DOI: 10.1002/cbic.200500143

Design and Synthesis of Oligosaccharides that Interfere with Glycoprotein Quality-control systems

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*Calnexin (CNX) and its soluble homologue calreticulin (CRT) are lectin-like molecular chaperones that help newly synthesized glycoproteins to fold correctly in the rough endoplasmic reticulum (ER). To investigate the mechanism of glycoprotein-quality control, we have synthesized structurally defined high-mannose-type oligosaccharides related to this system. This paper describes the synthesis of the non-natural undecasaccharide **2** and heptasaccharide **16**, designed as potential inhibitors of the ER quality-control system. Each possesses the key tetrasaccharide element (Glc_1Man_3) critical for the CNX/CRT binding, while lacking the pentamannosyl branch required for glucosidase II recognition. These oligosaccharides were evaluated for their ability to bind CRT by isothermal titration calorimetry (ITC). As expected, each of them had a significant affinity towards CRT. In addition, these compounds were shown to be resistant to glucosidase II digestion. Their activities in blocking the chaperone function of CRT were next measured by using malate dehydrogenase (MDH) as a substrate. Their inhibitory effects were shown to correlate well with their CRT-binding affinities, both being critically dependent upon the presence of the terminal glucose (Glc) residue.*

Introduction

The protein quality-control system in the endoplasmic reticulum (ER) ensures that only correctly folded proteins proceed along the secretory pathway.[1,2] The molecular chaperone calnexin (CNX) is a type I transmembrane protein, whilst calreticulin (CRT) is its soluble homologue. They share a lectin character, recognizing Asn-linked (Asn = asparagine; N-linked) high-mannose-type glycan chains, most typically $Glc_1Man_9GlcNAc_2$ (G1M9; **1a**), which is generated as the primary ligand from $Glc_3Man_9GlcNAc_2$ (G3M9) through sequential deglucosylation by glucosidases I (Glc-ase I) and II (Glc-ase II; Figure 1A). These chaperones are believed to promote protein folding by recruiting folding factors such as ERp57.[3,4] Glc-ase II further removes the α-1,3-linked Glc residue, to generate $Man_9GlcNAc_2$ (M9), which is inactive toward CNX/CRT. The correctly folded glycoproteins are subsequently transported to the Golgi apparatus, whereas the unfolded ones are reglucosylated by UDP-Glc:glycoprotein glucosyltransferase (UGGT) to regenerate the CNX/CRT ligand G1M9.[5] This constitutes the "CNX/CRT cycle", which plays the central role in glycoprotein quality control in the ER.[6,7] Two models to account for the chaperone activity of CNX/CRT have been proposed: the lectin-only model[8,9] and the dual-binding model.[10–12] The latter model hypothesizes that CNX and CRT each possess a polypeptide-binding site in addition to the lectin domain, while the former ascribes their function solely to the lectin domain. The precise role of the glycan chain in the chaperone cycle is still controversial.

Although the details remain ambiguous, a number of carbohydrate-binding proteins are considered to play important roles in the glycoprotein quality-control process; these include CNX/CRT, UGGT, Glc-ase I and II,[13] mannosidase-like proteins (EDEM, Mnlp1/Htm1p),[14–16] cargo receptors (e.g., ERGIC 53, VIP 36),[17] mannosidase I,[18] glycanase (PNGase),[19] and E3 ubiquitin ligase (Fbs).[20,21] In order to gain a clear understanding of the glycoprotein quality-control system, *structurally defined* high-mannose-type glycans would be valuable tools. As part of our effort toward the comprehensive synthesis of ER-related glycoprotein glycans,[22] we have previously achieved the first chemical synthesis of a monoglucosylated dodecasaccharide (G1M9),[23] the putative ligand for CNX/CRT. We have also reported the first synthesis of $Man_8GlcNAc_2$ (M8)—the proposed ligand of EDEM/Mnlp1/Htm1p—together with its monoglucosylated homologue $Glc_1Man_8GlcNAc_2$ (G1M8).[24]

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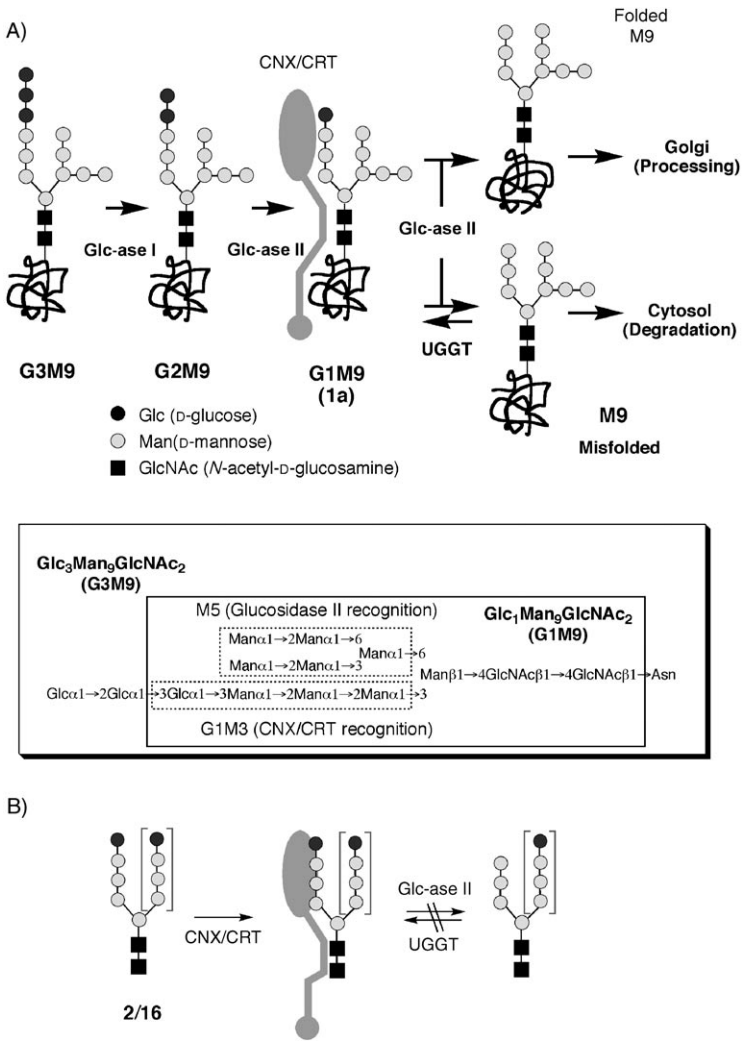


Figure 1. Glycoprotein processing and quality control in ER.

Most of the misfolded proteins are eliminated by the ubiquitin/proteasome-mediated degradation pathway. Under certain circumstances, however, misfolded proteins may escape the degradation process. Accumulation of such proteins results in the formation of aggregates and causes various "conformation-associated diseases", including Alzheimer's and Parkinson's diseases, amyloidoses, α_1-antitrypsin deficiency, and the prion encephalopathies.[25] α_1-Antitrypsin (α_1-AT) is a serum glycoprotein, secreted by the liver, and an α_1-AT deficiency mutation constitutes the genetic cause of liver disease in children, because of accumulation of the abnormally folded α_1-AT molecules in the ER lumen. A prolonged association of mutant peripheral myelin protein-22 (PMP-22) with CNX is found in a family of ER diseases termed heritable neuropathies.[26] Cells respond to such "ER stress" through the expression of chaperones and the nuclear transcriptional factor NF-κB, which is a central mediator of the immune response.

As a basis for exploration of mechanism-based therapies for "conformation-associated diseases", precise understanding of the above process is important. Since a number of proteins are involved in folding events in the ER, a specific inhibitor for each player would be helpful. The aim of this study was to synthesize non-natural high-mannose-like oligosaccharides (i.e., compounds **2** and **16**), designed as potential inhibitors of CNX/CRT (Figure 1B). Unlike inhibitors of glycoprotein processing, such as tunicamycin, which may cause global changes in glycoprotein composition, such compounds would be expected to perturb the folding event mediated by CNX/CRT in a specific manner.

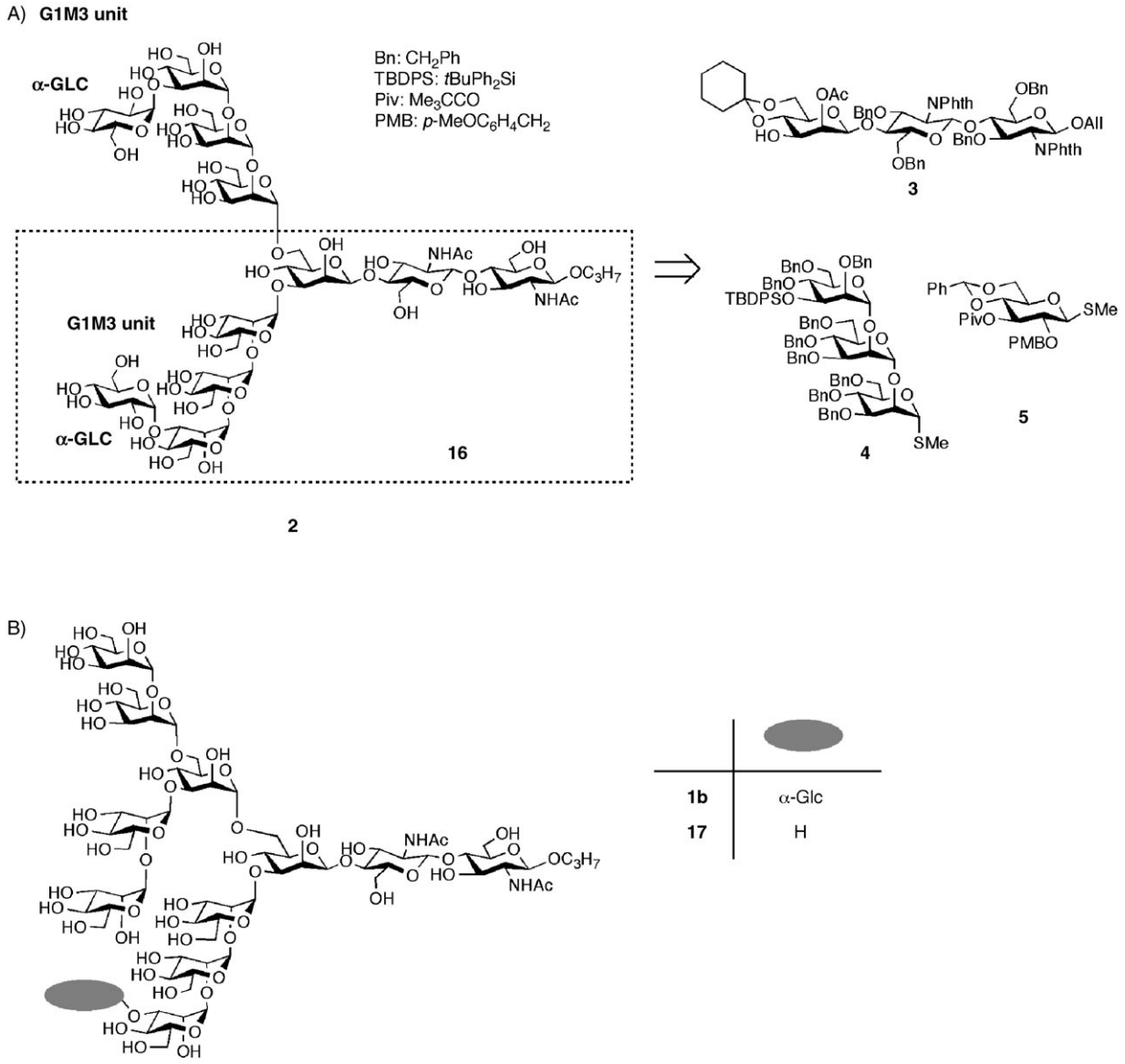
Results and Discussion

Design and synthesis of CNX/CRT inhibitors and related compounds

As depicted in Figure 1A, the native ligand of CNX/CRT (**1a**) contains both the CNX/CRT recognition site (G1M3) and a pentamannosyl branch (M5) critical for Glc-ase II recognition. Previous studies on the specificity of Glc-ase II have revealed that this enzyme requires the pentamannosyl branch (M5) for its full activity. The relative rates of Glc-ase II-catalyzed deglucosylation decline sharply as the number of mannose residues in the M5 branch decreases: the relative activities of $Glc_1Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$, $Glc_1Man_7GlcNAc_2$, $Glc_1Man_6GlcNAc_2$, and $Glc_1Man_5GlcNAc_2$ are 100, 21, 9, 5, and 3, respectively.[27] If the CNX/CRT ligand were to escape Glc-ase II trimming, it should remain associated with CNX/CRT to inhibit the glycoprotein quality-control system. In view of these factors, the non-natural undecasaccharide **2** and heptasaccharide **16** (Scheme 1) were designed. In these compounds the M5 branch of G1M9 (Figure 1B) has either been replaced by G1M3 (**2**) or has been removed (**16**). Since G1M3 itself has been known to display an affinity towards CRT,[28] it would be interesting to see whether compound **2**, which contains two G1M3 arms, would show an enhanced affinity relative to **16** and **1b**. In addition, **2** and **16** would be expected to be resistant to Glc-ase II and to bind to CNX/CRT in a stable fashion (Figure 1B).

For the convergent synthesis of the target oligosaccharides, fragments corresponding to $Man_1GlcNAc_2$ (**3**), Man_3 (**4**), and a glucosyl donor (**5**) were designed (Scheme 1A). The β-mannoside linkage of fragment **3** was constructed through PMB-assisted (PMB = *p*-methoxybenzyl) intramolecular aglycon delivery (IAD) as the key reaction, according to our standard procedure.[29,30] Trimannoside **4** and glucose donor **5** were prepared as reported previously.[23,24]

The synthesis of **2** was conducted as depicted in Scheme 2A. Coupling of **3** with trisaccharide donor **4** was achieved through the action of methyl trifluoromethanesulfonate (MeOTf) to afford the 3-*O*-glycosylated hexasaccharide **6** in 76% yield and as a single isomer, which was converted into the diol **7**. Regioselective glycosylation with donor **4** gave the

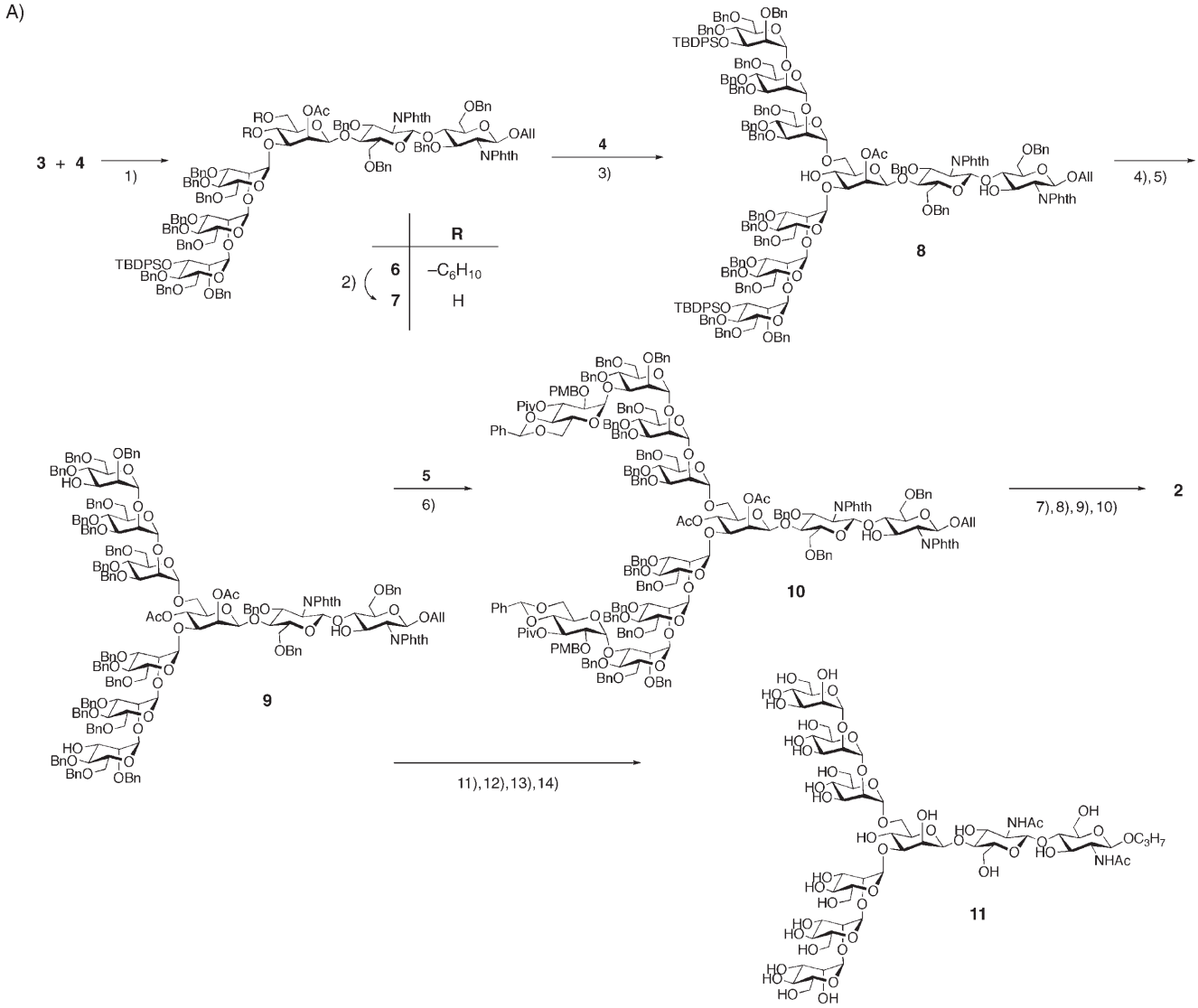


Scheme 1. A) Retrosynthetic plan for the non-natural oligosaccharides **2** and **16**. B) Other oligosaccharides used in this study.

6-*O*-glycosylated nonasaccharide **8** in 82% yield. After acetylation, removal of the TBDPS group under high-pressure conditions (1 GPa) with HF·pyridine[31] gave **9** in 84% yield. The same reaction under atmospheric pressure gave extremely poor results, presumably due to the steric hindrance of the reaction site. Two α-linked glucose residues were incorporated simultaneously to afford undecasaccharide **10** in 88% yield as the sole product. Thioglycoside **5** was highly suitable for the formation of a Glcα1→3Man linkage, as had been reported previously.[23] Complete deprotection gave the target compound **2** in 57% yield (four steps). Inspection of its ^{1}H NMR revealed the presence of two α-Glc [$\delta = 5.11$ (×2), $J = 3.4$ Hz] and six α-Man [$\delta = 5.20$, 5.16 (×2), 4.99, 4.89 Hz (×2)] residues, confirming its stereochemical integrity.

Previous studies have established that the α-linked terminal glucose residue is critical for recognition by CNX/CRT,[10,23,32] so for purposes of comparison a nonglucosylated congener (**11**) was synthesized. The preparation of **11** was achieved by successive deprotection of the nonasaccharide **9**, which was achieved in 29% overall yield.

Since compound **2** is bidentate in terms of G1M3, heptasaccharide **16** was synthesized in order to allow precise evaluation of the contribution of a single G1M3 arm (Scheme 2B). Acetylation of starting compound **7** thus gave **13** in 91% yield. The TBDPS group was removed uneventfully under high-pressure conditions to give compound **14** in 82% yield. Introduction of an α-glucose unit and full deprotection afforded heptasaccharide **16** in 35% yield over four steps.



Scheme 2. Synthesis of oligosaccharides **2**, **11**, and **16**. Conditions and yields: A: 1) MeOTf, MS (4 Å), $ClCH_2CH_2Cl$, 40 °C, 76%. 2) *p*-TsOH·H_2O, MeCN, 56%. 3) MeOTf, DTBMP, toluene, 82%. 4) Ac_2O, pyridine, DMAP, 91%. 5) HF·pyridine (10%), DMF, 1 GPa, 84%. 6) MeOTf, DTBMP, $ClCH_2CH_2Cl$, 88%. 7) Ethylenediamine, *n*BuOH. 8) Ac_2O, pyridine. 9) NaOMe/MeOH. 10) $Pd(OH)_2$, H_2, 50% AcOH, 57% (4 steps). 11) Ethylenediamine, *n*BuOH. 12) Ac_2O, pyridine, DMAP. 13) NaOMe/MeOH. 14) $Pd(OH)_2$, H_2, 50% AcOH, 29% (4 steps). B: 1) Ac_2O, pyridine, DMAP, 91%. 2) 10% HF·pyridine, DMF, 1 GPa, 82%. 3) MeOTf, DTBMP, $ClCH_2CH_2Cl$, 59%. 4) Ethylenediamine, *n*BuOH. 5) Ac_2O, pyridine. 6) NaOMe/MeOH. 7) $Pd(OH)_2$, H_2, 50% AcOH, 35% (4 steps).

Isothermal titration calorimetric studies of synthetic oligosaccharides

Isothermal titration calorimetry (ITC) has recently found use as a reliable tool to provide quantitative estimates of carbohydrate–protein interactions.[33] On the other hand, precise analysis of the binding between CNX/CRT and complex oligosaccharides have been limited,[28] mainly due to the poor availability of structurally defined high-mannose-type oligosaccharides

Table 1. Thermodynamic parameters for the binding of oligosaccharides to CRT.

Oligosaccharide	*n*	$K_b \times 10^{-6}$	$\Delta H^{[a]}$	$\Delta S^{[b]}$
2	0.719 ($\pm 3.6 \times 10^{-3}$)	1.73 (± 0.1)	−13.0	−16.0
16	0.989 ($\pm 2.0 \times 10^{-3}$)	1.86 (± 0.05)	−11.5	−10.7
11	–	n.b.[c]	–	–
1b	0.833 ($\pm 2.5 \times 10^{-2}$)	6.96 (± 0.5)	−12.7	−11.9
17	–	n.b.[c]	–	–

[a] kcal mol^{-1}. [b] cal mol^{-1} K^{-1}. [c] No binding under ITC measurement conditions.

or glycoproteins from biological sources. With synthetic oligosaccharides with natural (**1b**, **17**) or non-natural (**2**, **11**, **16**) structures now to hand, their abilities to interact with CRT were compared.

The binding of synthetic oligosaccharides to glutathione-*S*-transferase-fused CRT (GST-CRT) is characterized in Table 1 and Figure 2. G1M9 (**1b**) had a K_b of 6.96×10^6 M^{-1},[34] while non-glucosylated M9 (**17**) did not show binding. The binding constant of **2** was 1.73×10^6 M^{-1}. Under the same conditions, non-glucosylated glycan (**11**) was inactive. These results underscore the importance of the terminal glucose residue for CNX/CRT binding, consistent with previous studies. The affinity of the heptasaccharide **16** (1.86×10^6 M^{-1}) was essentially identical with that of **2**, showing that the divalency of the G1M3 arm did not enhance the affinity. While the ΔH and ΔS values for **2** and **16** were similar, the binding stoichiometry (*n*) of **2** was noticeably smaller than that of **16**. Although this might derive from the difference in G1M3 valency between **2** (divalent) and **16** (monovalent), the precise reason is not clear. Since G1M9 (**1b**) had a significantly higher affinity than **2**/**16**, the M5 arm of G1M9 might play an adjunct role in the interaction with CRT, although most of the affinity toward CRT derives from the G1M3 arm.

Glucosidase II treatment of oligosaccharides

Since non-natural oligosaccharides **2** and **16** are devoid of the M5 branch, they would be expected to be resistant to Glc-ase II. To confirm this, the membrane fraction of *Aspergillus oryzae* was isolated and used as the Glc-ase II source. It was first confirmed that G1M9 (**1b**) was smoothly converted to M9 (Figure 3A). In contrast, oligosaccharides **2** and **16** were completely resistant to Glc-ase II trimming, as judged from MALDI-TOF MS analyses (Figure 3B, C).

Chaperone inhibitory activities of synthetic oligosaccharides

Studies in vitro have shown that CNX and CRT act as molecular chaperones not only for glycoproteins but also for non-glycosylated proteins, although they were most efficient when a monoglucosylated high-mannose-type glycan-carrying protein was used as the substrate.[35,36] In these studies, the ability to suppress the aggregation of malate dehydrogenase (MDH), citrate synthase (CS), IgY, or α-mannosidase was taken as a measure of chaperone activity.[11,35,36] It was reported that binding to oligosaccharide (G1M3) inhibited the chaperone activity of CNX.[35,36] These results suggest the presence of overlap and/or

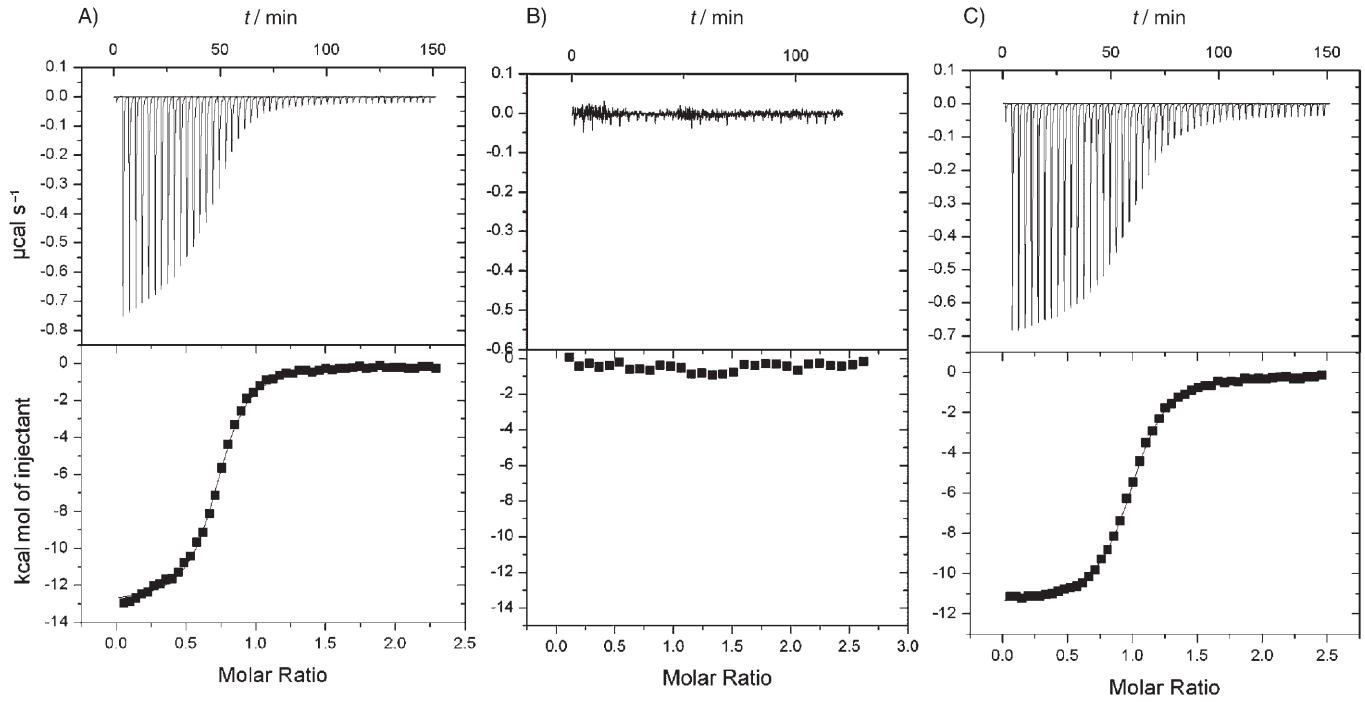


Figure 2. Isothermal calorimetric titration of calreticuline with oligosaccharides. Raw data were obtained after injections (6 µL) of sugar solutions (0.3 mM) into GST-CRT (30 µM) in MOPS buffer (10 mM) containing $CaCl_2$ (5 mM) and NaCl (150 mM) at 293 K (top). Molar heat values plotted as a function of the molar ratio (bottom). The solid line represents the best-fit binding isotherm obtained by use of a single-site model. A) **2**, $K_b = 1.73(\pm 0.1) \times 10^6$ M^{-1}, $n = 0.719 \pm 0.0036$. B) **11** and C) **16**, $K_b = 1.86(\pm 0.05) \times 10^6$ M^{-1}, $n = 0.989 \pm 0.0012$.

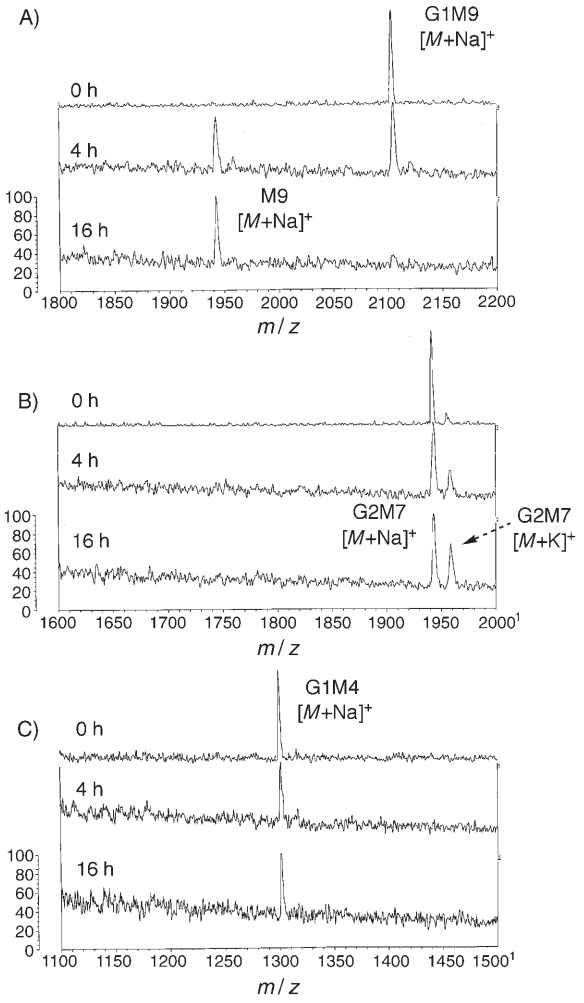


Figure 3. Glucosidase II treatment of oligosaccharides. Oligosaccharides **1b** (A), **2** (B), and **16** (C) were each treated with an *A. oryzae* membrane fraction containing glucosidase II. All experiments were conducted in Tris (pH 7.5, 10 mM) at 37 °C, in the presence of deoxymannojirimycin (10 mM). Aliquots were fractionated by ultrafiltration and the flow-through fractions were subjected to MALDI-TOF MS measurements.

crosstalk between the carbohydrate- and peptide-binding domains of CNX/CRT.

The inhibitory effects of the synthetic oligosaccharides on CRT chaperone activity were investigated by use of MDH, and the results are shown in Figure 4. MDH readily aggregated at 45 °C in MOPS buffer (10 mM) without prior denaturation. In the presence of GST-tagged (GST = glutathione-*S*-transferase) CRT (GST-CRT) (GST-CRT/MDH 3:1), the aggregation was suppressed to 10% of the control value. The synthetic oligosaccharides **2** and **16** were then evaluated for their abilities to interfere with the chaperone activity of GST-CRT, relative to G1M9 (**1b**) and M9 (**17**) (Figure 4). MDH (1 μM) was incubated in MOPS buffer (10 mM) at 45 °C in the presence of GST-CRT (1 μM) and the oligosaccharides (40 μM) for 60 min. After incubation, the aggregation was measured by light scattering at 360 nm. The undecasaccharide **2** inhibited the activity of GST-CRT, and a comparable result was observed with the heptasaccharide **16**. G1M9 (**1b**) also showed inhibitory activity, in line

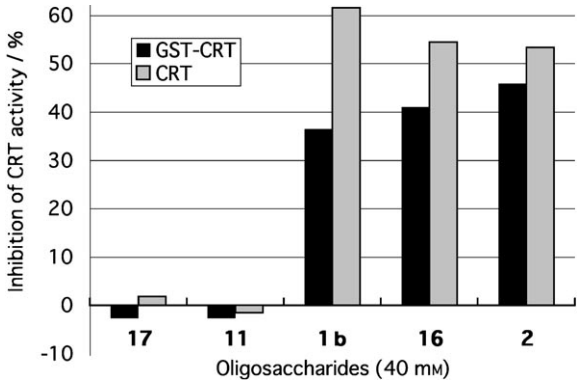


Figure 4. The inhibition of CRTs' aggregation-suppressing function by oligosaccharides. MDH (1 μM) was incubated in MOPS buffer (pH 7.4, 10 mM), $CaCl_2$ (5 mM), and NaCl (150 mM) with either GST-CRT (1 μM) or CRT (1 μM) at 45 °C in the presence of oligosaccharides (40 μM). The degree of aggregation was estimated by measurement of light scattering at 360 nm after 60 min.

with previous studies conducted with G1M3.[35,36] The presence of the terminal Glc was essential for the activity: neither M9 (**17**) nor **11** was effective. Overall, the inhibitory properties of oligosaccharides correlate extremely well with their CRT-binding properties. As suggested by Williams et al., the binding of oligosaccharide may cause a conformational change of CNX/CRT, abrogating the binding to denatured MDH.[11]

To eliminate the possibility that GST per se possesses the chaperone activity, a similar assay was conducted with GST-untagged CRT (Figure 4). Although the general tendency was similar, GST-CRT had a more robust chaperone activity and the effect on untagged CRT seemed to reflect the relative affinity more precisely, G1M9 (**1b**) being most potent.

Conclusion

We have designed and synthesized an undecasaccharide, **2**, and a heptasaccharide, **16**, as novel CNX/CRT inhibitors that lack a Glc-ase II recognition site. These oligosaccharides had binding affinity for CRT and inhibited the chaperone activity of CRT. Both were indeed resistant to Glc-ase II digestion; this implies their potential use as inhibitors of the glycoprotein quality-control system governed by the CNX/CRT-UGGT cycle. More judicious designs along these lines might result in the discovery of an effective inhibitor of CNX/CRT-mediated glycoprotein quality control.

Experimental Section

The GST-CRT construct was a generous gift from Dr. Shunji Natori (RIKEN) and Dr. Shunji Natsuka (Osaka University). The plasmid vector pGEX-6p-1 and the BL21(DE3) strain were purchased from Amersham Biosciences (Piscataway, NJ) and from Stratagene (La Jolla, CA), respectively. PreScission protease was purchased from Amersham Biosciences. Centrifugal concentrators (Centriprep) were obtained from Millipore Corp (Billerica, MA). A glutathione-Sepharose column (Amersham Biosciences) was used for the purification of GST-tagged proteins. Malate dehydrogenase (MDH) was purchased from Sigma (St. Louis, MO). MALDI-TOF mass spectra were

recorded in positive ion mode on an AXIMA-CFR Kompact MALDI instrument (Shimadzu/KRATOS). NMR spectra were obtained with a JEOL EX-400 spectrometer (^{1}H at 400 and ^{13}C at 100 MHz) at ambient temperature unless otherwise noted.

Expression and purification of recombinant GST-CRT: DNA encoding human calreticurin (CRT) with a N-terminal glutathione-*S*-transferase (GST) moiety was cloned into a pGEX-6p-1 plasmid vector. For expression, the plasmid was introduced into BL21 (DE3) cells. An overnight plateau phase culture was used to inoculate fresh LB medium containing ampicillin (50 μg mL^{-1}). Cells were grown at 25 °C to an A_{600} of 0.4–0.6, treated with isopropyl 1-thio-β-D-galactopyranoside (0.1 mM), and incubated for an additional 3 h. The cells were harvested and washed with PBS buffer and were then resuspended to be sonicated. Lysates were centrifuged at 12000 rpm for 20 min at 4 °C. The recombinant protein was purified from the filtered (0.45 μm, Whatman Inc. Clifton, NJ) supernatant with a glutathione-Sepharose column, concentrated by use of a Centriprep YM-3, and then dialyzed against a buffer (pH 7.4) consisting of MOPS (10 mM), $CaCl_2$ (5 mM), and NaCl (150 mM). GST-CRT was judged to be greater than 90% pure by SDS-PAGE. The molecular mass of GST-CRT was confirmed by MALDI-TOF mass spectrometry after desalting with Zip-Tip C4 (Millipore).

Purification of recombinant CRT: The release of CRT from GST was performed with PreScission protease. The protease was added to GST-CRT and incubated for 6 h at 4 °C in Tris-HCl (pH 7, 50 mM) containing NaCl (150 mM), EDTA (1 mM), and DTT (1 mM). CRT was purified on a glutathione-Sepharose column. CRT was further purified by UNO Q1 anion-exchange chromatography (BIO-RAD, Hercules, CA) with use of a linear gradient of NaCl (0.15 M to 1 M) in Tris-HCl (pH 8.0, 50 mM), and was dialyzed (pH 7.4) against MOPS (10 mM), $CaCl_2$ (5 mM), and NaCl (150 mM). CRT was judged by SDS-PAGE to be greater than 90% pure. The molecular mass was checked by MALDI-TOF mass spectrometry after desalting with Zip-Tip C4 (Millipore).

Isothermal titration calorimetry experiments: ITC experiments were performed with a VP-ITC calorimeter (Microcal, Northampton, MA). GST-CRT (30 μM; in 10 mM MOPS, 5 mM $CaCl_2$, and 150 mM NaCl, pH 7.4) in a sample cell was titrated with synthetic oligosaccharide (0.3 mM) in the same buffer at 293 K. The latter was added as 30–40 injections (6 μL). The heat of dilution values of the sugar solution were subtracted from the titration data. The nonlinear least-squares minimization method was used for the data fitting. The binding stoichiometry (n), binding constant (K_b), and changes in enthalpy (ΔH°_b) and entropy (ΔS) were determined by use of Origin software.

Aggregation assays: MDH (1 μM) was mixed with GST-fused CRT or CRT (1 μM) in MOPS buffer (pH 7.4, 10 mM) containing $CaCl_2$ (5 mM) and NaCl (150 mM) and incubated at 45 °C in the presence or absence of oligosaccharide (40 μM) for 60 min. The reaction mixture was then immediately cooled on ice. The degree of aggregation was assessed by light scattering at 360 nm.

Synthesis of oligosaccharides: The syntheses of fragments **3**–**5** and compound **7** have been reported previously.[23]

Compound 8: A mixture of compound **7** (26.0 mg, 9.5 μmol), compound **4** (20.0 mg, 12.6 μmol), 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP, 13.7 mg, 66.5 μmol), and molecular sieves (50 mg, type 4 Å) in dry toluene was stirred at −40 °C for 30 min, and MeOTf in $ClCH_2CH_2Cl$ (1 M, 83 μL, 83 μmol) was then added. After the mixture had been stirred for 15 h at RT, the reaction was quenched by the addition of triethylamine (~0.1 mL). This mixture was diluted with EtOAc and filtered through Celite. The filtrate was washed successively with sat. aq. $NaHCO_3$ and brine, the organic layer was dried over Na_2SO_4 and concentrated in vacuo, and the residue was purified by silica gel column chromatography (hexane/EtOAc 3:2) to give compound **8** as a colorless, amorphous solid (34.4 mg, 8.10 μmol, 85% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85–6.58 (m, arom), 5.68–5.52 (m, 1H; CH_2=C*H*-), 5.30 (d, J = 3.4 Hz, 1H), 5.24–5.12 (m, 3H), 3.10–2.95 (m, 1H), 1.94 (s, 3H; Ac), 1.09 ppm (s, 18H; *t*Bu×2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 101.01, 100.60, 99.99, 99.14, 96.94, 96.90 (C-1), 56.57, 55.59, 27.22, 20.95 ppm; elemental analysis calcd (%) for $C_{261}H_{272}N_2O_{49}Si_2$: C 73.29, H 6.41, N 0.65; found C 73.34, H 6.45, N 0.64.

Compound 9: Acetic anhydride (0.25 mL) was added at 0 °C to a stirred solution of compound **8** (84.0 mg, 19.6 μmol) and 4-dimethylaminopyridine (DMAP, 1.0 mg) in pyridine (0.50 mL). The mixture was stirred at 50 °C for 12 h, diluted with EtOAc, and successively washed with sat. aq. $CuSO_4$, brine, sat. aq. $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 3:1–3:2) to give the acetylated compound as a colorless, amorphous solid (76.6 mg, 17.7 μmol, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80–6.55 (m, arom), 5.66–5.50 (m, 1H; CH_2=C*H*-), 5.33 (d, J = 4.0 Hz, 1H), 3.10–3.00 (m, 1H), 1.95 (s, 3H; Ac), 1.88 (s, 3H; Ac), 1.07 ppm (s, 18H; *t*Bu×2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 101.06, 100.60, 99.26, 99.00, 96.95, 96.89 (C-1), 56.57, 55.62, 27.24, 20.99, 20.82 ppm.

The above acetylated compound (64.0 mg, 14.8 μmol), dissolved in DMF (0.8 mL) containing HF/pyridine (10%), was placed in a 1 mL Teflon reaction vessel, was compressed to 1.0 Gpa, and was left at 35 °C for 13 h. The resulting mixture was diluted with EtOAc and washed successively with sat. aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1) to give the title compound as a colorless, amorphous solid (48.0 mg, 12.5 μmol, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65–6.60 (m, arom), 5.64–5.50 (m, 1H; CH_2=C*H*-), 5.34 (d, J = 2.8 Hz, 1H), 3.26–3.19 (m, 1H), 3.18–3.14 (m, 1H), 3.05–2.92 (m, 1H), 2.32–2.18 (m, 2H; O*H*), 1.97 (s, 3H; Ac), 1.90 ppm (s, 3H; Ac); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 101.06, 100.77, 100.35, 99.27, 98.93, 98.44, 98.14, 96.96, 96.87 (C-1), 56.55, 55.62, 20.99, 20.90 ppm.

Compound 10: A mixture of compound **9** (26.0 mg, 6.74 μmol), compound **5** (14.4 mg, 28.8 μmol), DTBMP (11.6 mg, 56.6 μmol), and molecular sieves (143 mg, type 4 Å) in dry $ClCH_2CH_2Cl$ (0.75 mL) was stirred at 0 °C for 30 min, and MeOTf in $ClCH_2CH_2Cl$ (1 M, 47 μL, 47 μmol) was then added. After the mixture had been stirred for 12 h at 40 °C, the reaction was quenched by the addition of triethylamine (~0.1 mL). This mixture was diluted with EtOAc and filtered through celite, the filtrate was washed successively with sat. aq. $NaHCO_3$ and brine, and the organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by PTLC (hexane/EtOAc 3:2) to give compound **10** as a colorless, amorphous solid (28.1 mg, 5.9 μmol, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.60–6.50 (m, arom), 5.64 (t, J = 9.6 Hz, 1H; H-3 Glc), 5.63 (t, J = 9.8 Hz, 1H; H-3 Glc), 5.57–5.45 (m, 1H; CH_2=C*H*-), 5.34 (s, 2H; benzylidene C*H*), 3.61 (s; OMe), 3.60 (s; OMe), 1.91 (s, 3H; Ac), 1.83 (s, 3H; Ac), 1.10 ppm (s, 18H; *t*Bu×2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 101.10, 101.00, 100.64, 99.46, 99.25, 98.99, 96.96, 96.88 (C-1 and benzylidene *C*H), 62.88, 56.57, 55.64, 55.15, 38.85, 27.24, 21.02, 20.91 ppm; elemental analysis calcd (%) for $C_{283}H_{298}N_2O_{64}$: C 71.54, H 6.32, N 0.59; found C 71.35, H 6.33, N 0.54.

Compound 2: A solution of compound **10** (45.6 mg, 9.60 μmol) in *n*-butanol (6.1 mL) containing ethylenediamine (0.61 mL) was stirred at 80 °C for 10 h. Volatiles were removed by evaporation in vacuo and the residue was dissolved in pyridine (3.4 mL). Acetic anhydride (1.7 mL) was added at 0 °C and the mixture was stirred overnight at RT and then concentrated in vacuo. The residue was purified through a pad of silica gel (hexane/EtOAc 1/1 to EtOAc only) and concentrated to give the NHAc compound. This product was dissolved in MeOH (6.0 mL), and NaOMe/MeOH (28%, 0.4 mL) was added at 0 °C. After stirring at 75 °C for 50 h, the mixture was neutralized with Amberlyst 15 (H^+) resin and evaporated in vacuo. The residue was purified by PTLC (hexane/EtOAc 3:2) to give the *O*-deacylated product (27.0 mg, 6.24 μmol, 65% yield). The product (11.7 mg, 2.70 μmol) was dissolved in MeOH/H_2O (7:1, 2.5 mL) and hydrogenated in the presence of $Pd(OH)_2$ (20% on carbon, 11.0 mg) under H_2. After the mixture had been stirred for 30 min at RT, AcOH (0.9 mL) was added. The reaction mixture was stirred overnight at RT and was then filtered through a pad of celite. The filtrate was concentrated in vacuo and the residue was purified on a Sep-Pak C18 cartridge (3 cc, Waters) (H_2O only to H_2O/MeOH 5:1) to give the compound **2** (4.30 mg, 2.23 μmol, 83%) as a colorless, amorphous solid. ^{1}H NMR (400 MHz, D_2O): $\delta = 5.20$ (br s, 1H; H-1 Man), 5.16 (br s, 2H; H-1 Man×2), 5.11 (d, $J = 3.4$ Hz, 2H; H-1 Glc×2), 4.99 (br s, 1H; H-1 Man), 4.89 (br s, 2H; H-1 Man×2), 4.45 (d, $J = 7.6$ Hz, 1H; H-1 GlcNAc), 4.36 (d, $J = 6.8$ Hz, 1H; H-1 GlcNAc), 1.94 (s, 3H; NH*Ac*), 1.89 (s, 3H; NH*Ac*), 1.45–1.35 (m, 2H; CH_2), 0.72 ppm (t, $J = 7.4$ Hz, 3H; CH_3); LRMS (MALDI-TOF) calcd for $C_{73}H_{124}N_2O_{56}Na$ $[M+Na]^+$:1947.6; found 1947.7.

Compound 11: A solution of compound **9** (17.7 mg, 4.60 μmol) in *n*-butanol (3.0 mL) containing ethylenediamine (0.3 mL) was stirred at 80 °C for 15 h. Volatiles were removed by evaporation in vacuo and the residue was dissolved in pyridine (1.7 mL). Acetic anhydride (0.80 mL) was added at 0 °C and the mixture was stirred for 29 h at RT and for 21 h at 50 °C then concentrated in vacuo. The residue was dissolved in MeOH (3.0 mL) and NaOMe/MeOH (28%, 0.20 mL) was added at 0 °C. After stirring at 70 °C for 20 h, the mixture was neutralized with Amberlyst 15 (H^+) resin and evaporated in vacuo. The residue was purified by PTLC (hexane/EtOAc 3:2) to give the product without Ac and Piv (7.6 mg, 2.1 μmol, 46% yield). The product (7.6 mg, 2.1 μmol) was suspended in MeOH/H_2O (7:1, 3.0 mL) and hydrogenated in the presence of $Pd(OH)_2$ (20% on carbon, 5.0 mg) under an atmosphere of H_2. After 30 min of stirring at RT, AcOH (1.5 mL) was added to the clear solution. The reaction mixture was stirred for 17 h at RT then filtered through a pad of celite. The filtrate was concentrated in vacuo. The residue was purified on a Sep-Pak C18 cartridge (12 cc, Waters, H_2O only to H_2O/MeOH 5:1) to give the compound **11** (2.20 mg, 1.31 μmol, 62% yield) as a white, amorphous solid. ^{1}H NMR (400 MHz, D_2O): $\delta =$ 5.19 (s, 1H; H-1 Man), 5.15 (s, 2H; H-1 Man×2), 4.98 (s, 1H; H-1 Man), 4.89 (s, 2H; H-1 Man×2), 4.45 (d, 1H; $J = 8.0$ Hz, H-1 GlcNAc), 4.35 (d, 1H; $J = 6.8$ Hz, H-1 GlcNAc), 4.08 (br s, 1H), 1.94 (s, 3H; NHAc), 1.88 (s, 3H; NHAc), 1.40–1.35 (m, 2H; CH_2), 0.72 (t, 3H; $J =$ 7.4 Hz, CH_3); LRMS (MALDI-TOF) calcd for $C_{61}H_{104}N_2O_{46}Na$ $[M+Na]^+$: 1623.6; found 1624.1.

Compound 13: Acetic anhydride (0.12 mL) was added at 0 °C to a stirred solution of compound **7** (164.0 mg, 60.0 μmol) and DMAP (7.3 mg) in pyridine (0.20 mL). The mixture was stirred at RT for 15 h. The reaction mixture was diluted with EtOAc and was successively washed with sat. aq. $CuSO_4$, brine, sat. aq. $NaHCO_3$, and brine, and the organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1–1:2) to give the compound **13** as a white, amorphous solid (154.2 mg, 54.6 μmol, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.95$–6.78 (m, arom), 5.75–5.60 (m, 1H; $CH_2=CH$-), 5.43 (br s, 1H), 5.28 (d, $J = 8.0$ Hz, 1H; CH_2), 3.47–3.44 (m, 1H), 3.36–3.34 (m, 1H), 3.26–3.24 (m, 1H), 3.14–3.04 (m, 1H), 2.11 (s, 3H; Ac), 2.03 (s, 3H; Ac), 1.97 (s, 3H; Ac), 1.17 ppm (s, 9H; *t*Bu); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 101.3$, 100.62, 98.70, 96.89, 96.63 (C-1), 62.22, 56.43, 55.49, 27.10, 20.84, 20.52, 19.23 ppm; elemental analysis calcd (%) for $C_{168}H_{174}N_2O_{36}Si$: C 71.42, H 6.21, N 0.99; found C 71.37, H 6.21, N 0.95.

Compound 14: Compound **13** (24.0 mg, 8.50 μmol), dissolved in DMF (0.8 mL) containing HF/pyridine (10%), was placed in a 1 mL Teflon reaction vessel, compressed to 1.0 Gpa, and left at 35 °C for 17 h. The resulting mixture was diluted with EtOAc and washed successively with sat. aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo, and the residue was purified by silica gel column chromatography (hexane/EtOAc 2:1–3:2) to give the compound **14** as a white, amorphous solid (18.0 mg, 6.96 μmol, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 7.78–6.60 (m, arom), 5.57–5.47 (m, 1H; $CH_2=CH$-), 5.28 (d, $J =$ 3.2 Hz, 1H; H-2 Man), 5.13 (d, $J = 8.4$ Hz, 1H; CH_2), 5.08 (br s, 1H), 3.35–3.27 (m, 1H), 3.24–3.15 (m, 1H), 3.14–3.07 (m, 1H), 3.00–2.90 (m, 1H), 2.16 (d, $J = 9.2$ Hz, 1H; O*H*), 1.97 (s, 3H; Ac), 1.89 (s, 3H; Ac), 1.82 ppm (s, 3H; Ac); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 101.04$, 100.80, 98.74, 98.61, 96.99, 96.78 (C-1), 62.33, 56.52, 55.60, 31.55, 22.64, 20.94, 20.74, 20.61 ppm; LRMS (MALDI-TOF) calcd for $C_{152}H_{156}N_2O_{36}Na$ $[M+Na]^+$: 2609.7; found 2609.9 $[M+Na]^+$.

Compound 15: A mixture of compounds **14** (85.4 mg, 33.0 μmol) and **5** (35.0 mg, 69.0 μmol), DTBMP (28.0 mg, 0.14 mmol), and molecular sieves (603 mg, type 4 Å) in dry $ClCH_2CH_2Cl$/cyclohexane (1:5, 6.0 mL) was stirred at RT for 20 min, and MeOTf in $ClCH_2CH_2Cl$ (1 M, 0.12 mL, 47.2 μmol) was added. After the mixture had been stirred for 27 h at 50 °C, the reaction was quenched by the addition of TEA (0.1 mL). This mixture was diluted with EtOAc and filtered through celite, and the filtrate was washed with sat. aq. $NaHCO_3$ and then brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo, and the residue was purified by silica gel column chromatography (hexane/EtOAc 3:1–3:2) and PTLC (hexane/EtOAc 3:2) to give the compound **15** as a white, amorphous solid (58.9 mg, 19.4 μmol, α/β 93:7, 59% yield). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.88$–6.62 (m, arom), 5.73 (t, $J = 10.0$ Hz, 1H; H-3 Glc), 5.65–5.54 (m, 1H; $CH_2=CH$-), 5.43 (s, 1H; benzylidene C*H*), 5.37 (d, $J = 2.8$ Hz, 1H; H-2 Man), 3.69 (s, OMe), 3.32–3.24 (m, 1H), 3.21–3.15 (m, 1H), 3.07–3.00 (m, 1H), 2.05 (s, 3H; Ac), 1.98 (s, 3H; Ac), 1.90 (s, 3H; Ac), 1.19 ppm (s, 9H; Piv); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 101.27$, 101.01, 100.91, 99.51, 99.01, 98.77, 97.03, 96.84 (C-1×7 and benzylidene *C*H), 62.93, 62.38, 55.57, 55.15, 38.85, 27.23, 20.99, 20.77, 20.68 ppm; elemental analysis calcd (%) for $C_{178}H_{186}N_2O_{43}$: C 70.29, H 6.16, N 0.92; found C 69.90, H 6.15, N 0.81.

Compound 16: A solution of compound **15** (35.9 mg, 11.8 μmol) in *n*-butanol (7.0 mL) containing ethylenediamine (0.70 mL) was stirred at 75 °C for 17 h. Volatiles were removed by evaporation in vacuo and the residue was dissolved in pyridine (4.0 mL). Acetic anhydride (2.0 mL) was added at 0 °C and the mixture was stirred for 48 h at RT and was then concentrated in vacuo. The residue was purified by PTLC (AcOEt only) to give the *N*-acetylated compound (25.8 mg, 9.0 μmol, 76% yield). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.57$–6.79 (m, arom), 5.78–5.76 (m, 1H; $CH_2=CH$-), 5.74 (t, $J =$ 9.8 Hz, 1H; H-3 Glc), 5.43 (s, 1H; benzylidene C*H*), 5.31 (d, $J =$ 3.2 Hz, 1H), 3.25–3.15 (m, 1H), 3.05–2.95 (m, 1H), 2.04 (s, 3H; Ac), 2.01 (s, 3H; Ac), 1.954 (s, 3H; Ac), 1.951 (s, 3H; Ac), 1.73 (s, 3H; Ac), 1.19 ppm (s, 9H; Piv).

This compound (25.8 mg, 9.0 μmol) was dissolved in MeOH (6.0 mL), and NaOMe/MeOH (28%, 0.23 mL) was added at 0 °C. After stirring at 70 °C for 44 h, the mixture was neutralized with Amberlyst 15 (H^+) resin and evaporated in vacuo. The residue was purified by PTLC (AcOEt only) to give the product without Ac, Piv, and benzylidene groups (13.3 mg, 5.2 μmol, 58% yield). The product (11.0 mg, 4.3 μmol) was dissolved in MeOH/H_2O (5:1, 1.2 mL) and hydrogenated in the presence of $Pd(OH)_2$ (20% on carbon, 6.0 mg) under H_2. After the mixture had been stirred for 30 min at RT, AcOH (0.5 mL) was added to the clear solution. The reaction mixture was stirred for 14 h at RT and was then filtered through a pad of celite. The filtrate was concentrated in vacuo and the residue was purified on a Sep-Pak C18 cartridge (12 cc, Waters, H_2O only to H_2O/MeOH 20:1) to give compound **16** (5.40 mg, 4.2 μmol, 98% yield) as a white, amorphous solid. ^{1}H NMR (400 MHz, D_2O): $\delta = 5.19$ (s, 1H; H-1 Man), 5.15 (s, 1H; H-1 Man), 5.10 (d, $J = 3.9$ Hz, 1H; H-1 Glc), 4.88 (s, 1H; H-1 Man), 4.44 (d, $J = 7.2$ Hz, 1H; H-1 GlcNAc), 4.35 (d, $J = 7.6$ Hz, 1H; H-1 GlcNAc), 1.91 (s, 3H; NHAc), 1.88 (s, 3H; NHAc), 1.45–1.34 (m, 2H; CH_2), 0.71 ppm (t, $J = 7.4$ Hz, 3H; CH_3); LRMS (MALDI-TOF) calcd for $C_{49}H_{84}N_2O_{36}Na$ $[M+Na]^+$: 1300.1; found 1299.7.

Preparation of fungal membranous fraction: *Aspergillus oryzae* strain RIB40 was cultivated in DPY liquid medium (100 mL, 2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.5) at 30 °C for 18 h. Harvested mycelia (1.6 g wet weight) were homogenized in liquid nitrogen, and the total cell lysate was extracted with extraction buffer [6 mL, 50 mM Tris (pH 7.5), 2 mM PMSF, and 1:100 PIC (protein inhibitor cocktail)]. The debris was removed by centrifugation at 3000 g for 10 min at 4 °C. The supernatant was subjected to another round of centrifugation at 20000 g for 20 min at 4 °C. The membranous pellet was solubilized in 600 μL of the extraction buffer containing Triton X-100 (1%) and centrifuged at 20000 g for 20 min at 4 °C. The resultant supernatant was used for the assay.

Incubation of oligosaccharide with glucosidase II—typical procedure: Enzyme assay solution was prepared by mixing the following species: compound **2** (0.10 mg, 52 nmol, final conc. 0.52 mM), deoxymannojirimycin (10 mM, 4 μL, final conc 400 μM), fungal membranous fraction of *Aspergillus oryzae* (40 μL), and Tris·HCl (pH 7.5, 10 mM, 36 μL). The enzyme assay solution was incubated at 37 °C for 16 h. During the incubation, the Glc-trimming was monitored by MALDI-TOF MS as follows; an aliquot (20 μL) of the reaction mixture was fractionated by ultrafiltration (Millipore: Microcon YM10). The flow-through, small molecule, fraction was measured by MALDI-TOF MS with an α-cyano-4-hydroxycinnamic acid matrix.

Acknowledgements

Financial support in the form of a Grant-in-Aid for Young Scientists (B) (16790027) and a Grant-in-Aid for Creative Scientific Research (17GS0420) from the Japanese Ministry of Education, Science, Culture, Sports, and Technology (to M.A.A. and Y.I., respectively) is gratefully acknowledged. We thank Dr. Teiji Chihara and his staff for elemental analyses and high-pressure equipment, and Drs. Shunji Natori and Shunji Natsuka for providing the GST-CRT construct. M.A.A., S.H., and K.T. were recipients of RIKEN Special Postdoctoral Research Fellowships. The authors thank Akemi Takahashi for technical assistance.

Keywords: calnexin · calreticulin · carbohydrates · chaperone proteins · glucosidase · isothermal titration calorimetry

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Received: April 6, 2005
Published online on November 11, 2005

