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Synthesis of glycoprotein molecular probes for the analyses of protein quality control system

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Introduction

Recently, the functions of glycoprotein glycan chains in protein quality control are attracting particular attention [1] (Figure 1). Calnexin (CNX) and calreticulin (CRT) are ER-residing homologous chaperones, the former being membrane-bound and the latter being soluble [2,3]. It is believed that they have unique lectin properties, specifically recognizing $Glc_1Man_9GlcNAc_2$ (G1M9, **3a**), which is formed by stepwise removal of glucose residues from $Glc_3Man_9GlcNAc_2$(G3M9, **1a**). After further removal of the innermost glucose, glycoproteins possessing $Man_9GlcNAc_2$ (M9, **4a**) follow the diverged pathways. Namely, correctly folded proteins are transported to the Golgi for further processing, while misfolded ones are reglucosylated back to **3a** by the action of UDP-glucose:glycoprotein glucosyltransferase (UGGT) [4]. Fatally misfolded glycoproteins are delivered to the degradation pathway called ER-associated degradation (ERAD) [5]. Mannosidase-like proteins (MLPs), EDEM discovered from mammalian cells [6] and its yeast counterpart Mnl1p/Htm1p [7], are considered to have a lectin property and recognize glycoproteins having M8 oligosaccharides ($Man_8GlcNAc_2$, B-isomer, **5a**), which are formed by the action of ER mannosidase I from M9 [8,9]. Glycoproteins directed to ERAD are degraded by the proteasome [10–12].

In order to gain precise understanding of glycoprotein quality control, accesses to homogeneous and structurally defined oligosaccharides and glycoproteins are highly desired. In this minireview, we wish to summarize our recent efforts toward (1) the convergent synthesis of ER-related oligosaccharides and their partial structures, (2) the creation of glycoprotein mimetics, and (3) observation of the specific interaction with CRT.

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Synthesis of ER related high-mannose-type oligosaccharides

Considering the central importance of CNX/CRT in glycoprotein quality control, the chemical syntheses of monoglucosylated dodecasaccharide **3b** (Figure 2) was conducted first [13]. For comparative purpose, β-Glc-isomer (**7**) as well as nonglucosylated counterpart (M9, **4b**) were also prepared. Subsequently, octamannosylated decasaccharide (M8, **5b**)(the proposed ligand of MLPs) and its monoglucosylated homologue (**6b**) were synthesized [14].

For the convergent synthesis of target oligosaccharides, fragments corresponding to Glc (**8**,**9**), $Man_1GlcNAc_2$ (**10**), Man_4 (**11**) or Man_5 (**12**), and Man_3 (**14**) components were designed (Scheme 1). Fragment **10** was constructed from **13** by *p*-methoxybenzyl (PMB) assisted intramolecular aglycon delivery [15] developed in our laboratory as the key.

Octa- (**5b** and **6b**) and nonamannosylated glycan chain (**4b**, **3b** and **7**) were constructed as shown in Scheme 1. The coupling of **10** with **14** afforded 3-*O*-glycosylated hexasaccharide that was converted to the diol **15**. Regioselective glycosylation with tetra/pentasaccharide donor **11/12** gave deca- (**16a**) and undecasaccharide (**17a**), respectively. These compounds were then converted to **16b** and **17b**. For the incorporation of the α-linked glucose residue, glycosylation with the thioglucoside (**8**) proceeded satisfactorily and provided the desired undeca- (**18**) and dodecasaccharide (**19**), both as a single isomer. On the other hand, the stereoisomeric dodecasaccharide (**20**) carrying β-linked glucose residue was synthesized using glycosyl donor **9**. Finally, complete deprotection afforded M9 (**4b**), G1M9 (**3b**), M8 (**5b**), G1M8 (**6b**)gnd β-G1M9 (**7**), respectively. In addition,

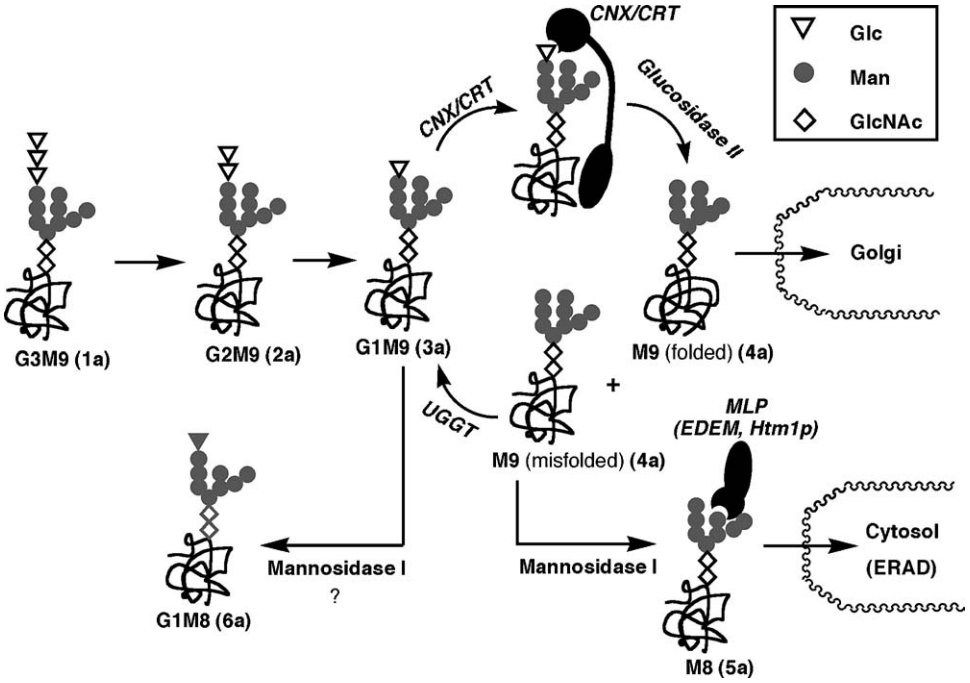
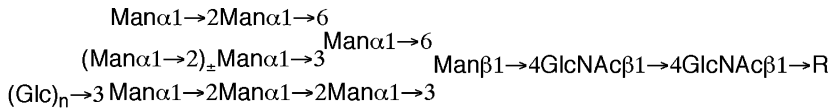


Figure 1. Glycoprotein processing and quality control in ER.



	$(Glc)_n$	(Manα1→2)
G3M9 (**1**)	Glcα1→2Glcα1→3Glcα1	+
G2M9 (**2**)	Glcα1→3Glcα1	+
α-G1M9 (**3**)	Glcα1	+
β-G1M9 (**7**)	Glcβ1	+
M9 (**4**)	H	+
M8 (**5**)	H	–
G1M8 (**6**)	Glcα1	–

	R
1~6a	Asn-Protein
1~6b, 7	OC_3H_7

Figure 2. Structures of high-mannose oligosaccharides associated with chaperone recognition.

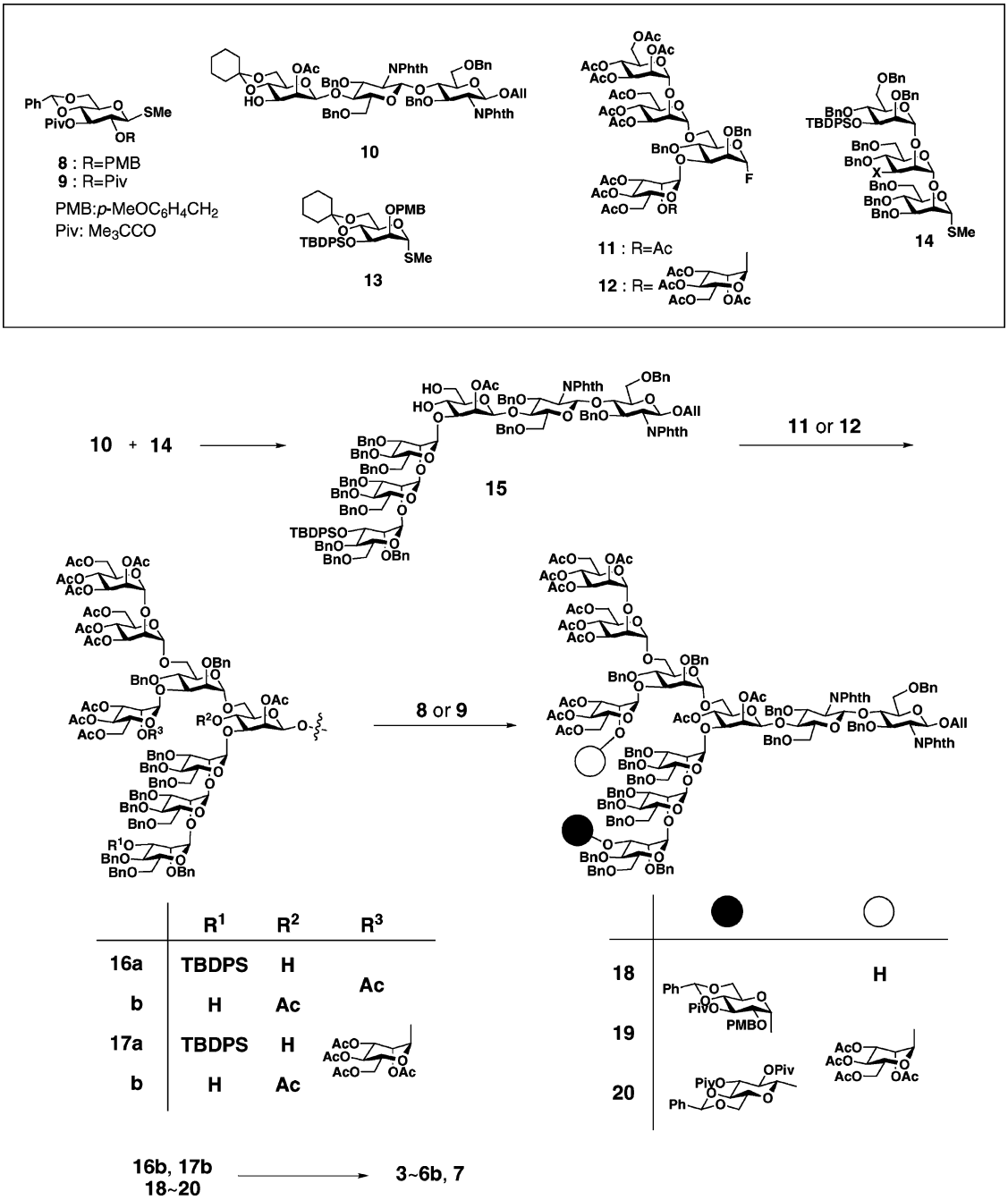
syntheses of G2M9 (**2b**) and G3M9 (**1b**) were accomplished recently [16].

Facile synthesis of the partial structure of ER-oligosaccharide

Syntheses of terminal trimannose (**21**, Figure 3) and monoglucosylated trimannose (**22**, Figure 3) portions of G1M9 were conducted as depicted in Scheme 2 [17]. For the temporary protection of 2- and 3-OH, pentafluoropropionyl (PFP) and trifluoroacetyl (TFA) groups were employed. Since the Glc_1Man_3 arm is considered to be play the primary role in CRT/CNX recognition, undecasaccharide **26** having bidentate Glc_1Man_3 was prepared, as well as its monodentate congener **25** (Figure 3) [18].

Approaches to artificial glycoproteins

Since naturally occurring glycoproteins are generally complex mixtures of various "glycoforms", glycoprotein mimetics having homogeneous and structurally defined oligosaccharides would be valuable alternatives [19]. In this respect, we



	R1	R2	R3
16a	TBDPS	H	Ac
b	H	Ac	
17a	TBDPS	H	
b	H	Ac	

18		H
19		
20		

Scheme 1. Synthesis of ER-related oligosaccharide.

turned our attention to the combination of dihydrofolate reductase (DHFR) [20] and its high-affinity ($K_D < 1$ nM) ligand methotrexate (MTX) (Figure 4) [21].

In the first place, pentasaccharide bifunctional ligand **27a** was prepared. Synthetic $Man_3GlcNAc_2$ was transformed to glycosylamine **27b** [22] and converted to glycine-linked oligosaccharide **27c** that was condensed with MTX(α^tBu) [23] and following deprotection of ^{t}Bu group to give **27a**. Undecasaccharide (M9)-MTX conjugate **28a** was prepared in a similar manner from M9 [24]. These sugar-MTX conjugates retained strong affinity to DHFR and their isolation were achieved by lectin beads.

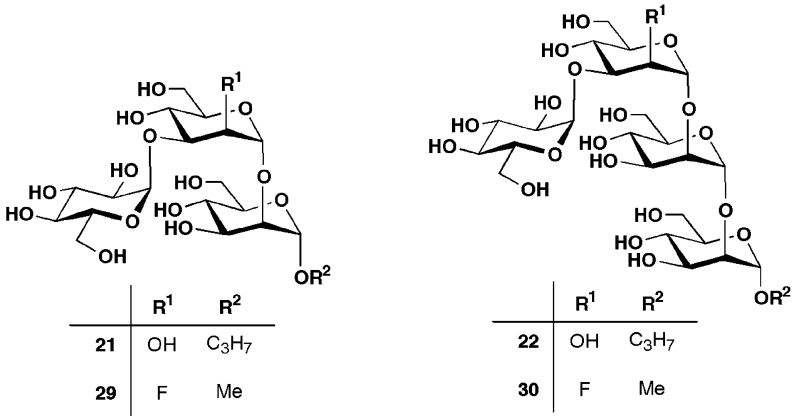
As the second approach to artificial glycoproteins, ligation of synthetic oligosaccharide with carbonic anhydrase (CA) was investigated. CA is a relatively small (27 kD) protein having a single Cys residue. Taking advantage of the reactivity of thiol, chemoselective ligation of oligosaccharide [25] was conducted. Thus, undecasaccharide **17b** was converted to iodoacetamide

Glcα1→3Manα1→2Manα1→2Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAcβ1→R

25

Glcα1→3Manα1→2Manα1→2Manα1→6
Glcα1→3Manα1→2Manα1→2Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAcβ1→R

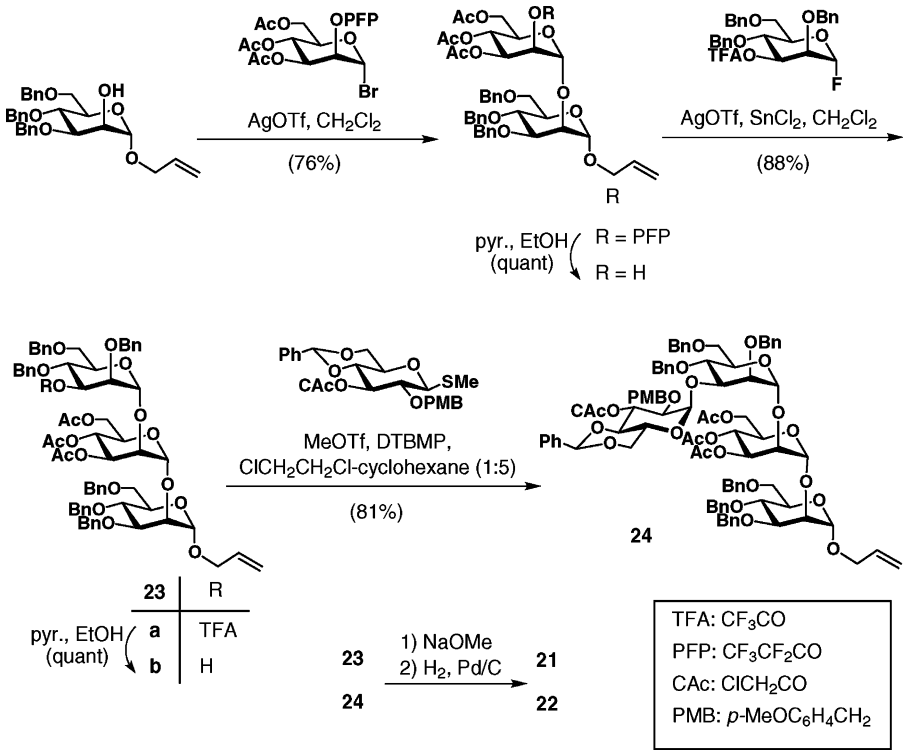
26



	R^1	R^2
21	OH	C_3H_7
29	F	Me

	R^1	R^2
22	OH	C_3H_7
30	F	Me

Figure 3. Synthetic analogues of high-mannose type oligosaccharides.



Scheme 2. Facile synthesis of Man_3 and Glc_1Man_3 using PFP and TFA as protecting groups.

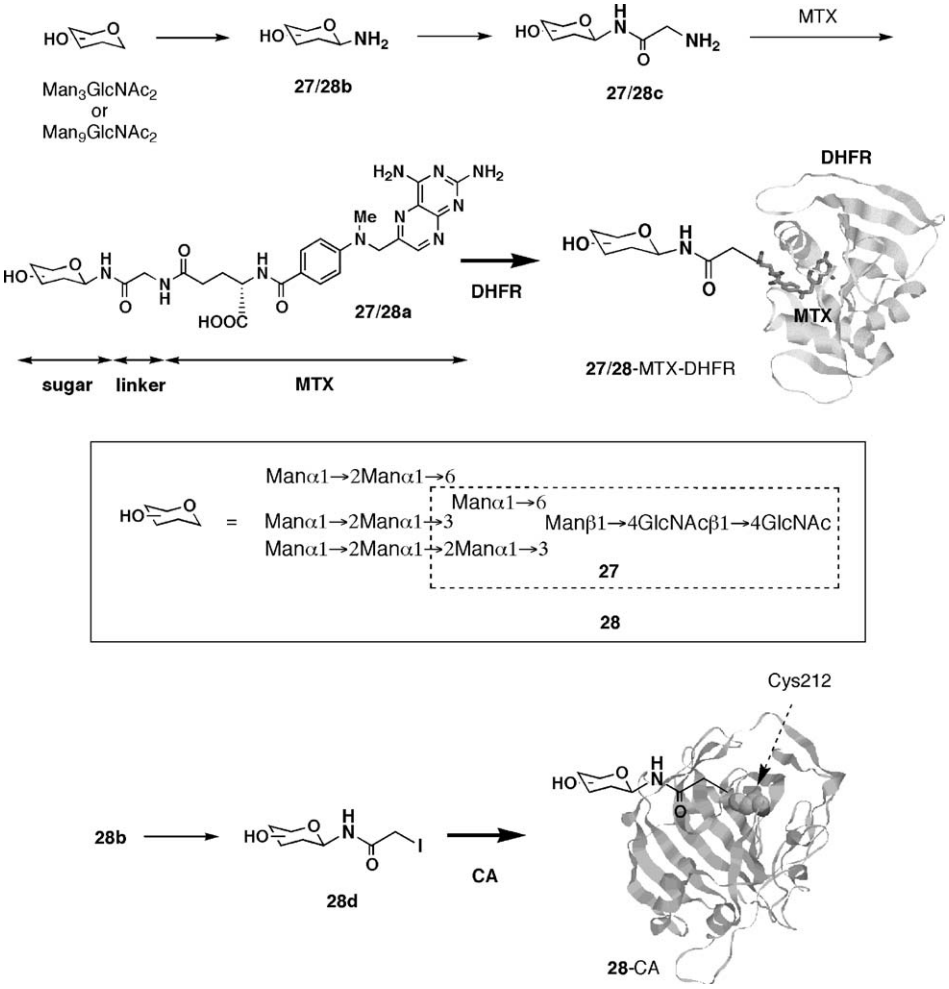


Figure 4. Preparation of artificial glycoproteins.

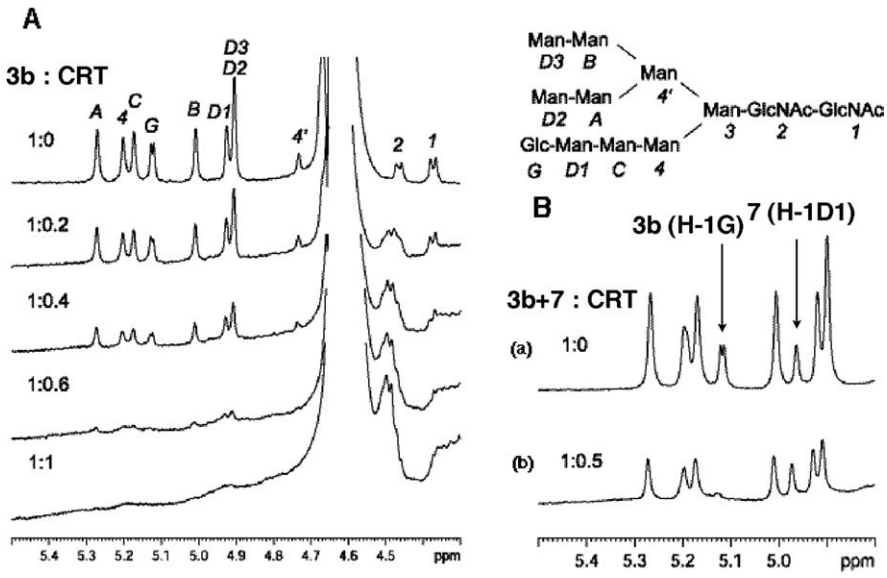


Figure 5. ^{1}H NMR spectra of $Glc_1Man_9GlcNAc_2$ + CRT; (A) anomeric region of 1D ^{1}H NMR spectra acquired of $\alpha Glc_1Man_9GlcNAc_2$ (**3b**) in $^{2}H_2O$, 10 mM Tris-HCl buffer, 10 mM $CaCl_2$ at pH 7.3. CRT was added to the sample by a step wise, final ratio of **3b** and CRT was 1:1. (B) anomeric region of 1D ^{1}H NMR spectra acquired of **3b** + **7** (1:1) without (a) and with 0.5 eq. of CRT(b).

28d via glycosylamine **28b**. Coupling was conducted under denaturing conditions to give M9 incorporated **28**-CA [26].

In both of above approaches, glycosylamines served as key intermediates. Because the conversion of complex glycan chain to corresponding glycosylamine is well-established [27], it is expected that various types of oligosaccharides can be incorporated to DHFR and/or CA to analyse the interaction with ER chaperones and lectins.

Analysis of interaction between synthetic oligosaccharides and proteins

Synthetic dodecasaccharide (Figure 2, **3b**) was subjected to binding experiment with CRT using ^{1}H-NMR. In the presence of recombinant CRT, all anomeric signals were strongly suppressed by extensive broadening, implying that **3b** binds tightly with CRT under these conditions (Figure 5A) [28]. Using 1:1 mixture of **3b** and its stereoisomer **7**, in the presence of 0.5 equiv. (with respect to the mixture of **3b** and **7**) of CRT, only peaks derived from **3b** were strongly suppressed, implying that the CRT recognizes the fine structure of G1M9 (Figure 5B) [13].

Oligosaccharide-CRT interactions were subjected to quantitative measurement using isothermal titration calorimetry (ITC) [29]. Preliminary results strongly support the current view of the specificity of CRT recognition. Namely, G1M9 (**3b**) had a strong affinity ($K_B \sim 5.2 \times 10^6$ M^{-1}) to CRT, while no interactions with G2M9 (**2b**) and M9 (**4b**) were observed within the detection limit of ITC. Judging from the slightly reduced affinities of G1M8 (**6b**) ($K_B \sim 5.2 \times 10^6$ M^{-1}) and Glc_1Man_3 (**22**, $K_B \sim 1.6 \times 10^6$ M^{-1}), Man_5 branch seems to play an adjunct role in CRT binding. Further interaction analyses of the mono/bidentate hepta/undecasaccharides **25/26**, and fluorine-substituted Glc_1Man_2 (**29**) and Glc_1Man_3 (**30**) are under way.

Summary and future outlook

Convergent and stereoselective synthetic route to $Man_8GlcNAc_2$, α-$Glc_1Man_8GlcNAc_2$ $Man_9GlcNAc_2$, α-$Glc_1Man_9GlcNAc_2$ and its stereoisomer were established. Using ^{1}H-NMR and ITC, we observed the interaction with CRT. Glycoprotein mimetics were created by employing specific binding of MTX to DHFR and chemoselective ligation at Cys^{212} of CA. These oligosaccharides and artificial glycoproteins would be useful as molecular probes to analyze glycoprotein quality control. Further studies are in progress along this line and will be reported in due course.

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