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Synthesis of fluorine substituted oligosaccharide analogues of monoglucosylated glycan chain, a proposed ligand of lectin-chaperone calreticulin and calnexin

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As a part of a exploring the N-glycan-mediated glycoprotein quality control in endoplasmic reticulum, 2-fluorinated derivative Glcα1 → 3Man(F) 1, Glcα1 → 3Man(F)α1 → 2Man2, and Glcα1 → 3Man(F)α1 → 2Manα1 → 2Man 3 were synthesized in a concise manner. These oligosaccharides were subjected to binding studies with calreticulin by using isothermal titration calorimetry. It was revealed that disaccharide 1 was a poor ligand, while tri- (2) and tetrasaccharide (3) had observable affinity.
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Introduction

Among various functions of glycoprotein glycan chains [1], pivotal roles of asparagine (Asn)-linked oligosaccharides in glycoprotein quality control are attracting particular attention (Figure 1) [2–4]. Asn-linked glycan chain is first incorporated as tetradecasaccharide to nascent polypeptide by the action of multisubunit enzyme, oligosaccharyl transferase (OST) that catalyze the transfer of $Glc_3Man_9GlcNAc_2$ (G3M9) from dolichol diphosphate (Dol-PP) to Asn residue of nascent protein [5]. Subsequently it is converted, by the action of glucosidase-I and -II, to monoglucosylated dodecasacchraide ($Glc_1Man_9GlcNAc_2$, G1M9), which is proposed to be the key structure in glycoprotein quality control [6–8].

Calnexin (CNX) and calreticulin (CRT) are endoplasmic reticulum (ER)-residing molecular chaperones [9–11]. They are homologous to each other, the former being membrane-bound and the latter being soluble. Both of them form complexes with ERp57, which catalyze the formation and cleavage of Cys-Cys disulfide linkages, thereby assist protein folding. Very characteristic feature of CNX and CRT is their lectin property. They are believed to recognize aforesaid dodecasaccharide G1M9 as the primary ligand in a Ca^{2+}-dependent manner.

After removal of the innermost Glc by glucosidase-II, misfolded proteins carrying $Man_9GlcNAc_2$ (M9) are reglucosylated back to G1M9 by the action of UDP-glucose: glycoprotein glucosyltransferase (UGGT) [12–14]. Thus, CNX/CRT, glucosidase-II and UGGT act cooperatively to constitute so-called "calnexin cycle". Ultimately, correctly folded proteins are transported to Golgi for further processing, while "fatally misfolded" glycoproteins are delivered to the degradation pathway called ER-associated degradation (ERAD) [15–18]. Mannosidase like proteins (MLPs) [19], EDEM discovered from mammalian cells and its yeast counterpart Mn11p/Htm1p [20], are hypothesized to recognize misfolded glycoproteins having M8 oligosaccharides ($Man_8GlcNAc_2$, B-isomer), which are formed from M9 by the action of ER mannosidase-I [21]. Glycoproteins directed to ERAD are dislocated to cytosol, ubiquitinated, deglycosylated and degraded by proteasome [22–25].

The exact mode of CNX/CRT chaperone action is elusive, in light of the fact that they prevent aggregation of both glycosylated as well as non-glycosylated proteins. However, it has been revealed clearly that they chaperone glycosylated

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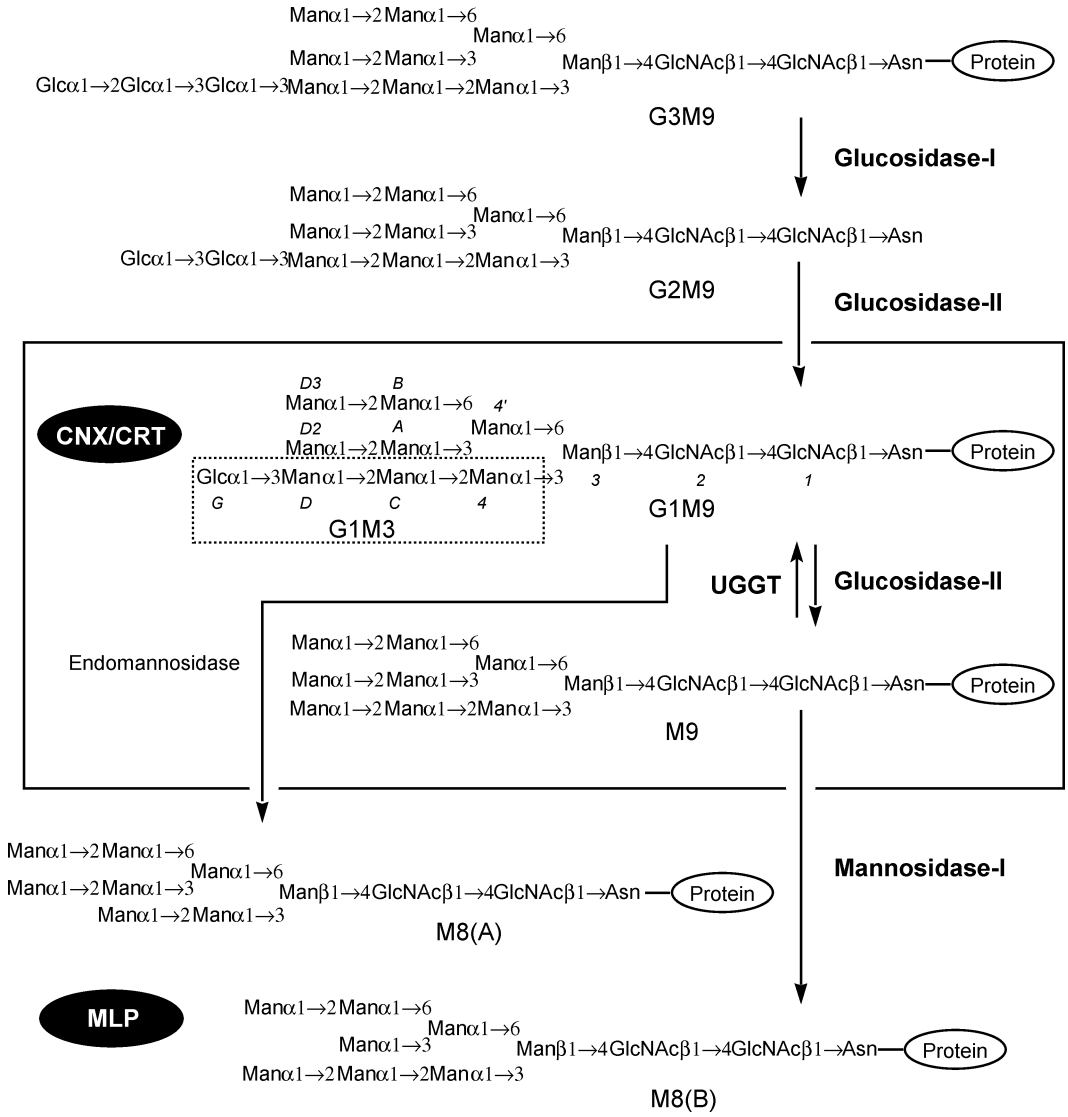


Figure 1. Glycoprotein processing and quality control in ER.

proteins with much higher efficiency [26]. Isothermal titration calorimetry (ITC) [27] and surface plasmon resonance (SPR) [28] studies estimated the affinities toward G1M9 was in $\sim\mu$M range. CNX and CRT consist of globular N-domain and arm-like P-domain. The former is proposed to have a lectin property, while the latter is involved in polypeptide binding and heterodimer formation with ERp57 [29–31]. As a prerequisite, carbohydrate binding specificity of CRT/CNX should be strict, in a sense that they recognize monoglucosylated glycans such as G1M9 and G1M8 but not G3M9, G2M9, M9, or M8 [32–34]. It was revealed that α-configured Glc residue is critical for CRT binding; β-$Glc_1Man_9GlcNAc_2$ was a poor ligand of CRT [35]. On the other hand, branched pentasaccharide linked to *C*-6 of β-Man is not playing a significant role in CRT/CNX recognition. In fact, smaller oligosaccharide Glcα1 → 3Manα1 → 2Manα1 → 2Manα (G1M3) was shown to bind CNX and CRT with similar avidity to the full structure of G1M9.

In this paper, we wish to report the synthesis of fluorinated analogue of Glcα1 → 3Manα(**1**) Glcα1 → 3Manα1 → 2Manα (**2**) and G1M3 (**3**) (Figure 2). Because glucosidase II requires the presence of branched pentamannose (Man-4′, -*A*, -*B*, -D_2, and -D_3) for its activity [36], compounds **1**–**3** are expected to be resistant to this enzyme. Fluorine substitution of Man-*D* should also provide resistance to endomannosidase [37], which cleaves αMan1 → 2 Man linkage of G1M9 that escaped CRT/CNX-UGGT cycle. Therefore, it can be expected that these compounds may stall the CNX/CRT-UGGT cycle to inhibit glycoprotein quality control. As the first measure, affinities of these compounds with CRT were evaluated by using ITC, in comparison with G1M3 and G1M9.

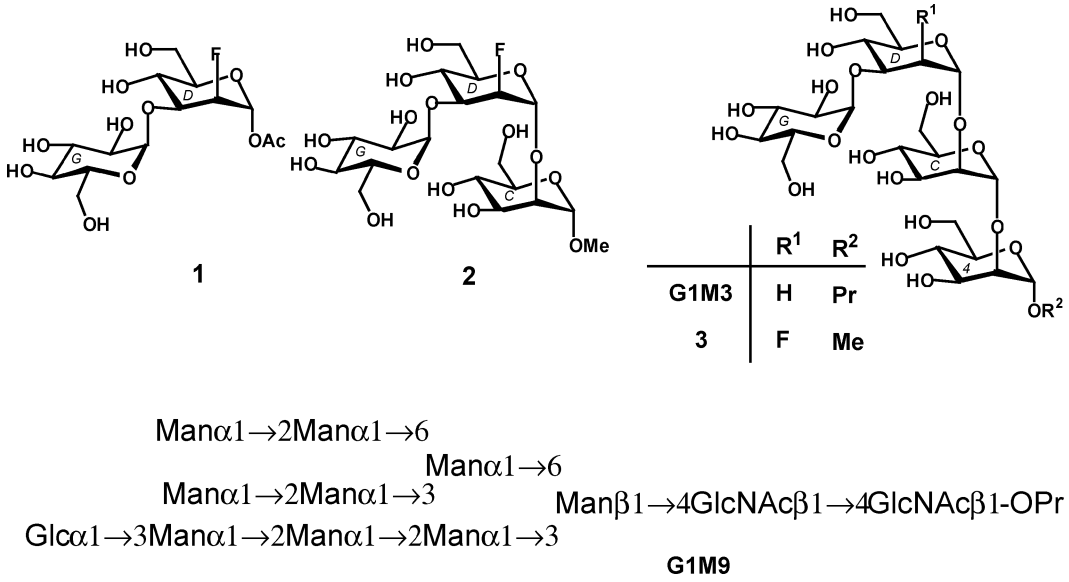


Figure 2. Structures of fluorine substituted oligosaccharide analogues.

Materials and methods

General methods

Starting materials and reagents were purchased from standard vendors and used without purification unless otherwise noted. All reactions sensitive to air and/or moisture were carried out under nitrogen or argon atmosphere with anhydrous solvents. Analytical thin layer chromatography (0.25 mm) was developed on silica gel 60 F plates (Merck, Darmstadt, Germany). Silica gel column chromatography was performed on Silica gel 60 N (40–100 mesh or 100–210 mesh, Kanto Kagaku Co., Ltd., Japan). NMR spectra were obtained on a JEOL EX-EX-400spectrometer (^{1}H at 400 and ^{13}C at 100 MHz) at ambient temperature unless otherwise noted.

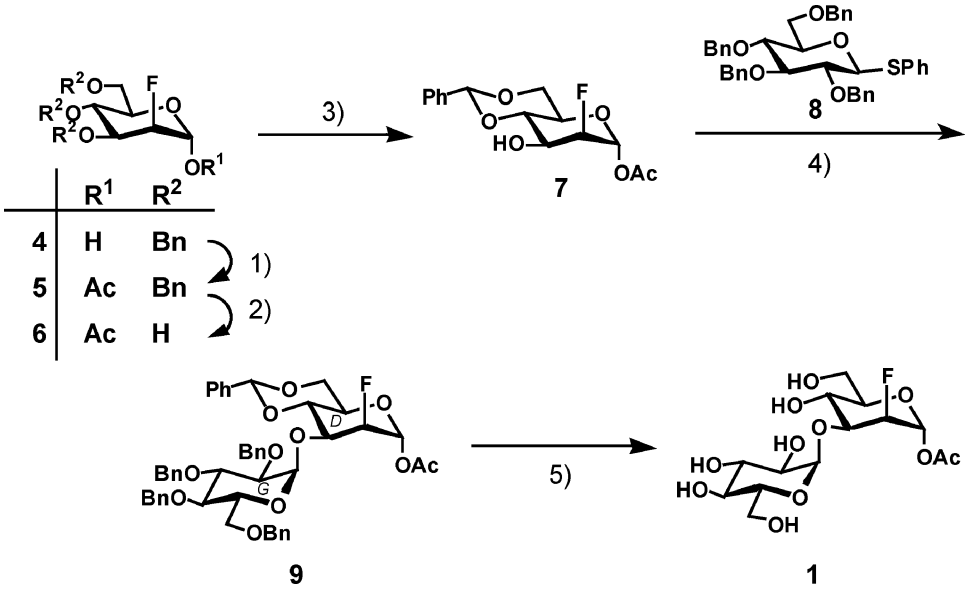
3,4,6-Tri-O-benzyl-2-deoxy-2-fluoro-α-D-mannopyranosyl acetate (**5**)

3,4,6-Tri-O-benzyl-2-deoxy-2-fluoro-α-D-mannopyranose (**4**) was acetylated under standard conditions. Separation by silica gel column chromatography (hexane-AcOEt, 4:1) afforded the title compound; ^{1}H-NMR ($CDCl_3$) 7.9–7.1 (15 H, m, Ar), 6.252 (1 H, dd, *J* 6.1 and <1 Hz, H-1), 4.860 (1 H, d, *J* 10.7 Hz, benzyl), 4.762 (1 H, d, *J* 10.7 Hz, benzyl), 4.724 (1 H, d, *J* 11.7 Hz, benzyl), 4.668 (1 H, dt, *J* 49.0 and <1 Hz, H-2), 4.642 (1 H, *J* 11.9 Hz, benzyl), 4.535 (1 H, *J* 10.5 Hz, benzyl), 4.514 (1 H, d, *J* 12.2. Hz, benzyl), 4.029 (1 H, t, *J* 9.9 Hz, H-4), 2.043 (3 H, s, Ac); ^{13}C-NMR ($CDCl_3$) δ 168.25 (Ac), 90.88 (J_{C-F} 30.7 Hz, C-1), 85.63 (J_{C-F} 179.1 Hz, C-2), 77.86 (J_{C-F} 17.4 Hz, C-3), 75.42 (CH_2), 74.04, 73.58, 73.52 (CH_2), 72.26 (CH_2)), 68.31 (CH_2), 20.89 (Ac).

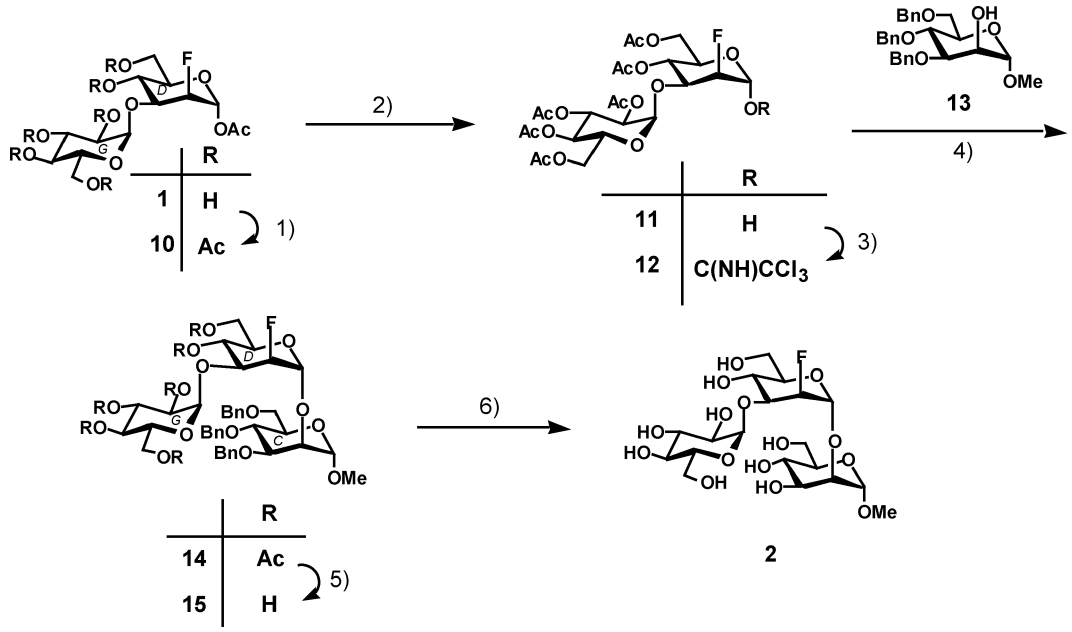
4,6-O-Benzylidene-2-deoxy-2-fluoro-α-D-mannopyranosyl acetate (**7**)

A solution of compound **5** (909 mg, 1.84 mmol) in MeOH (20 ml) was hydrogenated over $Pd(OH)_2$ (50 mg) under atmospheric pressure at room temperature. After 5 h, insoluble material removed by filtration and the filtrate was evaporated *in vacuo*. The residue was co-evaporated with *N*,*N*-dimethylformamide (DMF), and the residue was re-dissolved in DMF (10 ml). The solution was treated with benzaldehyde dimethylacetal (0.48 ml, 3.2 mmol) and camphorsulfonic acid (10 mg, 0.04 mmol) and the mixture was stirred at room temperature with occasional co-evaporation with acetonitrile (∼5 ml × 3). After 18 h, the mixture warmed to 50°C and stirred for 3 h. The mixture was diluted with ethyl acetate (AcOEt), washed with aq. $NaHCO_3$, water (×2) and brine, successively. The organic layer was dried over anhydrous $MgSO_4$, evaporated *in vacuo* and the residue crystallized from ether-hexane (1:1) to afford the title compound as a white powder (393 mg, 68%); m.p. 177–178°C; ^{1}H-NMR ($CDCl_3$) δ 7.5–7.3 (5 H, m, aromatic), 6.243 (1 H, dd, *J* 7.3 and 1.7 Hz, H-1), 5.589 (1 H, s, benzylidene), 4.742 (1 H, ddd, *J* 48.1, 2.9 and 1.7 Hz, H-2), 4.291 (1 H, dd, *J* 10.2 and 4.5 Hz, H-6), 4.143 (1 H, m, H-3), 3.958 (1 H, ddd, *J* 9.6, 9.6 and 1.5 Hz, H-4), 3.881 (1 H, ddd, *J* 9.8, 9.6 and 4.5 Hz), 3.791 (1 H, dd, *J* 10.2 and 9.8 Hz, H-6′), 2.482 (1 H, d, *J* 5.9 Hz), 2.144 (3 H, s, Ac); ^{13}C-NMR ($CDCl_3$)δ 168.19 (Ac), 136.69, 129.30, 128.30. 126.12, 102.24 (benzylidene), 90.96 (J_{C-F} 33.2 Hz, C-1), 88.52 (J_{C-F} 179.1 Hz, C-2), 78.16, 68.34 (C-6), 67.93 (J_{C-F} 17.4 Hz, C-3), 65.64, 20.91 (Ac).

Anal. Calcd for $C_{15}H_{17}FO_6$: C 57.69, H 5.49. Found: C 57.11, H 5.46. $[\alpha]_D$ + 64.4 (*c*0.93, $CHCl_3$).



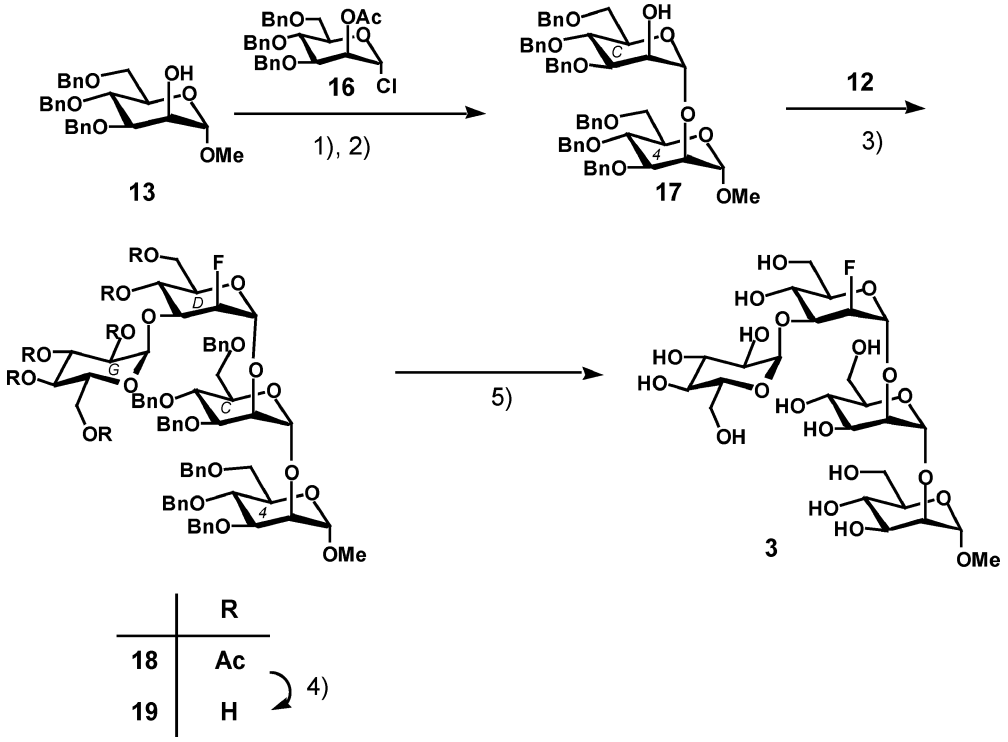
Scheme 1. Synthesis of disaccharide: (1) Ac_2O-pyridine, (2) H_2, $Pd(OH)_2$, (3) $PhCH(OMe)_2$, CSA, (4) NIS, AgOTf, CH_2Cl_2-Et_2O, (5) H_2, $Pd(OH)_2$.



Scheme 2. Synthesis of trisaccahride: (1) Ac_2O-pyridine, (2) NH_2NH_2-AcOH, (3) CCl_3CN, Cs_2CO_3, (4) TMSOTf, (5) NaOMe, MeOH, (6) H_2, $Pd(OH)_2$.

O-(2,3,4,6-Tetra-O-benzyl-α-D-glucopyranosyl)-(1 $\rightarrow$ 3)-4,6-O-benzylidene-2-deoxy-2-fluoro-α-D-mannopyranosyl acetate (**9**)

A mixture of compounds **8** (1.45 g, 2.29 mmol) and **7** (583 mg, 1.87 mmol) was co-evaporated with dioxane and dried under high vacuum. It was transferred to a flask containing *N*-iodosuccinimide (NIS, 0.60 g, 2.7 mmmol) and powdered molecular sieves 4A (3.0 g) as a solution in CH_2Cl_2-ether (1:1, 30 ml), and the mixture cooled down to –40°C with stirring. Silver trifluoromethanesulfonate (AgOTf, 50 mg, 0.19 mmol) was added and the mixture stirred at the same temperature. After 1 h, the reaction was quenched with $NaHCO_3$, $Na_2S_2O_3$ and ice-water, diluted with ethyl acetate and stirred at ambient temperature for 0.5 h. Filtered through Celite and the filtrate was washed with water ($\times$2) and brine, successively, dried ($MgSO_4$) and evaporated *in vacuo*. The residue was crystallized from hexane-ether ($\sim$3:1) to afford the title compound



Scheme 3. Synthesis of tetrasaccharide: (1) AgOTf, (2) NaOMe, MeOH, (3) TMSOTf, (4) NaOMe, (5) H_2, $Pd(OH)_2$.

as a white solid (1.28 g, 82%); m.p. 147–149°C; $[\alpha]_D$ +72.5 (c 0.80, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 6.151 (1 H, d, *J* 7.6 and 1.5 Hz, H-1^D), 5.560 (1 H, s, benzylidene), 5.272 (1 H, d, *J* 3.9 Hz, H-1^G), 2.020 (3 H, s, Ac); ^{13}C-NMR ($CDCl_3$) δ 168.14 (Ac), 102.33 (benzylidene), 98.43 (C-1^G), 91.13 (J_{C-F} 33.2 Hz, C-1^D), 88.31 (J_{C-F} 180.8 Hz, C-2^D), 81.40, 78.87, 77.59, 75.66 (benzyl), 75.15 (benzyl), 73.48 (benzyl), 73.45 (J_{C-F} 16.6 Hz, C-3^D), 71.18 (benzyl), 70.75, 68.95 (C-6), 68.45 (C-6), 66.08, 20.82 (Ac).

Anal. Calcd for $C_{49}H_{51}FO_{11}$: C 70.49, H 6.16. Found: C 70.51, H 6.17. $[\alpha]_D$ + 72.5 (*c* 0.80, $CHCl_3$).

β-Isomer; ^{1}H-NMR ($CDCl_3$) δ 6.265 (1 H, dd, *J* 7.3 and 2 Hz, H-1^D), 5.597 (1 H, s, benzylidene), 4.962 (1 H, d, *J* 10.7 Hz, benzyl), 4.922 (1 H, d, *J* 11.0 Hz, benzyl), 4.771 (1 H, dt, *J* 47.6 and 2 Hz, H-2^D), 4.615 (1 H, d, *J* 7.6 Hz, H-1^G), 2.092 (3 H, s, Ac); ^{13}C-NMR ($CDCl_3$) δ 102.00, 101.86, 90.93 (J_{C-F} 33.2 Hz, C-1^D), 86.59 (J_{C-F} 180.8 Hz, C-2^D), 84.63, 81.89, 77.61, 76.65, 75.52 (CH_2), 75.20, 74.93, 74.02 (J_{C-F} 16.6 Hz, C-3^D), 73.45, 68.83 (CH_2), 68.29 (CH_2), 66.05, 20.85 (Ac).

O-(α-D-Glucopyranosyl)-(1 → 3)-2-deoxy-2-fluoro-α-D-mannopyranosyl acetate (**1**)

A solution of compound **9** (1.58 g, 1.90 mmol) in AcOEt-EtOH-H_2O (4:2:1, 40 ml) was hydrogenated in the presence of $Pd(OH)_2$ (0.3 g) for 24 h. Catalyst removed by filtration and the filtrate evaporated *in vacuo* and co-evaporated with EtOH (×3). The residue was lyophylized from water to afford **1** (740 mg, quantitative); ^{1}H-NMR (D_2O) δ 6.188 (1 H, dd, *J* 6.8 and 2.0 Hz, H-1^D), 5.229 (1 H, d, *J* 3.9 Hz, H-1^D), 4.996 (1 H, dt, *J* 47.8 and 2 Hz, H-2^D), 3.572 (1 H, dd, *J* 10.2 and 3.9 Hz, H-2^G), 3.397 (1 H, t, *J* 9.5 Hz), 2.158 (3 H, s, Ac); ^{13}C-NMR (D_2O) δ 172.24 (Ac), 101.40 (C-1^G), 91.17 (J_{C-F} 31.5 Hz, C-1^D), 88.67 (J_{C-F} 175.8 Hz, C-2^D), 78.62 (J_{C-F} 16.6 Hz, C-3^D), 75.15, 73.43, 73.01, 72.26, 70.22, 65.56, 61.26, 60.69, 20.89 (Ac).

O-(2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl)-(1 → 3)-4,6-di-O-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl acetate (**10**)

Compound **1** (740 mg, 1.90 mmol) was dissolved in pyridine (20 ml). Ac_2O (10 ml) was added and the mixture left at room temperature overnight. It was evaporated, co-evaporated with EtOH (×4), diluted with AcOEt, washed with water and brine, successively, and passed through a short pad of $MgSO_4$-silican gel that was eluted with AcOEt. The filtrate was evaporated *in vacuo* and the residue was triturated with hexane to give the title compound as a white solid (1.21 g, quantitative); m.p. 156–157°C; $[\alpha]_D$ + 93.8 (*c* 1.1, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 6.255 (1 H, dd, *J* 6.7 and 2.3 Hz, H-1^D), 5.42 (2 H, m), 5.236 (1 H, d, *J* 3.9 Hz, H-1^G), 5.020 (1 H, t, *J* 9.5 Hz), 4.814 (1 H, dd, *J* 10.2 and 3.9 Hz, H-2^G), 4.808 (1 H, ddd, *J* 77.6, 2.4 and 2.2 Hz, H-2^D), 4.28–4.15 (3 H, m), 4.145–3.90 (4 H, m), 2.133, 2.080, 2.056, 2.046, 2.037, 2.018 and 1.978 (3 H each, s, Ac); ^{13}C-NMR ($CDCl_3$)δ 170.63, 170.31, 170.19, 169.53, 168.87, 167.78, 97.16 (C-1^G), 90.16 (J_{C-F}30.7 Hz, C-1^D), 86.79 (J_{C-F} 182.5 Hz, C-2^D), 76.15 (J_{C-F} 17.4 Hz, C-3^D), 70.81, 69.61,

68.22, 65.89, 61.90 (CH_2), 61.74 (CH_2), 20.90, 20.80, 20.73, 20.78.

Anal. Calcd for $C_{26}H_{35}FO_{17}$: C 48.90, H 5.52. Found: C 49.03, H 5.44.

O-(2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl)-(1 → 3)-4,6-di-O-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl trichloroacetimidate (**12**)

To a solution of **10** (276 mg, 0.432 mmol) in DMF (4 ml) was added hydrazine acetate (60 mg, 0.65 mmol) and the mixture was stirred at room temperature for 2 h. Resulting mixture was diluted with AcOEt, washed with water (×2) and brine, successively, dried ($MgSO_4$) and evaporated *in vacuo*. The residue was exposed to high vacuum to afford crude hemiacetal **11** (260 mg, quantitative). It was dissolved in CH_2Cl_2 (6 ml) and treated with trichloroacetonitrile (0.66 ml, 6.6 mmol) and Cs_2CO_3 (14 mg, 0.043 mmol) under ice-water cooling. After being stirred at 0°C∼r.t. for 2 h, the mixture was diluted with AcOEt, washed with water and brine, successively, dried ($MgSO_4$) and evaporated *in vacuo*. The residues was dried under high vacuum to give the title compound (307 mg, 96%); $[\alpha]_D$ + 90.5 (*c* 1.3, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 8.818 (1 H, s, NH), 6.443 (1 H, dd, *J* 5.6 and <1 Hz), 5.50–5.39 (2 H, m), 5.222 (1 H, d, *J* 3.7 Hz, H-1^G), 5.039 (1 H, t, *J* 9.6 Hz), 4.981 (1 H, dt, *J* 46.1 and <1 Hz, H-2^D), 4.840 (1 H, dd, *J* 10.3 and 3.7 Hz, H-2^G), 4.30–3.95 (7 H, m), 2.07–1.97 (18 H, Ac); ^{13}C-NMR ($CDCl_3$) δ 170.40, 170.18, 169.95, 169.43, 169.33 and 168.80 (CO), 159.41 (C=NH), 97.22 (C-1^G), 93.92 (J_{C-F} 31.7 Hz, C-1^D), 86.30 (J_{C-F} 181.8 Hz, C-2^D), 76.12 (J_{C-F} 17.5 Hz, C-3^D), 71.32, 70.66, 69.69, 68.19, 68.12, 65.74, 61.73 (CH_2), 61.67 (CH_2), 20.82, 20.79 and 20.74 (Ac).

Anal. Calcd for $C_{26}H_{35}Cl_3FNO_{16}$: C 42.15, H 4.49, N 1.89. Found: C 42.30, H 4.44, N 1.92.

Methyl O-(α-D-glucopyranosyl)-(1 → 3)-(2-deoxy-2-fluoro-α-D-mannopyranosyl)-(1 → 2)-3,4,6-tri-O-benzyl-α-D-mannopyranoside (**15**)

A mixture of compounds **12** (154 mg, 0.208 mmol) and **13** (126 mg, 0.271 mmol) was added as a solution in CH_2Cl_2 (4 ml) to a flask containing powdered molecular sieves 4A (0.35 g), and was cooled down to −40°C. Then, trimethylsilyl trifluoromethanesulfonate (TMSOTf, 0.44 M in 1,2-dichloroethane, 95 μl, 0.042 mmol) was added and the mixture was stirred for ∼1 h, with gradual warming to ∼0°C. Then, an additional amount of TMSOTf (0.042 mmol) was added and stirring continued for 1 h, while the mixture warmed to room temperature. It was diluted with AcOEt, quenched with $NaHCO_3$ and ice chips, stirred for 0.5 h, and filtered though Celite. The filtrate was washed with water and brine, successively, dried ($MgSO_4$) and evaporated *in vacuo*. The residue was dissolved in MeOH (10 ml) and treated with NaOMe (0.49 M in MeOH, 50 μl, 0.025 mmol) at room temperature for 4 h. The mixture was quenched with a drop of acetic acid (phenolphthalein negative), evaporated in *vacuo*, and purified by silica gel column chromatography (AcOEt-EtOH-H_2O, 25:2:1) to give 82.5 mg (45%) of the title compound, together with corresponding β-isomer (23.1 mg, 13%). Compound **15**; $[\alpha]_D$ + 72.0 (c 0.75, MeOH); ^{1}H-NMR (CD_3OD) δ 7.34–7.17 (15 H, m, aromatic), 5.106 (1 H, dd, *J* 7.3 and <1 Hz, H-1^D), 5.011 (1 H, d, *J* 3.7 Hz, H-1^G), 5.011 (1 H, dt, *J* 49.3 and <1 Hz), 4.880 (1 H, s, H-1^C), 4.782 (1H, d, *J* 11.0 Hz, benzyl), 4.69–4.58 (3 H, m, benzyl), 4.525 (1 H, d, *J* 10.7 Hz, benzyl), 4.511 (1 H, d, *J* 12.0 Hz, benzyl), 4.069 (1 H, bt, H-2^C), 3.351 (3 H, s, Me); ^{13}C-NMR ($CDCl_3$) δ 102.92 (C-1), 101.05 (C-1), 100.47 (J_{C-F} 29.9 Hz, C-1^D), 89.92 ($J_{C\wedge F}$ 175.8 Hz, C-2^D), 81.66 (J_{C-F} 17.4 Hz, C-3^D), 80.98, 76.64, 76.04 (CH_2), 75.92, 74.99, 74.95 (CH_2), 74.35, 73.88 (CH_2), 73.34, 72.75, 71.36, 70.23 (CH_2), 67.24, 62.71, 62.40 (CH_2), 55.39 (Me).

β-Isomer; ^{1}H-NMR (CD_3OD, 50°C) δ 7.4–7.1 (15 H, m, Ar), 5.092 (1 H, d, *J* 3.9 Hz, H-1^G), 4.985 (1 H, dd, *J* 51.2 and 2.5 Hz, H-2^D), 4.804 (1 H, d, *J* 2 Hz, H-1^C), 4.275 (1 H, t, *J* 2 Hz, H-2), 3.368 (3 H, s, Me); ^{13}C-NMR (CD_3OD) δ 103.03 (C-1), 100.09 (C-1), 98.75 (J_{C-F} 14.9 Hz, C-1^D), 90.93 (J_{C-F} 185.8 Hz, C-2^D), 83.69 (J_{C-F} 16.6 Hz, C-3^D), 79.30, 78.13, 75.72 (CH_2), 75.67, 74.89, 72.70, 71.88 (CH_2), 71.51, 70.32 (CH_2), 67.16, 62.72 (CH_2), 62.45 (CH_2), 55.43 (Me).

Methyl O-(α-D-glucopyranosyl)-(1 → 3)-(2-deoxy-2-fluoro-α-D-mannopyranosyl)-(1 → 2)-α-D-mannopyranoside (**2**)

A solution of compound **15** (49.2 mg, 0.056 mmol) in MeOH (5 ml) was hydrogenated in the presence of $Pd(OH)_2$ (15 mg) at room temperature for 20 h. Catalyst removed by filtration and the filtrate evaporated *in vacuo*. The residue was dissolved in water, passed through Sep-Pac C_{18} cartridge that was eluted with water. The solution was lyophylized to give the title compound (31.7 mg, 93%); ^{1}H-NMR (D_2O) 5.239 (1 H, dd, *J* 7.6 and 1.8 Hz, H-1^D), 5.216 (1 H, d, *J* 3.9 Hz, H-1^G), 5.064 (1 H, dt, *J* 49.0 and 2 Hz, H-2^D), 4.967 (1 H, d, *J* 1.5 Hz, H-1^C), 3.988 (1 H, dd, *J* 3.3 and 1.8 Hz, H-3^D), 3.649 (1 H, dd, *J* 10.0 and 9.3 Hz, H-3^G), 3.545 (1 H, dd,*J* 10.0 and 3.9 Hz, H-2^G), 3.380 (3 H, s, Me); ^{13}C-NMR (D_2O) δ 101.18 (C-1). 99.75 (C-1), 99.69 (J_{C-F} 30.7 Hz, C-1^D), 89.29 (J_{C-F} 174.1 Hz, C-2^D), 79.41, 78.69 (J_{C-F} 16.6 Hz, C-3^D), 73.66, 73.41, 73.18, 72.88, 72.25, 70.73, 70.25, 67.52, 66.40, 61.53 (CH_2), 61.31 (CH_2), 61.22 (CH_2), 55.45.

Methyl O-(3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-(1 → 2)-3,4,6-tri-O-benzyl-α-D-mannopyranoside (**17**)

A solution of compounds **16** (265 mg, 0.519 mmol) and **13** (154 mg, 0.332 mmol) in CH_2Cl_2 (8 ml) was added to a mixture of AgOTf (160 mg, 0.622 mmol) and molecular sieves 4A (1.0 g) in CH_2Cl_2 (2 ml) at −15°C. The mixture was stirred at −15°C ∼ 0°C for 2 h and at room temperature for 0.5 h. Diluted with AcOEt, stirred with $NaHCO_3$ (100 mg)-ice water (0.5 ml) for 0.5 h. Anhydrous $MgSO_4$ was added and stirred for

10 min. The mixture was filtered through Celite and the filtrate was evaporated *in vacuo*. The residue was treated with NaOMe (0.49 M, 10 ml) at room temperature for 3 d. Neutralized with AcOH, evaporated *in vacuo* and purified by silica gel column chromatography (hexane-AcOEt 5;1) to afford 227 mg (76%) of the title compound; $[\alpha]_D + 24.3$ (*c* 0.59, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 5.129 (1 H, d, *J* 1 Hz, H-1^C), 4.800 (1 H, d, *J* 2 Hz, H-1^4), 4.116 (1 H, dd, *J* 2 and 1 Hz, H-2), 4.024 (1 H, t, *J* 2 Hz, H-2), 3.229 (3 H, s, Me); ^{13}C-NMR ($CDCl_3$) δ 101.04 (C-1), 99.75 (C-1), 79.97, 79.76, 75.04 (CH_2), 75.00 (CH_2), 74.72, 74.38, 73.40 (CH_2), 73.30 (CH_2), 72.22 (CH_2), 72.12 (CH_2), 71.70, 71.55, 69.30 (CH_2), 69.16 (CH_2), 68.52, 54.71 (Me).

Anal. Calcd for $C_{55}H_{60}O_{11}$: C 73.64, H 6.74. Found: C 73.70, H 6.81.

Methyl O-(α-D-glucopyranosyl)-(1 $\rightarrow$ 3)-(2-deoxy-2-fluoro-α-D-mannopyranosyl)-(1 $\rightarrow$ 2)-(3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-(1 $\rightarrow$ 2)-3,4,6-tri-O-benzyl-α-D-mannopyranoside (**19**)

A mixture of disaccharide **17** (62.8 mg, 0.070 mmol), trichloroacetimidate **12** (58.4 mg, 0.079 mmol) and powdered molecular sieves 4A (0.25 g) in CH_2Cl_2 (3 ml) was stirred at 0°C. TMSOTf (0.44 M in 1,2-dichloroethane, 30 μl, 0.013 mmol) was added and the mixture stirred for 1 h. Then, a second portion of TMSOTf (0.013 mmol) was added and stirring continued at 0°C to room temperature for 2 h. The mixture was diluted with AcOEt, $NaHCO_3$ ($\sim$100 mg) and H_2O (0.5 ml) were added, and stirred for 10 min. Anhydrous Na_2SO_4 was added and the mixture was filtered through Celite. The filtrate was evaporated *in vacuo* and the residue was exposed to high vacuum to afford crude tetrasaccharide **18** that was dissolved in MeOH (0.5 ml). NaOMe (0.49 M in MeOH, 0.2 ml, 0.1 mmol) was added and stirred for 4 h. The reaction was quenched with acetic acid and evaporated *in vacuo*. The residue was chromatographed over silica gel (AcOEt-EtOH-H_2O, 25:2:1) to give the title compound (25.8 mg, 30%); ^{1}H-NMR (CD_3OD, 50°C) δ 7.4–7.1 (30 H, m, Ar), 5.238 (1 H, d, *J* 1.7 Hz, H-1^C), 5.054 (1 H, d, *J* 3.4 Hz, H-1^G), 4.967 (1 H, dt, *J* 51.5 and 2 Hz, H-2^D), 4.807 (1 H, d, *J* 11.2 Hz, benzyl), 4.786 (1 H, d, *J* 11.2 Hz, benzyl), 4.694 (1 H, d, *J* 11.7 Hz, benzyl), 4.107 (1 H, t, *J* 2 Hz, H-2), 3.227 (3 H, s, Me); ^{13}C-NMR (CD_3OD) δ 102.97, 102.00, 100.96, 100.49 (J_{C-F} 29.9 Hz, C-1^D), 89.96 (J_{C-F} 175.8 Hz, C-2^D), 81.77 (J_{C-F} 16.6 Hz, C-3^D), 80.60, 80.31, 77.33, 77.07, 76.18 (CH_2), 76.11, 75.92 (CH_2), 75.84, 75.04, 74.89, 74.41 (CH_2), 74.26 (CH_2), 73.95, 73.44 (CH_2), 73.20, 73.02, 72.79 (CH_2), 71.46, 70.76 (CH_2), 70.13 (CH_2), 67.06, 62.55 (CH_2), 62.48 (CH_2), 55.41 (Me).

Methyl O-(α-D-glucopyranosyl)-(1 $\rightarrow$ 3)-(2-deoxy-2-fluoro-α-D-mannopyranosyl)-(1 $\rightarrow$ 2)-(α-D-mannopyranosyl)-(1 $\rightarrow$ 2)-α-D-mannopyranoside (**3**)

Compound **19** (14.6 mg, 0.0119 mmol) was hydrogenated over $Pd(OH)_2$ (7 mg) in MeOH (3 ml) at room temperature and processed as described for compound **2** to give 8.2 mg (quantitative) of the title compound.

^{1}H-NMR (D_2O) δ 5.262 (1 H, d, *J* 1.5 Hz, H-1^C), 5.244 (1 H, dd, *J* 7.6 and 2 Hz, H-1^D), 5.209 (1 H, d, *J* 3.9 Hz, H-1^G), 5.054 (1 H, dt, *J* 48.5 and 2 Hz, H-2^D), 4.953 (1 H, d, *J* 1.5 Hz, H-1^4), 4.128 (1 H, dd, *J* 2.9 and 2 Ha, H-2), 3.366 (3 H, s, Me); ^{13}C-NMR (D_2O) δ 101.06 (C-1), 100.95 (C-1), 99.71 (C-1), 99.54 (J_{C-F} 34.0 Hz, C-1^D), 89.15 (J_{C-F} 173.5 Hz, C-2^D), 79.35, 79.16, 78.60 (J_{C-F} 16.6 Hz, C-3^D), 73.77, 73.39, 73.25, 73.02, 72.75, 72.10, 70.66, 70.31, 70.11, 67.48, 67.41, 66.20, 61.58, 61.39, 61.17, 61.01, 55.32 (Me).

Expression and purification of recombinant calreticulin

The DNA fragment encoding human calreticulin (CRT) was cloned into the pGEX-6P-1 plasmid vector (Amersham Pharmacia Biotech, Piscataway, NJ) with a N-terminal GST moiety. The construct was transformed into Escherichia coli BL21 (DE3) strain (Stratagene, La Jolla, CA). An overnight plateau phase culture was used to inoculate fresh LB broth containing 50 μg/ml ampicillin, at a dilution of 1:100 (1 L). Cells were grown at 30°C to an A600 of 0.6–0.8 and were added 0.1 mM isopropyl-1-thio-D-galactopyranoside and 2 mM calcium chloride followed by incubation for an additional 3 h. The cells were harvested and resuspended in a 50 mL of lysis buffer (10 mM PBS, 150 mM NaCl, pH 7.5) and sonicated on ice. The lysates were loaded onto GST-agarose (equilibrated with 2 bed volumes of lysis buffer). The fusion protein was eluted by 1 mM glutathione (10 mM Tris.HCl, 150 mM NaCl, pH 8.0). The eluate was concentrated using a Centriprep (Milipore) and dialyzed against a 10 mM MOPS, 5 mM $CaCl_2$, 150 mM NaCl, pH 7.5, buffer. The concentration of CRT calculated A280 1.73 as 1-mg ml^{-1} CRT solution.

Isothermal titration calorimetry (ITC)

Calorimetric measurements were carried out with a VP-ITC titration calorimeter (MicroCal). The experiment was conducted by adding 6 μl of synthetic oligosaccharide (300 μM) every 3 min into a MOPS buffer (10 mM, pH 7.4) containing NaCl (150 mM), $CaCl_2$ (5 mM), and GST-CRT (0 or 28 μM) at 20°C. Heats of dilution were measured in blank titrations by injecting oligosaccharide into the buffer and were subtracted from the binding heats. Thermodynamic parameters were determined by nonlinear least-squares methods, using routines included in the Origin software package (MicroCal, version 7.0).

Results and discussion

Synthesis of oligosaccharides

Synthesis of target molecules commenced with 2-fluoro-2-deoxy-D-mannose derivative **4**, which was reported previously [38]. It was first converted to acetate **5**, which was debenzylated with Pearlman's catalyst under atmospheric pressure of hydrogen. Resultant **6** was treated with benzaldehyde dimethylacetal

and camphorsulphonic acid to give 4,6-*O*-benzylidene derivative **7**. For the introduction of α-linked glucose, thioglycoside **8** was used as the donor and was activated with *N*-iodosuccinimide (NIS) and a catalytic amount of silver trifluoromethanesulfonate (AgOTf) [39]. While the reaction in CH_2Cl_2 proceeded with modest selectivity (α:β = 1.7:1, 87% yield), more selective formation of the α-isomer (α:β = 10:1) was achieved when a medium was supplemented with Et_2O. Under these conditions, anomerically pure **9** was isolated in 82% yield without recourse of chromatography. The product **9**, obtained as a crystalline solid was subjected to catalytic hydrogenation to simultaneously remove benzyl and benzylidene groups to give 2-fluorinated Glcα1 $\rightarrow$ 3Man **1**.

To synthesize tri- (**2**) and tetrasaccharide (**3**), compound **1** was once acetylated to give **10** that was, without purification, subjected to anomeric deacetylation with NH_2NH_2·AcOH. Resultant hemiacetal **11** was pure enough to be used directly for the subsequent transformation.

Previously, glycosylation with 2-fluoro-substituted glycosyl bromide was reported to proceed with poor stereoselectivity [38]. Possibly, the presence of strongly electronegative fluorine discouraged the formation of oxocarbenium ion-like intermediate.

Recognizing the difficulty associated with the use of glycosyl donor having 2-fluoro substitution, we selected trichloroacetimidate as the leaving group [40,41], with its excellent ability as a leaving group in mind. Thus, the aforementioned hemiacetal **11** was subjected to the treatment with trichloroacetonitrile and Cs_2CO_3, which provided the trichloroacetimidate **12** with an excellent quality. Its anomeric configuration was assigned to be α, based on the J_{C-F} value (5.6 Hz). This material obtained in 96% overall yield from **1**, was used without purification for the coupling with 2-*O*-unprotected mannose derivative **13**. The glycosylation was conducted in CH_2Cl_2 in the presence of a trimethylsilyl trifluoromethanesulfonate (TMSOTf) at -40°C. Since the isolation of the product **14** seemed to be difficult at this stage, the crude mixture was deacetylated with NaOMe to **15**, which was isolated in 45% yield, together with corresponding β-isomer (13%). Finally, debenzylation under standard conditions gave **2**.

For the synthesis of tetrasaccharide **3**, dimannosyl fragment **17** was prepared from chloride **16** and acceptor **13** [42]. Glycosylation promoted by AgOTf in dichloromethane (-20°C$\sim$r.t.) was followed by deacetylation to provide **17** in 76% yield. Coupling with disaccharide donor **12** was conducted with TMSOTf (0.4 equiv.) in CH_2Cl_2. Deacetylation of the crude mixture allowed the isolation of tetrasaccharide **19** in 30% yield from **17**. Corresponding β-isomer could not be identified in this case. Hydrogenolysis with $Pd(OH)_2$ in methanol gave **3**.

Measurement of oligosaccharide-CRT interaction by isothermal titration calorimetry

Isothermal titration calorometry (ITC) is a valuable tool and has been widely used to estimate association constants and thermodynamic parameters of biomolecule interactions, such as lectin-carbohydrate binding. It directly measures the amount of heat released or absorbed, which accompanies with protein-protein or protein-ligand interaction. Determination of K_a, ΔH, and $T\Delta S$ values, without modifying or immobilizing the ligands or proteins of interest. Estimated strength of CRT-carbohydrate interactions is in a range ($\sim 10^6$) suitable for ITC measurements.

Table 1. Thermodynamic parameters for the binding of oligosaccharides to CRT obtained by ITC experiments

ligand	*Ka* $\times 10^{-6}$ *(M^{-1})*	*H (kcal/mol)*	*S (cal/mol/K)*
1	N.D.	N.D.	N.D.
2	0.99	−9.18	−3.89
3	0.41	−13.6	−20.7
G1M3	1.65	−8.08	0.88
G1M9	5.26	−11.8	−9.64

By using recombinant CRT, expressed as a fusion protein with glutathion S-transferase (GST), 2-fluorinated di- (**1**), tri- (**2**), and tetrasaccharide (**3**) were subjected to ITC measurements, in comparison with G1M3 [43] and G1M9 [35]. As summarized in Table 1, compounds **2** and **3** had detectable affinities toward CRT. Somewhat unexpectedly, the affinity of tetrasaccharide analogue **3** was slightly smaller than trisaccharide **2**. On the other hand, binding of Glcα1 $\rightarrow$ 3Man analogue **1** was not detectable. In agreement with previous reports, [27] the affinity of G1M3 was somewhat weaker than G1M9 (the natural ligand of CRT/CNX).

Discussion

CRT and CNX have been revealed to have a lectin activity and recognize G1M9 as the ligand. It has been known that these lectin-chaperons mainly recognize the C-3 arm of this oligosaccharide and G1M3 has a similar level of avidity. We designed compounds **1**$\sim$**3**, which are the 2-fluorinated analogues of Glcα1 $\rightarrow$ 3Man, Glcα1 $\rightarrow$ 3Manα1 $\rightarrow$ 2Man, and Glcα1 $\rightarrow$ 3Manα1 $\rightarrow$ 2Manα1 $\rightarrow$ 2Man, respectively. These compounds were synthesized in a concise manner and subjected to binding studies with CRT by using ITC. It was revealed that disaccharide **1** was a poor ligand, while tri- (**2**) and tetrasaccharide (**3**) had observable affinity. Somewhat unexpectedly, the G1M2 analogue (**2**) seemed to be more potent ligand than G1M3 analogue (**3**). Although we do not have an explanation, this result is pragmatically fortuitous. Among 11 steps to synthesize **2**, only two of them require chromatograhic separation. Structural modification, such as the introduction of functionalized aglycon and/or incorporation to multivalent systems would provide higher-affinity ligand of CNX/CRT, which may be useful as a novel inhibitor of glycoprotein quality control.

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