistry, The Pennsylvania State University
104 Chemistry Building, University Park, PA 16802 (USA)
Fax: (+1) 814-863-5319
E-mail: guc11@psu.edu

[b] *J. Hu, Prof. Dr. Y. Wang*
Department of Biochemistry and Molecular Biology
The Pennsylvania State University
454A North Frear Laboratory, University Park, PA 16802 (USA)

[c] *Dr. J. Wu*
College of Pharmaceutical Sciences, Capital Medical University
Beijing, 100069 (China)

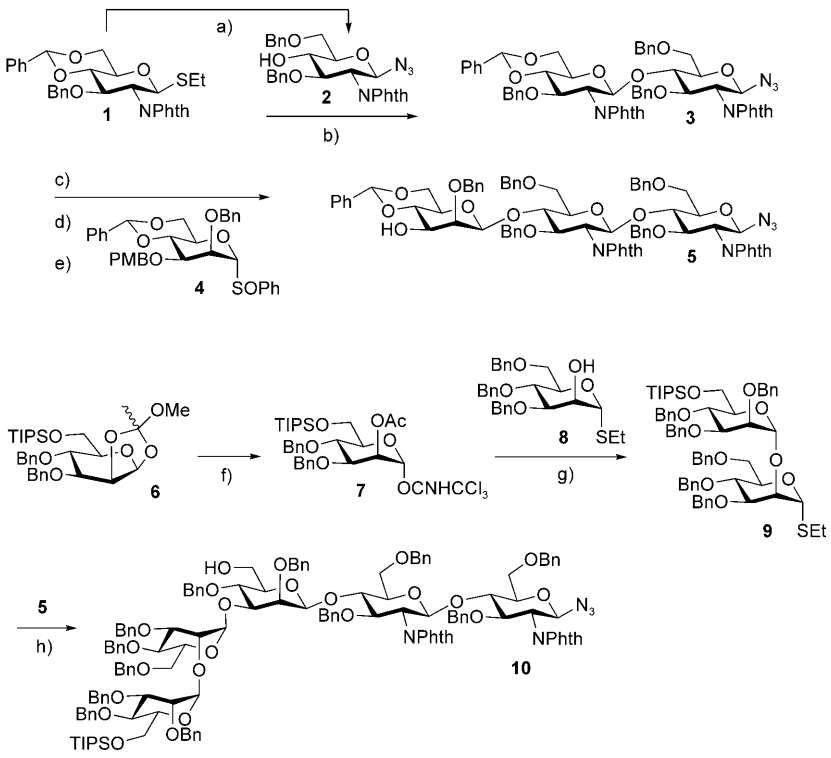
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000785.

through a a highly efficient late-stage phosphorylation strategy. In addition, we have constructed a monosubstituted neoglycoprotein platform based on human carbonic anhydrase II (HCAII) in a site-specific manner. Finally, using a non-covalent fluorescent tag that binds HCAII, we have successfully demonstrated that a single bivalent M6P N-glycan can facilitate MPR-mediated cellular uptake of the neoglycoprotein by Hep G2 cells.

The majority of naturally occurring M6P N-glycans carry one or two phosphate groups at different mannoses, and multivalent M6P N-glycans generally bind more tightly to the MPRs.[1] As a result of this, a bis-phosphorylated octasaccharide N-glycan was chosen as our initial synthetic target (Scheme 1 B). Multiphosphorylated M6P N-glycan poses a greater synthetic challenge than ordinary N-glycans. New synthetic strategies had to be delicately orchestrated and executed in order to incorporate labile phosphate groups.[7,8] In addition, due to the difficulties associated with the purificationof intermediates and the final product, high-yielding and stereoselective transformations are necessary for all the key steps, especially those in the late stages. In principle, two general synthetic strategies can be employed to prepare the target multi-phosphorylated N-glycans (Scheme 1 B). A global phosphorylation strategy can be applied to install the phosphate moieties at a late stage of the synthesis. Though this approach offers a convenient solution to preparing a variety of M6P N-glycans, we were initially concerned that it would suffer from potential complications due to incomplete multi-phosphorylation steps near the end of the synthesisand difficulties in purifying partially phosphorylated products from our target.

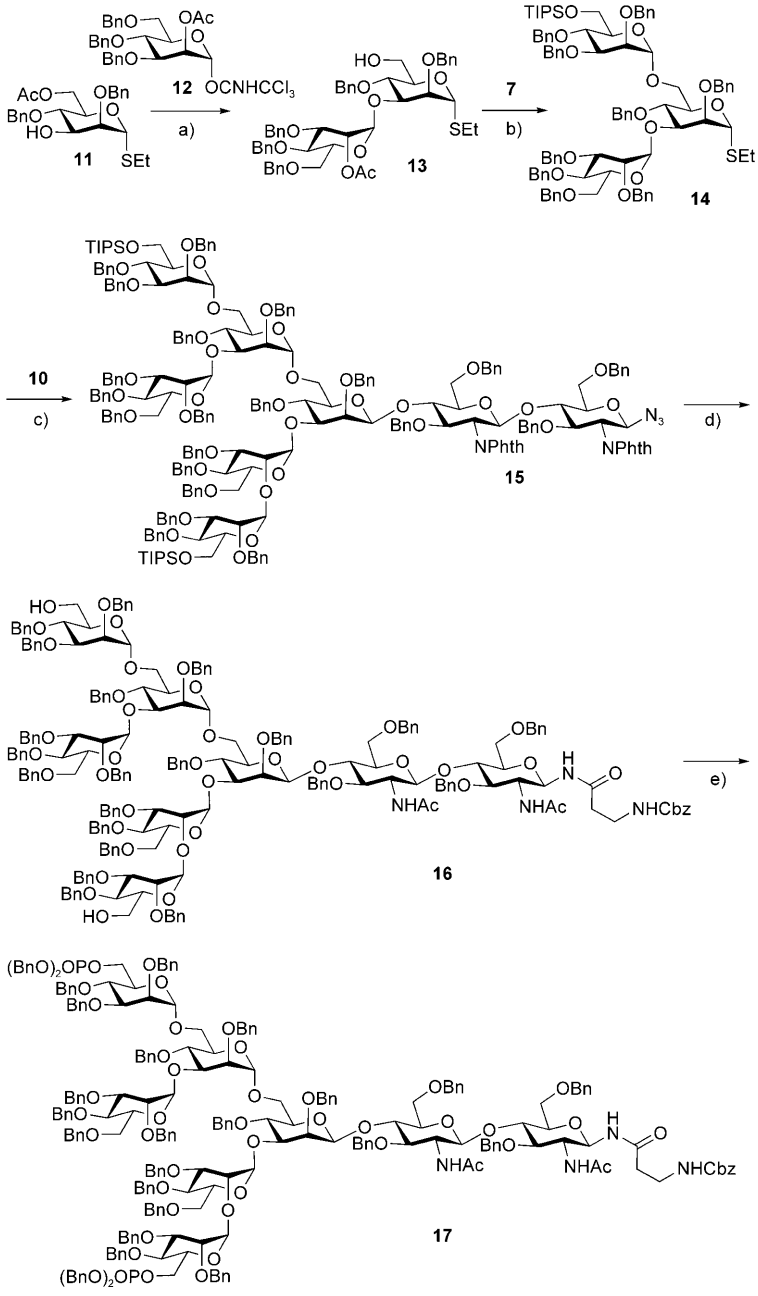
Alternatively, a properly protected phosphate moiety could be first incorporated into simple mannose building blocks, which could be assembled in a modular fashion to provide the final multi-phosphorylated product. These protected phosphate moieties should withstand glycosylation and other procedures, and can be quantitatively removed at the end. Due to its convergence, the pre-phosphorylation strategy was tested in our initial synthesis of the octasaccharide M6P N-glycan. O-Benzyl groups were chosen for the protection of all the hydroxyl and phosphate groups; an azido group was used to mask the nitrogen on the reducing end, and phthalimide (Phth) groups were used to protect the amino groups of glucosamines. However, it was soon found that the benzyl-protected phosphate groups failed to survive all attempts to remove the Phth group of glucosamine with any nucleophilic treatment tested.[9] The chemical lability of the Bn-protected phosphate group in this N-glycan system forced us to pursue the seemingly riskier late-stage phosphorylation strategy.

Accordingly, chitobiose **3**[10] was prepared from the easily accessible benzylidene-protected glucosamine thioether donor **1** and glucosamine acceptor **2** under promotion by MeOTf (Tf = triflate, Scheme 2). Glycosylation of mannose sulfoxide donor **4** and the chitobiose acceptor under Crich–Kahne conditions gave the desired trisaccharide intermediate in satisfying yield and β-selectivity.[11] Upon oxidative deprotection of the *p*-methoxybenzyl group, the core trisaccharide acceptor **5** was readily prepared in large quantity. The triisopropylsilyl (TIPS) protecting group, which tolerates the acidic treatment required for the glycosylation and the benzylidene opening steps, was chosen to mask the latent C6-OH phosphorylation site on the mannose residues (Scheme 2).[12] Man_2 thioether donor **9** was prepared and then stereoselectively installed onto trisaccharide core **5** with *N*-iodosuccinimide (NIS)/TfOH. Upon benzylidene opening with $Et_3SiH/PhBCl_2$, pentasaccharide acceptor **10** was obtained in excellent yield.



Scheme 2. Synthesis of compound **10**. Reagents and conditions: a) $TMSN_3$, MeOTf, MS 4 Å, CH_2Cl_2, RT, 92%; b) MeOTf, MS 4 Å, CH_2Cl_2, RT, 82%; c) $NaBH_3CN$, HCl (1 M in Et_2O), MS 3 Å, THF, RT, 93%; d) **4**, Tf_2O, TTBP, MS 3 Å, CH_2Cl_2, −40 °C; e) CAN, CH_3CN, phosphate buffer (pH 7.0), RT, 50% over two steps; f) i: $HgBr_2$, EtSH, CH_3CN, RT, 90%; ii: NBS, acetone/H_2O, −20 °C, 92%; iii: DBU, CCl_3CN, CH_2Cl_2, −20 °C, 92%; g) i: **8**, TMSOTf, MS 4 Å, CH_2Cl_2, −20 °C, 80%; ii: NaOMe, MeOH, RT, 94%; iii: BnBr, NaH, TBAI, DMF, RT, 89%; h) i: **5**, NIS, TfOH, Et_2O, MS 4 Å, −20 °C→RT, 92%; ii: $PhBCl_2$, Et_3SiH, CH_2Cl_2, −78 °C, 92%. $TMSN_3$ = trimethylsilyl azide, MS = molecular sieves, TTBP = tri-*tert*-butylpyridine, CAN = cerium ammonium nitrate, NBS = *N*-bromosuccinimide, DBU = 1,5-diazabicyclo-(5.4.0)undec-5-ene, TMSOTf = trimethylsilyl triflate, TBAI = tetrabutylammonium iodide.

TIPS-protected Man_3 branch **14** was prepared in the thioether form by using standard protocols (Scheme 3). After an extensive screening, the NIS/AgOTf conditions were found to be superior in facilitating a highly efficient and stereoselective installation of the upper-branch **14** to acceptor **10** ($a/b > 15:1$, stereochemistry was confirmed by $^1J_{1CH}$ of Man3[13]). In order to minimize the number of post-phosphorylation synthetic steps, the N-Phth groups of the resulting octasaccharide were transformed to NH-Ac groups, and the azido group was reduced by propanedithiol and coupled with Cbz-β-alanine by HATU.[14] TIPS groups were then removed with tetrabutylammonium fluoride (TBAF) to give compound **16**. Gratifyingly, final bisphosphorylation of **16** through dibenzyl *N*,*N*-diisopropyl phosphoramidite/*m*CPBA oxidation gave the desired bisphosphorylated product **17** in near quantitative yield.[15] The final global deprotection of Bn groups and the Cbz group proceeded cleanly by first catalytic hydrogenolysis with $Pd(OH)_2$ in MeOH/EtOAc/H_2O (15:8:3), followed by Pd/C in MeOH/H_2O under H_2 (1 atm; Figure 1 A).[16] Analytically pure N-glycan product **18** bearing an amino group handle was obtained in near quantitative yield without chromatographic purification (Figure 1 B, see the Supporting Information for ^{13}C, ^{31}P NMR and MS characterization).



Scheme 3. Synthesis of compound **17**. Reagents and conditions: a) i: TMSOTf, CH_2Cl_2, MS 4 Å, −20 °C, 79%; ii: thiourea, 2,6-lutidine, MeOH, 70 °C, 98%; b) i: TMSOTf, MS 4 Å, CH_2Cl_2, −20 °C, 70%; ii: NaOMe, MeOH, RT, 80%; iii: BnBr, NaH, TBAI, DMF, RT, 94%; c) **10**, NIS, AgOTf, Et_2O, MS 4 Å, −20 °C-RT, 72%, ($\alpha/\beta > 15:1$); d) i: ethylenediamine, *n*-butanol, 90 °C; ii: Ac_2O, pyridine, RT, 63% over two steps; iii: propanedithiol, DIEA, MeOH, RT; iv: Cbz-β-alanine, HATU, DIPEA, *N*-methyl-2-pyrrolidone, RT, 50% over two steps; v: TBAF, THF, RT, 90%; e) i: $(BnO)_2PN(iPr)_2$, tetrazole, MS 4 Å, CH_2Cl_2, RT; ii: *m*CPBA, RT, 91% over two steps. HATU = 2-(1*H*-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium, DIPEA = *N*, *N*-diisopropylethylamine, $(BnO)_2PN(iPr)_2$ = dibenzyl *N*, *N*-diisopropylphosphoramidite, *m*CPBA = *m*-chloroperoxybenzoic acid.

With this M6P N-glycan in hand, we constructed a neoglycoprotein in order to evaluate the contribution of the N-glycan in ligand/CI-MPR interactions and cellular uptake. As stated before, preparing homogenous, naturally occurring glycoprotein by using current methodologies remains a formidable technical challenge.[17] We feel that a chemically well defined neoglycoprotein that carries a monosubstitution of M6P N-glycan linked to a protein molecule at a precise location would provide an excellent model with which to study the function of an individual N-glycan.[18]

The resulting neoglycoprotein could be further fluorescently tagged and utilized to track the M6P N-glycan-directed protein-transport process inside cells by using fluorescence imaging. Human carbonic anhydrase II (HCAII), a 29 kD globular protein that carries an unpaired Cys206, is particularly suited for this purpose for two reasons.[19] Firstly, the SH group of Cys206 is remote from the active site and so can be used to conjugate agents carrying an iodoacetamide group; the resulting HCAII conjugate retains its catalytic and ligand-binding ability.[20] Secondly, HCAII forms a tight 1:1 noncovalent complex with simple arylsulfonamide-derived ligands. A fluorescent tag tethered to an arylsulfonamide ligand can be used to complex the HCAII conjugate to provide a fluorescently tagged neoglycoprotein probe.[21]

Accordingly, we prepared the monosubstituted neoglycoprotein probe with the synthetic M6P N-glycan. M6P N-glycan **19** bearing an α-iodoacetamide handle was obtained in high yield by treating glycan **18** with iodoacetic anhydride (Figure 1 A). The conjugation reaction between **19** (10 equiv) and Cys206 of HCAII was effected by using a well-known strategy, firstly under denaturing conditions (5 M guanidine HCl, pH 7.6) for 12 h at 25 °C.[22] Upon dilution (to 0.3 M guanidine) and standing, the modified HCAII

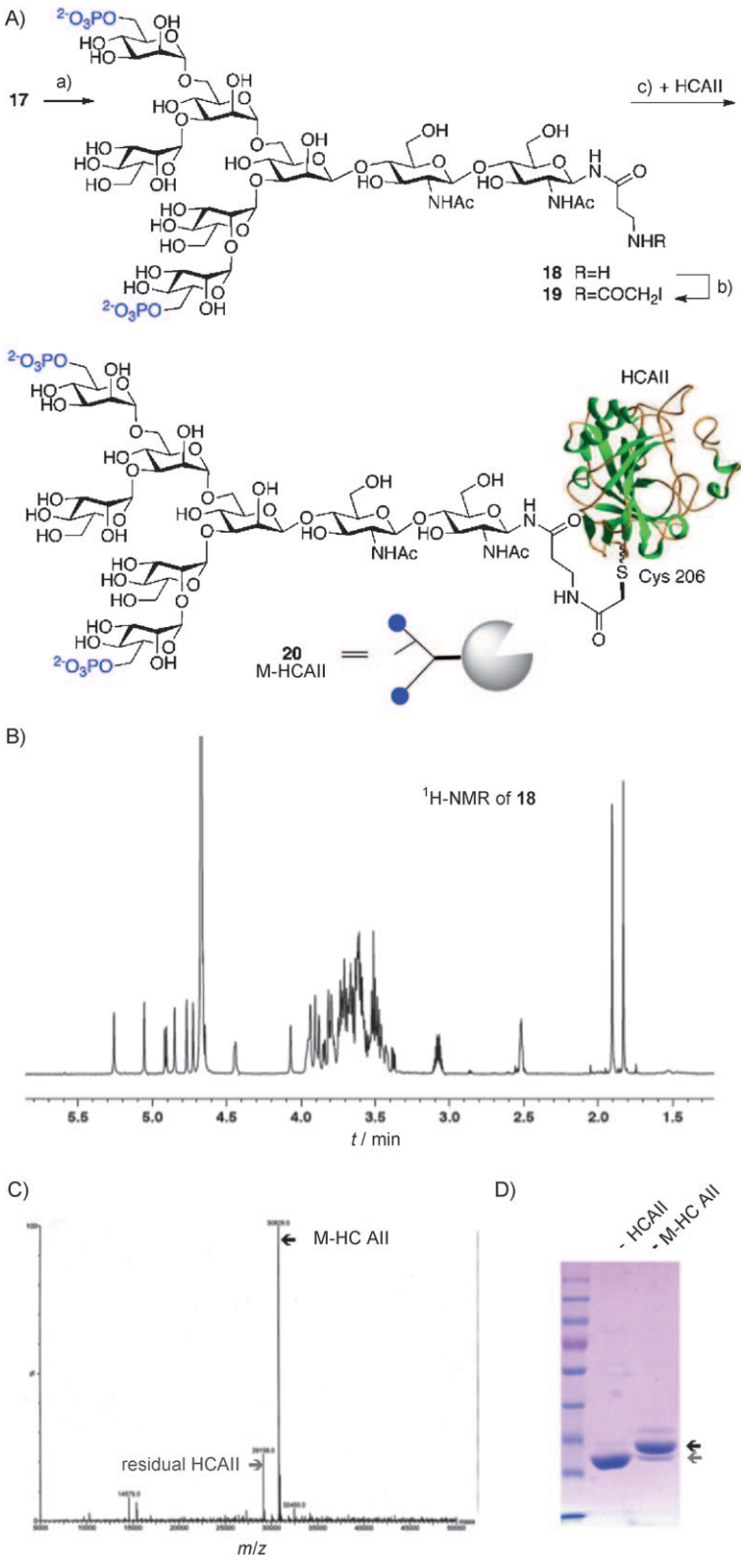


Figure 1. Global deprotection and monoconjugation of the M6P N-glycan with HCAII. A) Reagents and conditions: a) i: $Pd(OH)_2$, H_2 (1 atm), MeOH/EtOAc/H_2O (15:8:3), RT, 8 h; ii: 10% Pd/C, H_2 (1 atm), MeOH/H_2O, RT, 12 h, 92% over two steps; b) iodoacetic anhydride, $NaHCO_3$, H_2O, RT, 12 h, 91%; c) HCAII labeling: HCAII, **19** (10 equiv), guanidine HCl (5 M), Tris buffer pH 7.6, 25 °C, 12 h; then quenched with 2-mercaptoethanol and diluted into guanidine (0.3 M) for HCAII refolding; B) ^1H NMR spectrum of **18** (D_2O, 850 MHz); C) ESR-TOF MS of M-HCAII conjugate; D) SDS-PAGE of HCAII and M-HCAII (stained by coomassie blue).

readily refolded, and its enzymatic activity was restored.[23] After the removal of excess **19** and buffer exchange, the desired monolabeled product (M-HCAII) was obtained in ~70% purity based on protein gel staining (Figure 1C, D). ESR-TOF MS analysis confirmed that a single N-glycan molecule was conjugated to HCAII.

The arylsulfonamide-derived fluorescent affinity ligand (LF) was easily prepared by standard amide couplings (Figure 2A). *p*-Sulfonamidobenzoic acid (**21**) was first extended with the linker hexane-1,6-diamine (**22**) and then capped with Texas Red succinimidyl ester. Upon mixing LF and M-HCAII in Tris buffer (100 mM, pH 7.6) for 2 h at room temperature, a tight protein complex (M-HCAII-LF) formed that was readily purified on a short Sephadex G25 column (Figure 2B); the stoichiometry of the ligand/protein complex was estimated to be 0.7:1 by UV spectrometry (Supporting Information). A control probe (without the N-glycan-modification, HCAII-LF) was also prepared by using the same procedure. The dissociation constants, K_d, of LF/M-HCAII (90 nM) and LF/HCAII (65 nM) complexes were determined from fluorescence-polarization assays (Supporting Information).

The fluorescently labeled protein probes were then subjected to cell-based internalization assay. CI-MPR at the cell membrane is responsible for the receptor-mediated endocytosis process for extracellular proteins bearing M6P N-glycans.[24] Hep G2 hepatocarcinoma cells were used as a model in our initial assays because high levels of CI-MPR were detected on the membrane of these cells.[25] Briefly, Hep G2 cells were incubated with the M-HCAII-LF and HCAII-LF probes respectively for 4 h, washed extensively with blank buffer, and analyzed by confocal fluorescence microscopy. The fluorescence imaging study indicated that M-HCAII-LF was readily internalized and accumulated in endocytotic compartments, while HCAII-LF showed only background levels of internalization (Figure 2C). These experiments clearly demonstrated that the HCAII-based neoglycoprotein platform will allow us to carry out well-controlled, cell-based assays in order to investigate the cellular function of the individual M6P N-glycan and to analyze the dynamics of this M6P-directed protein-transport process.

In summary, we have accomplished the first chemical synthesis of a fully elaborated bis-M6P triantennary N-glycan through a highly efficient, late-stage phosphorylation strategy. The synthetic N-glycan was then monoconjugated with the model protein HCAII to provide a functional neoglycoprotein that was further fluorescently tagged by facile, noncovalent complexation with a fluorescent affinity ligand. Confocal fluorescence imaging analysis of the cell-based internalization assays was successfully carried out to evaluate the M6P-facilitated cellular uptake of the neo-

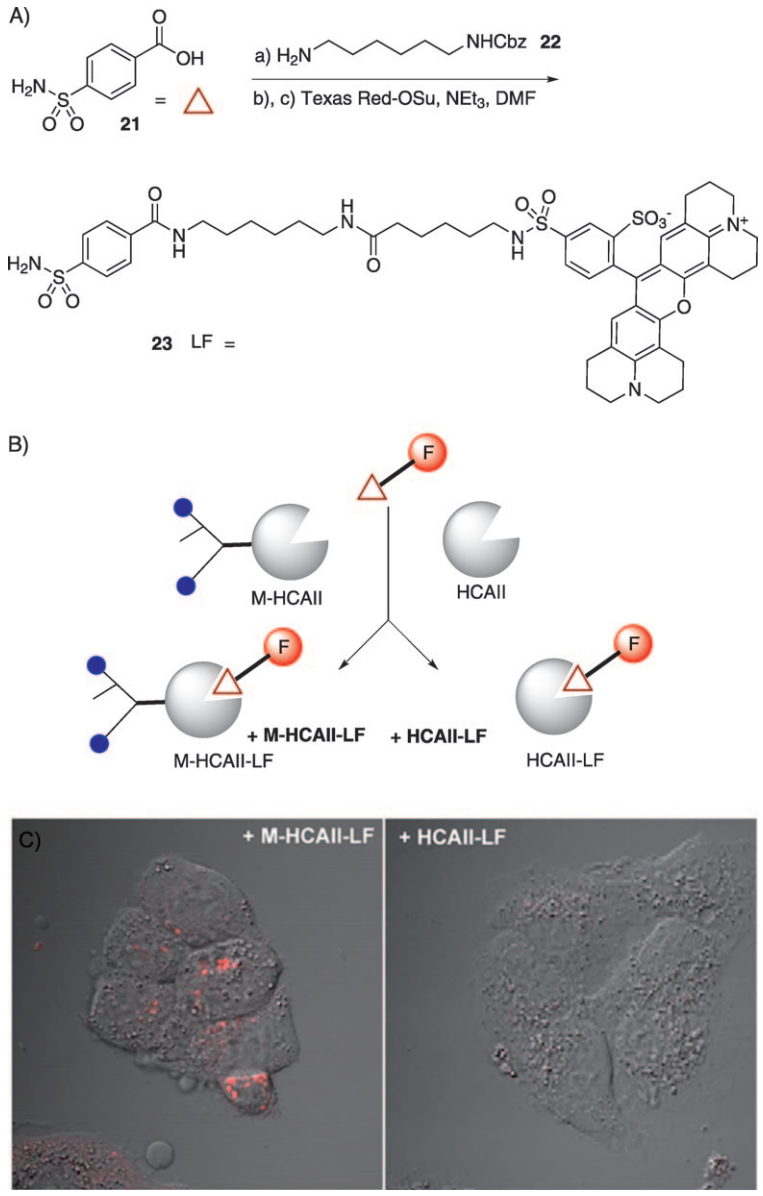


Figure 2. Cell-based internalization assays with the fluorescently tagged protein probe. A) Synthesis of affinity ligand LF: a) **24**, EDC, HOBt, DMF, DIPEA, RT, 90%; b) $Pd(OH)_2$, H_2 (1 atm), $MeOH/CH_2Cl_2$, RT; c) Texas Red-OSu, TEA, DMF, RT, 92% over two steps; B) Preparation of M-HCAII-LF complexes: mixing of M-HCAII (or HCAII) and LF (20 equiv) in Tris buffer (100 mM, pH 7.6) for 2 h at RT, purified by short gel filtration column filled with Sephadex G25; C) Fluorescent images of M-HCAII-LF and HCAII-LF uptake by Hep G2 cells. Cells were grown on glass coverslips then incubated with the protein probes for 4 h. After extensive washing to remove extracellular probes, fluorescent images were observed under an Olympus FluoView 1000 confocal microscope. Differential interference contrast (DIC) images were simultaneously obtained to show cell morphology. EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, DMF = *N*,*N*-dimethylformamide, TEA = triethylamine.

glycoprotein probe. We believe that this study has laid a solid foundation to allow us to test systematically the structure-activity relationships of M6P N-glycans. Highly effective M6P N-glycan ligands revealed by those studies will find broad application in MPR-targeted therapy and diagnosis.

Acknowledgements

We thank the Pennsylvania State University for its support of this work.

Keywords: fluorescence imaging · HCAII · mannose-6-phosphate · MPR · neoglycoproteins · N-glycans

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Received: December 23, 2010
Published online on February 18, 2011

