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High-mannose-type glycan modifications of dihydrofolate reductase using glycan–methotrexate conjugates

Kiichiro Totani,[a,b] Ichiro Matsuo,[a,b] Yoshito Ihara[b,c] and Yukishige Ito[a,b,*]

[a]*RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*
[b]*CREST, Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-1102, Japan*
[c]*Department of Biochemistry and Molecular Biology of Disease, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Science, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*

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Abstract—Various high-mannose-type glycan modifications of dihydrofolate reductase (DHFR) were achieved by ligand-based approach using glycan–methotrexate (MTX) conjugates as tight binding glycan bearing ligands for DHFR. The resulting glycan–MTX conjugates and the corresponding artificial glycoproteins could be useful as oligosaccharide- and glycoprotein-probes to perform quantitative analysis of glycan recognizing protein such as lectins, glycosyltransferases or glycosidases. Moreover, artificial glycoproteins having two different high-mannose-type glycans were developed for the first time by a combination of two different types of glycan modification strategies.

1. Introduction

A majority of eukaryotic proteins are N-glycosylated on Asn residues. Glycoprotein oligosaccharides play pivotal roles in various biological events.[1] In particular, functions of high-mannose-type glycans in glycoprotein processing and maturation in the endoplasmic reticulum (ER) are attracting recent attention.[2] In order to gain a precise understanding of their functions, structurally defined glycoproteins are desired. However, a variation of homogeneous glycoproteins derived from natural sources is limited and not optimally suitable for this purpose.[3,4] Addressing this limitation, artificial glycan modification of protein has been widely investigated.[5,6] However, the efficiency of glycan incorporation is relatively low in most cases. A large excess of functionalized oligosaccharide is required and introduction of complex oligosaccharide has been scarcely reported. We recently reported the ligand-based approach, which allowed the immobilization of glycan chain on dihydrofolate reductase (DHFR).[7] Namely, glycan–methotrexate conjugates (CHO–MTX) can be converted to CHO–MTX–DHFR complexes, by virtue of tight binding ($K_a \sim 10^{10}M^{-1}$)[8] between DHFR and MTX, the strong inhibitor of the enzyme (Fig. 1A). Although the binding is tight enough to keep complexes stable under physiological conditions, CHO–MTX can be released once treated with a high concentration (0.8 M) of NaCl.

In our preliminary report, we demonstrated that mono-, di-, and trisaccharide as well as core pentasaccharide (Man3GlcNAc2), which is common to Asn-linked glycoporteins, could be incorporated to DHFR. We wish to report herein the comprehensive syntheses of MTX conjugates of ER-residing high-mannose-type oligosaccharides and measurement of their affinities to DHFR. It was found that efficiency of the glycan incorporation was insensitive to the glycan structure, and incorporation of these biologically important oligosaccharides can be conducted readily as required.

2. Results and discussion

2.1. Synthesis of glycan–MTX conjugates

Figure 1B depicts a series of CHO–MTX conjugates (**2a–i**) and corresponding DHFR complexes (**3a–i**), which were prepared in this study. Scheme 1 summarizes the diagram of this study. The protected oligosaccharides

Keywords: Glycoprotein; Methotrexate; Dihydrofolate reductase; High-mannose-type oligosaccharide.
* Corresponding author. Tel.: +81 48 467 9430; fax: +81 48 462 4680; e-mail: yukito@riken.jp

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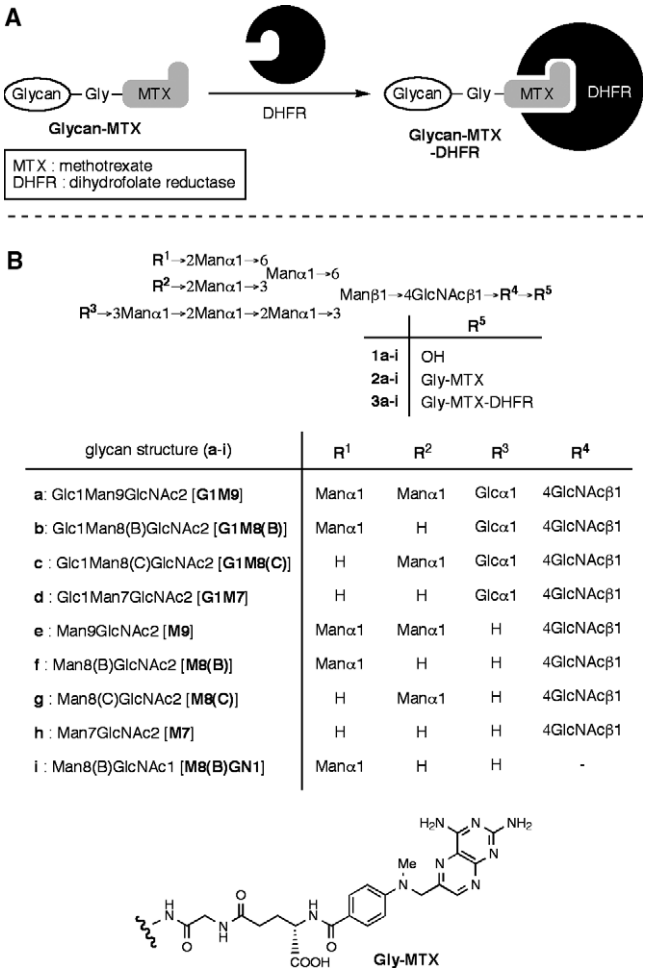


Figure 1. The synthetic high-mannose-type oligosaccharide- and glycoprotein-probes. (A) The principle of ligand-based approach to generate artificial glycoprotein-probes. (B) Structures of the ER glycoprotein related synthetic molecular probes.

4a–**h** were synthesized based on our generalized strategy,[9] parts of which were reported previously for Glc1Man9GlcNAc2,[10] GlcMan8(B)GlcNAc2,[11] Man9GlcNAc2,[10] and Man8(B)GlcNAc2.[11] Removal of protecting groups was conducted in a unified manner: (1) conversion of phthalimide into acetamide, (2) de-allylation using Ir complex, (3) hydrogenolysis of benzyl-, *p*-methoxybenzyl-, and benzylidene-groups, and (4) de-acylation with NaOMe. Interestingly, alkaline treatment of the reducing sugar **1b** under stronger conditions (100 mM NaOH at 50 °C, 48 h) caused peeling[12–14] of GlcNAc to give Man8(B)GlcNAc1 (**1i**) cleanly.

Oligosaccharides thus obtained (**1a**–**i**) were converted to glycosylamines **5a**–**i** under Kochetkov's conditions[15] using saturated aqueous NH_4HCO_3. After removal of the residual ammonium salt by lyophilization, they were quickly condensed with FmocGlyCl[16] in the presence of $NaHCO_3$ and deprotection of Fmoc group gave Gly-conjugates **7a**–**i** in good yields. Subsequent coupling with MTX(α^tBu)[7] was conducted with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM),[17] the water compatible condensing agent, and ^{t}Bu removal completed the syntheses of glycan–MTX conjugates (**2a**–**i**).

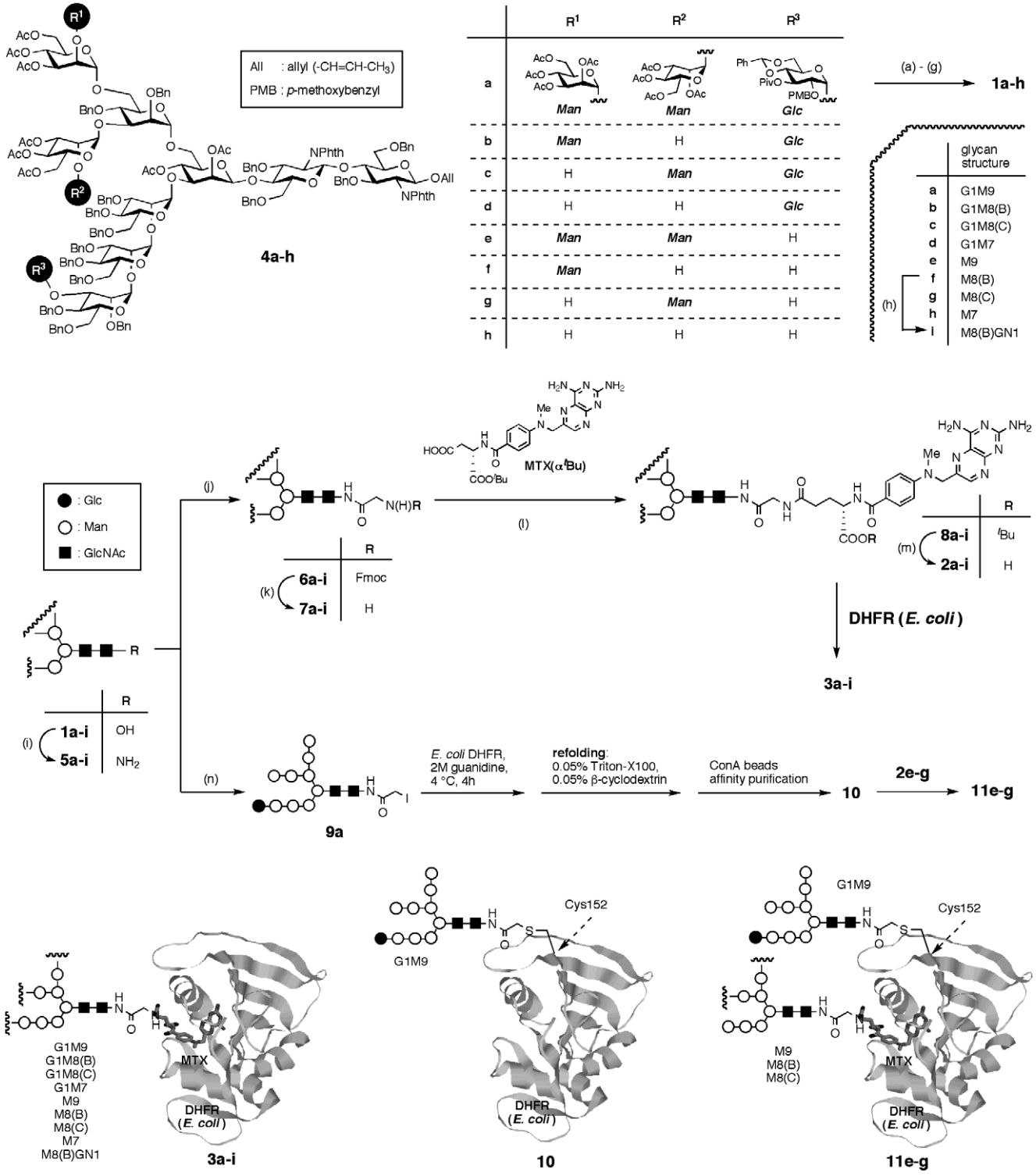
Besides their ability to form complexes with DHFR, compounds **2a**–**i** themselves have proven to be valuable as substrates for the specificity analysis of lectins and glycosyltransferase, because they carry a UV-detectable tag MTX, which has a specific absorption at 304 nm. For instance, **2e**–**h** were revealed to be excellent substrates of UDP-glucose: glycoprotein glucosyltransferase (UGGT)-mediated Glc-transfer reaction.[18] UGGT was known to have a highly unique feature, accepting Man9-7GlcNAc2, only when they are covalently bound to incompletely folded proteins.[19] Interestingly, compound **2i**, which lacks innermost GlcNAc, was not accepted as a substrate, indicating that the presence of chitobiose is essential for the recognition by UGGT.[18]

2.2. Generations of DHFR complexes

To evaluate the affinity of CHO–MTX conjugates to DHFR (*Escherichia coli*), inhibitory activities of **2a**–**i** were measured (Scheme 1 and Fig. 2A). Enzyme activities were measured based on the absorption change of coenzyme NADPH as reported.[20] Results indicated that their affinities to DHFR were nearly identical with MTX ($K_a \sim 10^{10}$ M^{-1}),[8] suggesting the formation of tight binding complexes **3a**–**i**. To gain an additional proof, interaction analysis between Man9GlcNAc2–MTX–DHFR **3e** and ConA was conducted by a pull-down assay. Thus, as shown in Figure 2B, the complex **3e** (lane 1) was retained on ConA-agarose (lanes 2 and 3), and was retrieved by eluting with a high concentration (1 M) of methyl mannoside–methyl glucoside combined elution buffer (lane 4), indicating that the glycan chain was immobilized on DHFR.

When artificial glycoproteins **3** were used as substrates of glycan processing enzymes, it was possible to monitor the transformation by HPLC after heat-induced inactivation of the enzyme. As an example, the HPLC profile of enzymatic Glc-trimming reaction by Glc'ase II[19] for the artificial glycoprotein **3a** is shown in Figure 3.

Subsequently, our attempt was directed to the covalent incorporation of glycan chains to DHFR. In this respect, we focused on a surface-exposed cystein (Cys) residue at the position 152. Reactions with iodoacetylated derivatives with Cys are well precedented in the modification of various proteins.[21] To incorporate G1M9, iodoacetamide **9a** (G1M9-IAc) was prepared from glycosylamine **5a**. Thus, crude **5a** was reacted with $(ICH_2CO)_2O$ in DMF in the presence of $NaHCO_3$ to provide **9a** in 74% yield from **1a**. Conjugation of **9a** with DHFR proceeded under denaturing conditions (2 M guanidine). That the dodecasaccharide (G1M9) was incorporated was proven by SDS–PAGE and MALDI-TOF-MS analysis (Figs. 4A; lane 2 and B; (b)). Then, the mixture was incubated under refolding conditions (Triton X-100, β-cyclodextrin),[22] and affinity chromatographic purification by ConA-agarose gave G1M9-grafted DHFR **10** in a homogeneous form



Scheme 1. Syntheses of the glycan–MTX probes (**2a**–**i**) and the corresponding artificial glycoproteins (**3a**–**i**, **10**, and **11e**–**g**). Reagents and conditions: (a) ethylenediamine, *n*-BuOH, 80 °C; (b) Ac_2O, pyridine, DMAP, ~94% (two steps); (c) $[Ir(COD)(PMePh_2)_2]PF_6$, H_2, THF; (d) I_2, $NaHCO_3$, H_2O–THF; (e) Ac_2O, pyridine, ~97% (three steps); (f) $Pd(OH)_2/C$, H_2, 60% AcOH–EtOH; (g) NaOMe, MeOH, ~89% (two steps); (h) 100 mM NaOH, 50 °C, 92%; (i) satd aq NH_4HCO_3, 40 °C; (j) FmocGlyCl, $NaHCO_3$, H_2O–dioxane, 0 °C; (k) piperidine, DMF, ~79% (three steps); (l) MTX(α'Bu), DMT-MM, H_2O–MeOH; (m) CF_3COOH, ~65% (two steps); (n) $[ICH_2CO]_2O$, $NaHCO_3$, DMF, 0 °C, 74% (two steps).

(Figs. 4A; lane 3 and B; (c)). The enzymatic activity of purified **10** was essentially identical with unmodified DHFR, implying that glycan modified DHFR **10** was refolded correctly. Furthermore, its activity was inhibited by a series of glycan–MTX conjugates **2e**–**g**, to the same extent as by MTX (Fig. 4C). These results imply that bis-glycosylated DHFR **11e**–**g** can be generated from **10** and **2a**–**g**.

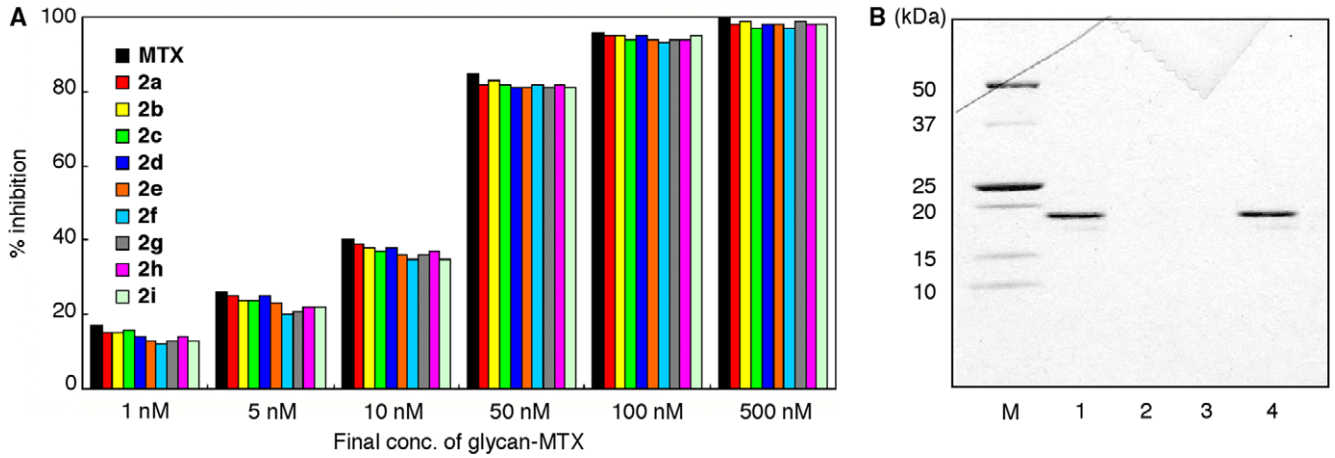


Figure 2. Formation of artificial glycoproteins **3a**–**i**. (A) DHFR inhibition by glycan–MTX (**2a**–**i**). (B) SDS–PAGE of lectine binding experiment of **3e**: lane 1, **3e** in 0.05% Tris–HCl (pH 7.5) (buffer A); lane 2, **3e** + ConA-Aga (flow through fraction); lane 3, washings. lane 4, elution fraction (eluent: 1.0 M methyl mannoside, 1.0 M methyl glucoside in buffer A).

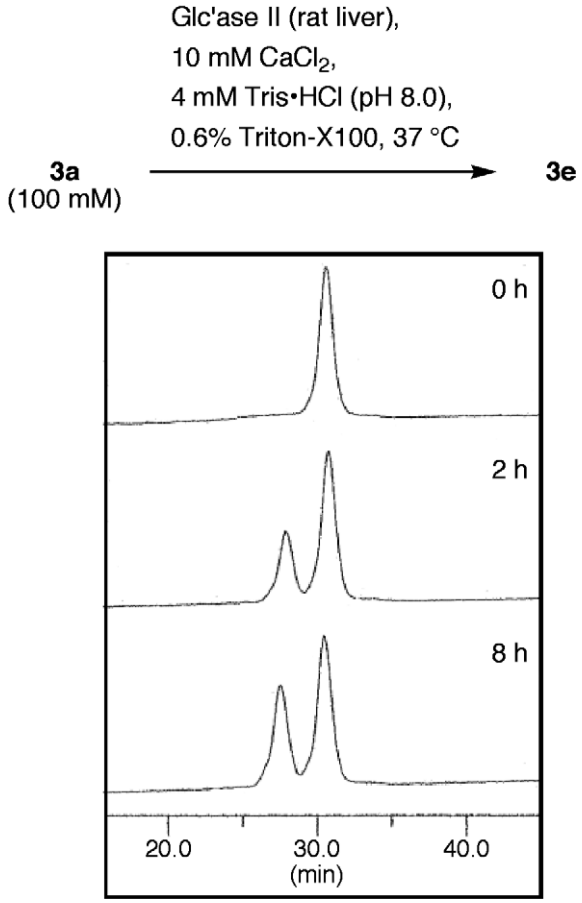


Figure 3. HPLC analysis of glc'ase II catalyzed trimming of **3a**.

3. Conclusion

Various high-mannose-type oligosaccharides in ER were synthesized and conjugated with MTX via a glycine linker. They were readily incorporated to DHFR by way of its strong affinity to MTX. The enzymatic glycan processing reaction of the artificial glycoproteins could be monitored directly by detecting released glycan–MTX using HPLC system. Moreover, we explored the strategy for the incorporation of two different high-mannose-type glycans to DHFR by the combination of glycan modification at Cys and the MTX-mediated ligand-based glycan incorporation. Both of CHO–MTX and CHO–IAc derivatives can be prepared from glycosylamines, preparation of which is well established, the strategy developed in this study can be readily applied to other types of glycans.

While a combination of biotinated glycan (CHO-Bio) and streptavidin (SA) has been investigated,[23] it would seem to have a difficulty controlling the reversible binding-release of the ligand (CHO-Bio), because of the extremely tight binding ($K_a \sim 10^{15}\ M^{-1}$) between Bio and SA.[24] In addition, the resulting CHO-Bio-SA exists as a tetramer, which may have a tendency to exaggerate the ability of oligosaccharide to interact with lectins or enzymes of interest.

A number of natural glycoproteins are known to have plural glycan chains. Therefore, variously glycosylated proteins developed would be effective for analysis of complicated biological system. Further studies are in progress along this line and will be reported in due course.

4. Experimental

4.1. General

Glc'ase II was obtained from rat liver, as described.[25] ^1H NMR spectra were recorded at 400 or 600 MHz in D_2O solution (JEOL AL-400 spectrometer, JEOL ECA-600 spectrometer). MALDI-TOF MS spectra were recorded in the positive ion mode on an AXIMA-CFR Kompact MALDI (Shimadzu/KRATOS). HPLC was performed on a Waters 2690 (separation module) and Waters 996 (photodiode array detector) with a TSK-GEL Amide-80 column (TOSOH). UV absorption was recorded on a SPECTRA MAX190 microplate reader (Molecular Devices).

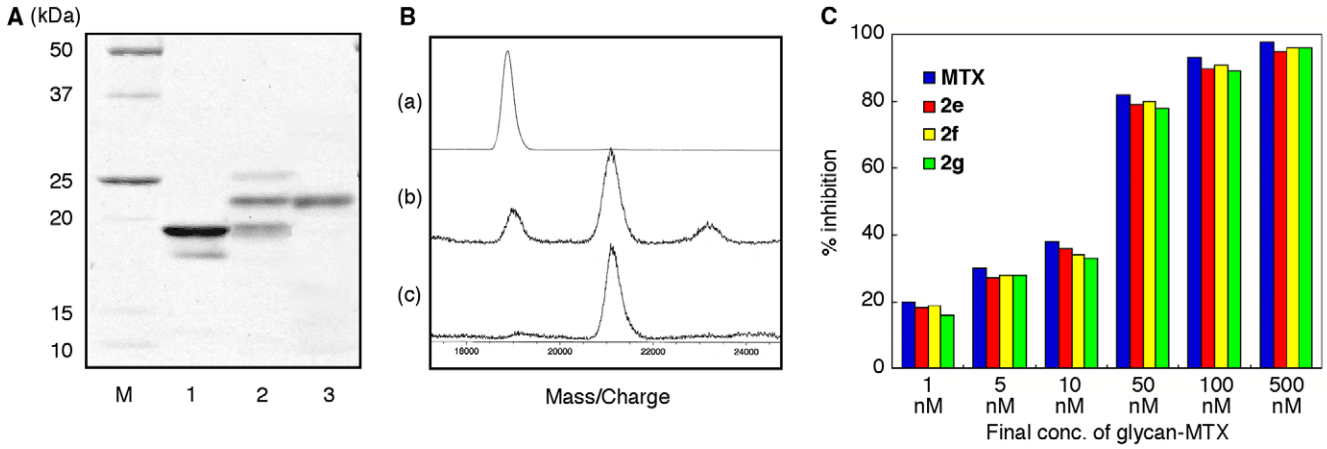


Figure 4. Formation of artificial glycoproteins carrying two glycan chains. (A) SDS–PAGE of synhthesis of **10**: lane 1, *Escherichia coli* DHFR; lane 2, reaction mixture; lane 3, after ConA-aga affinity purification. (B) MALDI-TOF MS of synthesis of **10**: (a) *E. coli* DHFR, (b) Reaction mixture, (c) After ConA-aga affinity purification. (C) G1M9-DHFR (**10**) inhibition by glycan–MTX (**2e**–**g**).

4.2. Synthesis of compound 1 (typical experimental procedure)

To a solution of **4a** (463 mg, 0.101 mmol) in *n*-BuOH (16 mL) was added ethylenediamine (4 mL). After being stirred at 80 °C for 22 h, the solvent was removed by concentration in vacuo and co-evaporated with toluene. The residue was dissolved in pyridine (10 mL), then Ac_2O (5 mL) and DMAP (1.2 mg, 0.010 mmol) were added at 0 °C. After being stirred at room temperature for 16 h, the mixture was quenched with MeOH at 0 °C and concentrated in vacuo. The residue was diluted with EtOAc (50 mL) and washed with aqueous Cu_5O_4 (25 mL), brine (25 mL), saturated aqueous $NaHCO_3$ (25 mL), and brine (25 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by chromatography on silica gel (EtOH/toluene, 1:10) to give N-acetylated compound. $[Ir(COD)(PMePh_2)_2]PF_6$ (8.8 mg, 10.1 mmol) was dissolved in degassed THF (5 mL) and stirred under H_2 atmosphere for 15 min then stirred under N_2 atmosphere for 1 min. A solution of the N-acetylated compound in THF (10 mL) was added at 0 °C and stirred under N_2 atmosphere at room temperature for 1.5 h. Then, water (2.5 mL), $NaHCO_3$ (339.7 mg, 4.044 mmol), and iodine (51.3 mg, 0.202 mmol) were added at 0 °C. After being stirred at room temperature for 15 min, the mixture was diluted with EtOAc (50 mL) and washed with saturated aqueous $Na_2S_2O_3{\cdot}5H_2O$ (25 mL) and brine (25 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was dissolved in pyridine (10 mL), then Ac_2O (5 mL) was added at 0 °C. After being stirred at room temperature for 12 h, the reaction mixture was quenched with MeOH at 0 °C and concentrated in vacuo. The residue was diluted with EtOAc (50 mL) and washed with aqueous Cu_5O_4 (25 mL), brine (25 mL), saturated aqueous $NaHCO_3$ (25 mL) and brine (25 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by chromatography on silica gel (EtOH/toluene, 1:20) to give 1-O-acetylated compound. To the 1-O-acetylated compound in 60% AcOH/EtOH (3:1) (20 mL) was added $Pd(OH)_2$ (20% on carbon, 1.63 g). The mixture was stirred under H_2 atmosphere for 14 h then filtered through a pad of Celite. The filtrate and washings were concentrated in vacuo. The residue was dissolved in MeOH (30 mL), and NaOMe (28% in MeOH, 300 μL) was added at 0 °C. After being stirred at room temperature for 27 h, the mixture was neutralized with Amberlyst 15E (H^+). The mixture was filtered and concentrated. The residue was purified by reverse-phase chromatography (Waters Sep-Pak® C_{18}, water) to give 144.1 mg (81%) of **1a** (α/β, 2:1)

Glc1Man9GlcNAc$_2$ (**1a**): TLC, R_f 0.34 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O, α-isomer) δ 1.89 (s, 3H), 1.92 (s, 3H), 3.25 (t, J = 9.3 Hz, 1H), 3.39–4.01 (m, 69H), 4.08 (br s, 2H), 4.45 (d, J = 7.4 Hz, 1H, H-1^{GlcNAc}), 4.56 (d, J = 3.1 Hz, 1H, H-1^{GlcNAc}), 4.72 (br s, 1H, H-1^{Man}), 4.88 (3br s, 3H, 3× H-1^{Man}), 4.91 (br s, 1H, H-1^{Man}), 4.99 (br s, 1H, H-1^{Man}), 5.11 (d, J = 3.7 Hz, 1H, H-1^{Glc}), 5.16 (br s, 1H, H-1^{Man}), 5.19 (br s, 1H, H-1^{Man}), 5.26 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{76}H_{128}N_2O_{61}Na$ $(M+Na)^+$ *m/z*: 2067.7, found: 2067.9.

Glc1Man8(B)GlcNAc2 (**1b**): TLC, R_f 0.36 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O, α-isomer) δ 1.91 (s, 3H), 1.95 (s, 3H), 3.28 (t, J = 9.3 Hz, 1H), 3.42–3.99 (m, 54H), 4.01 (br s, 1H), 4.11 (br s, 1H), 4.45 (d, J = 7.3 Hz, 1H, H-1^{GlcNAc}), 4.59 (d, J = 3.0 Hz, 1H, GlcNAc), 4.75 (br s, 1H, H-1^{Man}), 4.93 (2br s, 2H, 2× H-1^{Man}), 4.94 (br s, 1H, H-1^{Man}), 5.00 (br s, 1H, H-1^{Man}), 5.06 (br s, 1H, H-1^{Man}), 5.10 (d, J = 3.6 Hz, 1H, H-1 Glc), 5.18 (br s, 1H, H-1^{Man}), 5.20 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{70}H_{118}N_2O_{56}Na$ $(M+Na)^+$ *m/z*: 1906.7, found: 1906.8.

Glc1Man8(C)GlcNAc2 (**1c**): TLC, R_f 0.36 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O, α-isomer) δ 1.92 (s, 3H), 1.94 (s, 3H), 3.26 (t, J = 9.4 Hz, 1H), 3.42–4.03 (m, 63H), 4.11 (br s, 2H), 4.48 (d, J = 7.3 Hz, 1H, H-1^{GlcNAc}), 4.58 (d, J = 3.2 Hz, 1H, H-1^{GlcNAc}), 4.75 (br s, 1H, H-1^{Man}), 4.78 (br s, 1H, H-

1^{Man}), 4.92 (br s, 1H, H-1^{Man}), 4.93 (br s, 1H, H-1^{Man}), 5.07 (br s, 1H, H-1^{Man}), 5.13 (d, J = 3.2 Hz, 1H, H-1^{Glc}), 5.18 (br s, 1H, H-1^{Man}), 5.21 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{70}H_{118}N_2O_{56}Na$ $(M+Na)^+$ m/z: 1906.7, found: 1906.4.

Glc1Man7GlcNAc2 (**1d**): TLC, R_f 0.34 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O, α-isomer) δ 1.93 (s, 3H), 1.95 (s, 3H), 3.27 (t, J = 9.4 Hz, 1H), 3.50–3.99 (m, 48H), 4.02 (br s, 1H), 4.11 (br s, 1H), 4.49 (d, J = 7.4 Hz, 1H, H-1^{GlcNAc}), 4.60 (d, J = 3.2 Hz, 1H, H-1^{GlcNAc}), 4.74 (br s, 1H, H-1^{Man}), 4.80 (br s, 1H, H-1^{Man}), 4.92 (br s, 1H, H-1^{Man}), 4.98 (br s, 1H, H-1^{Man}), 5.08 (br s, 1H, H-1^{Man}), 5.10 (d, J = 3.0 Hz, 1H, H-1^{Glc}), 5.19 (br s, 1H, H-1^{Man}), 5.24 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{64}H_{108}N_2O_{51}Na$ $(M+Na)^+$ m/z: 1743.6, found: 1743.6.

Man9GlcNAc2 (**1e**): TLC, R_f 0.34 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O, α-isomer) δ 2.04 (s, 3H), 2.07 (s, 3H), 3.60–4.10 (m, 64H), 4.15 (br s, 1H), 4.23 (br s, 1H), 4.59 (d, J = 7.2 Hz, 1H, H-1^{GlcNAc}), 4.79 (d, J = 3.1 Hz, 1H, H-1^{GlcNAc}), 4.86 (br s, 1H, H-1^{Man}), 5.04 (2br s, 2H, 2× H-1^{Man}), 5.05 (br s, 1H, H-1^{Man}), 5.14 (br s, 1H, H-1^{Man}), 5.18 (br s, 1H, H-1^{Man}), 5.30 (br s, 1H, H-1^{Man}), 5.33 (br s, 1H, H-1^{Man}), 5.40 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{70}H_{118}N_2O_{56}Na$ $(M+Na)^+$ m/z: 1906.7, found: 1906.6.

Man8(B)GlcNAc2 (**1f**): TLC, R_f 0.35 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O, α-isomer) δ 1.92 (s, 3H), 1.94 (s, 3H), 3.51–3.98 (m, 58H), 4.02 (br s, 1H), 4.11 (br s, 1H), 4.47 (d, J = 7.3 Hz, 1H, H-1^{Man}), 4.65 (d, J = 3.2 Hz, 1H, H-1^{Man}), 4.75 (br s, 1H, H-1^{Man}), 4.92 (2br s, 2H, 2× H-1^{Man}), 4.96 (br s, 1H, H-1^{Man}), 5.02 (br s, 1H, H-1^{Man}), 5.06 (br s, 1H, H-1^{Man}), 5.18 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{64}H_{108}N_2O_{51}Na$ $(M+Na)^+$ m/z: 1743.6, found: 1743.8.

Man8(C)GlcNAc2 (**1g**): TLC, R_f 0.35 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O, α-isomer) δ 1.92 (s, 3H), 1.95 (s, 3H), 3.50–3.98 (m, 58H), 4.03 (br s, 1H), 4.11 (br s, 1H), 4.48 (d, J = 7.1 Hz, 1H, H-1^{GlcNAc}), 4.60 (d, J = 3.1 Hz, 1H, H-1^{GlcNAc}), 4.75 (br s, 1H, H-1^{Man}), 4.79 (br s, 1H, H-1^{Man}), 4.93 (br s, 1H, H-1^{Man}), 4.94 (br s, 1H, H-1^{Man}), 5.07 (br s, 1H, H-1^{Man}), 5.18 (br s, 1H, H-1^{Man}), 5.21 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{64}H_{108}N_2O_{51}Na$ $(M+Na)^+$ m/z: 1743.6, found: 1743.4.

Man7GlcNAc2 (**1h**): TLC, R_f 0.36 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O, α-isomer) δ 1.93 (s, 3H), 1.96 (s, 3H), 3.53–3.99 (m, 52H), 4.03 (br s, 1H), 4.12 (br s, 1H), 4.49 (d, J = 7.2 Hz, 1H, H-1^{GlcNAc}), 4.65 (d, J = 3.0 Hz, 1H, H-1^{GlcNAc}), 4.76 (br s, 1H, H-1^{Man}), 4.80 (br s, 1H, H-1^{Man}), 4.93 (br s, 1H, H-1^{Man}), 4.98 (br s, 1H, H-1^{Man}), 5.08 (br s, 1H, H-1^{Man}), 5.19 (br s, 1H, H-1^{Man}), 5.23 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{58}H_{98}N_2O_{46}Na$ $(M+Na)^+$ m/z: 1581.5, found: 1581.4.

4.3. Synthesis of Man8(B)GlcNAc1 (1i)

Compound **1f** (5 mg) was treated with 100 mM aqueous NaOH (10 mL). After being stirred at 50 °C for 48 h, the mixture was neutralized with Amberlyst 15E (H^+). The mixture was filtered and concentrated. The residue was purified by reverse phase chromatography (Waters Sep-Pak® C_{18}, water) to give 4.1 mg (92%) of **1i** (α/β, 2:1). TLC, R_f 0.38 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O, α-isomer) δ 1.91 (s, 3H), 3.47–3.97 (m, 52H), 4.01 (br s, 1H), 4.10 (br s, 1H), 4.66 (d, J = 3.1 Hz, 1H, H-1^{GlcNAc}), 4.73 (br s, 1H, H-1^{Man}), 4.90 (2s, 2H, 2× H-1^{Man}), 4.95 (br s, 1H, H-1^{Man}), 5.01 (br s, 1H, H-1^{Man}), 5.11 (br s, 1H, H-1^{Man}), 5.17 (br s, 1H, H-1^{Man}), 5.20 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{56}H_{95}NO_{46}Na$ $(M+Na)^+$ m/z: 1540.5, found: 1540.6.

4.4. Synthesis of compound 7 (typical experimental procedure)

Compound **1a** (26.1 mg, 0.0128 mmol) was dissolved in saturated aqueous NH_4HCO_3 (2 mL) and stirred at 40 °C for 48 h then the mixture was concentrated and co-evaporated with H_2O in vacuo. The residue was dissolved in dioxane/H_2O (1:1) (2 mL) then $NaHCO_3$ (5.1 mg, 0.061 mmol) and FmocGlyCl (9.7 mg, 0.031 mmol) were added at 0 °C. The mixture was stirred at 0 °C for 1 h then $NaHCO_3$ (2.6 mg, 0.031 mmol) and FmocGlyCl (4.9 mg, 0.016 mmol) were added. After being stirred at 0 °C for 1 h, the mixture was diluted with water (10 mL) and washed with EtOAc (5× 5 mL). The aqueous layer was concentrated, and the residue was purified by reverse phase chromatography (Waters Sep-Pak® C_{18}, water for elution of recoverd **1a** then water/MeOH (1:1) for elution of FmocGly-linked saccharide **6a**) to give mixture of FmocGly-linked saccharide (**6a**) and excess FmocGly-derivatives. The mixture was dissolved in DMF (2 mL), and piperidine (0.4 mL) was added at 0 °C. After being stirred at room temperature for 4 h, the mixture was concentrated in vacuo. The residue was diluted with water (10 mL) and washed with EtOAc (5× 5 mL). The aqueous layer was concentrated in vacuo, and the residue was purified by gel filteration (Sephadex G15, water) to give 21.3 mg (79%) of **7a**.

Glc1Man9GlcNAc2-Gly (**7a**): TLC, R_f 0.11 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.89 (s, 3H), 1.95 (s, 3H), 3.29 (t, J = 9.3 Hz, 1H), 3.42–4.04 (m, 77H), 4.11 (br s, 2H), 4.49 (d, J = 7.1 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.75 (br s, 1H, H-1^{Man}), 4.92 (2br s, 2H, 2× H-1^{Man}), 4.94 (br s, 1H, H-1^{Man}), 4.98 (d, J = 9.8 Hz, 1H, H-1^{GlcNAc}), 5.02 (br s, 1H, H-1^{Man}), 5.14 (d, J = 3.7 Hz, 1H, H-1^{Glc}), 5.19 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}); ^{13}C NMR (100 MHz, D_2O) δ 22.63, 22.85, 55.45, 2× 61.30, 2× 61.50, 2× 61.64, 2× 61.73, 4× 61.75, 4× 67.48, 3× 70.30, 2× 70.36, 6× 70.57, 3× 70.68, 3× 70.86, 5× 70.92, 2× 72.38, 2× 72.92, 3× 73.42, 3× 73.77, 6× 73.89, 2× 78.94, 5× 79.20, 3× 100.95, 3× 101.17, 101.64, 4× 102.78, 3× 181.89; MS (MALDI-TOF) calcd for $C_{78}H_{132}N_4O_{61}Na$ $(M+Na)^+$ m/z: 2123.7, found: 2123.8.

Glc1Man8(B)GlcNAc2-Gly (**7b**): TLC, R_f 0.13 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.88 (s, 3H), 1.94 (s, 3H), 3.27 (t, J = 9.4 Hz, 1H), 3.43–4.05 (m, 71H), 4.11 (br s, 2H), 4.47 (d, J = 7.3 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.74 (br s, 1H, H-1^{Man}), 4.91 (2br s, 2H, 2× H-1^{Man}), 4.96 (br s, 1H, H-1^{Man}), 5.00 (d, J = 10.0 Hz, 1H, H-1^{GlcNAc}), 5.02 (br s, 1H, H-1^{Man}), 5.13 (d, J = 3.9 Hz, 1H, H-1^{Glc}), 5.18 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{72}H_{122}N_4O_{56}Na$ $(M+Na)^+$ *m*/*z*: 1961.7, found: 1961.4.

Glc1Man8(C)GlcNAc2-Gly (**7c**): TLC, R_f 0.13 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.88 (s, 3H), 1.94 (s, 3H), 3.27 (t, J = 9.7 Hz, 1H), 3.42–3.98 (m, 70H), 4.02 (br s, 1H), 4.10 (br s, 2H), 4.49 (d, J = 7.2 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.74 (br s, 1H, H-1^{Man}), 4.78 (br s, 1H, H-1^{Man}), 4.91 (br s, 1H, H-1^{Man}), 4.93 (br s, 1H, H-1^{Man}), 4.99 (d, J = 9.3 Hz, 1H, H-1^{GlcNAc}), 5.12 (d, J = 3.6 Hz, 1H, H-1^{Glc}), 5.18 (br s, 1H, H-1^{Man}), 5.21 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{72}H_{122}N_4O_{56}Na$ $(M+Na)^+$ *m*/*z*: 1961.7, found: 1961.9.

Glc1Man7GlcNAc2-Gly (**7d**): TLC, R_f 0.11 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.89 (s, 3H), 1.95 (s, 3H), 3.27 (t, J = 9.6 Hz, 1H), 3.49–4.01 (m, 59H), 4.03 (br s, 1H), 4.13 (br s, 1H), 4.52 (d, J = 7.4 Hz, 1H, H-1^{GlcNAc}), 4.69 (br s, 1H, H-1^{Man}), 4.78 (br s, 1H, H-1^{Man}), 4.82 (br s, 1H, H-1^{Man}), 4.96 (br s, 1H, H-1^{Man}), 5.02 (br s, 1H, H-1^{Man}), 5.03 (d, J = 9.0 Hz, 1H, H-1^{GlcNAc}), 5.19 (d, J = 3.8 Hz, 1H, H-1^{Glc}), 5.23 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{66}H_{112}N_4O_{51}Na$ $(M+Na)^+$ *m*/*z*: 1799.6, found: 1799.7.

Man9GlcNAc2-Gly (**7e**): TLC, R_f 0.11 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.89 (s, 3H), 1.95 (s, 3H), 3.49–3.98 (m, 66H), 4.04 (br s, 1H), 4.11 (br s, 1H), 4.49 (d, J = 7.1 Hz, 1H, H-1^{GlcNAc}), 4.68 (br s, 1H, H-1^{Man}), 4.75 (br s, 1H, H-1^{Man}), 4.92 (2br s, 2H, 2× H-1^{Man}), 4.94 (br s, 1H, H-1^{Man}), 4.99 (d, J = 9.5 Hz, 1H, H-1^{GlcNAc}), 5.02 (br s, 1H, H-1^{Man}), 5.19 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}); ^{13}C NMR (100 MHz, D_2O) δ 22.65, 22.85, 54.33, 60.64, 5× 61.66, 4× 61.76, 66.20, 3× 67.53, 70.21, 7× 70.58, 2× 70.68, 7× 70.93, 71.82, 2× 73.28, 3× 73.77, 2× 73.86, 3× 73.89, 74.79, 75.18, 2× 76.82, 78.95, 2× 79.05, 3× 79.22, 79.32, 79.45, 79.55, 80.32, 98.59, 4× 101.16, 101.91, 4× 102.79, 174.93, 175.25, 181.89; MS (MALDI-TOF) calcd for $C_{72}H_{122}N_4O_{56}Na$ $(M+Na)^+$ *m*/*z*: 1961.7, found: 1961.6.

Man8(B)GlcNAc2-Gly (**7f**): TLC, R_f 0.12 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.87 (s, 3H), 1.93 (s, 3H), 3.42–3.96 (m, 60H), 4.01 (br s, 1H), 4.10 (br s, 1H), 4.46 (d, J = 7.2 Hz, 1H, H-1^{GlcNAc}), 4.68 (br s, 1H, H-1^{Man}), 4.73 (br s, 1H, H-1^{Man}), 4.91 (2br s, 2H, 2× H-1^{Man}), 4.95 (br s, 1H, H-1^{Man}), 4.97 (d, J = 9.8 Hz, 1H, H-1^{GlcNAc}), 5.01 (br s, 1H, H-1^{Man}), 5.17 (br s, 1H, H-1^{Man}), 5.20 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{66}H_{112}N_4O_{51}Na$ $(M+Na)^+$ *m*/*z*: 1799.6, found: 1799.4.

Man8(C)GlcNAc2-Gly (**7g**): TLC, R_f 0.12 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.87 (s, 3H), 1.94 (s, 3H), 3.40–3.97 (m, 60H), 4.02 (br s, 1H), 4.10 (br s, 1H), 4.48 (d, J = 6.8 Hz, 1H, H-1^{GlcNAc}), 4.68 (br s, 1H, H-1^{Man}), 4.74 (br s, 1H, H-1^{Man}), 4.78 (br s, 1H, H-1^{Man}), 4.92 (2br s, 2H, 2× H-1^{Man}), 4.95 (d, J = 9.8 Hz, 1H, H-1^{GlcNAc}), 5.17 (br s, 1H, H-1^{Man}), 5.20 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{66}H_{112}N_4O_{51}Na$ $(M+Na)^+$ *m*/*z*: 1799.6, found: 1800.0.

Man7GlcNAc2-Gly (**7h**): TLC, R_f 0.13 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.93 (s, 3H), 1.96 (s, 3H), 3.54–4.03 (m, 54H), 4.07 (br s, 1H), 4.15 (br s, 1H), 4.53 (d, J = 7.6 Hz, 1H, H-1^{GlcNAc}), 4.69 (br s, 1H, H-1^{Man}), 4.80 (br s, 1H, H-1^{Man}), 4.83 (br s, 1H, H-1^{Man}), 4.97 (br s, 1H, H-1^{Man}), 5.02 (br s, 1H, H-1^{Man}), 5.03 (d, J = 8.2 Hz, 1H, H-1^{GlcNAc}), 5.23 (br s, 1H, H-1^{Man}), 5.26 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{60}H_{102}N_4O_{46}Na$ $(M+Na)^+$ *m*/*z*: 1637.6, found: 1637.3.

Man8(B)GlcNAc1-Gly (**7i**): TLC, R_f 0.13 (water/*i*-PrOH, 1:2); ^{1}H NMR (400 MHz, D_2O) δ 1.90 (s, 3H), 1.93 (s, 3H), 3.40–3.93 (m, 51H), 4.01 (br s, 1H), 4.10 (br s, 1H), 4.69 (br s, 1H, H-1^{Man}), 4.72 (br s, 1H, H-1^{Man}), 4.91 (2br s, 2H, 2× H-1^{Man}), 4.94 (br s, 1H, H-1^{Man}), 4.97 (d, J = 9.7 Hz, 1H, H-1^{GlcNAc}), 4.99 (br s, 1H, H-1^{Man}), 5.17 (br s, 1H, H-1^{Man}), 5.21 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{58}H_{99}N_3O_{46}Na$ $(M+Na)^+$ *m*/*z*: 1596.5, found: 1596.3.

4.5. Synthesis of 2 (typical experimental procedure)

To a cold (−0 °C) solution of **7a** (21.3 mg, 10.1 μmol) and MTX(α^tBu)[7] (6.2 mg, 12 μmol) in water/MeOH (1:1) (1 mL) was added DMT-MM[17] (3.4 mg, 12.2 μmol). The mixture was stirred at room temperature for 12 h, then concentrated in vacuo. The residue was purified by reverse phase chromatography (Waters Sep-Pak® C_{18}, water/MeOH, 1:0–1:1) to give $Glc_1Man9GlcNAc_2$-MTX(α^tBu). $Glc_1Man9GlcNAc_2$-MTX(α^tBu) was dissolved in CF_3COOH (1 mL) at 0 °C. After being stirred at room temperature for 1 h, the mixture was concentrated and co-evaporated with toluene in vacuo. The residue was purified by HPLC (TSK-GEL Amide-80, 40 °C, 3% AcOH–Et_3N aq (pH 7.3)/CH_3CN, 35:65–50:50) to give 17.1 mg (65%) of **2a**.

Glc1Man9GlcNAc2-MTX (**2a**): TLC, R_f 0.42 (water/*i*-PrOH, 1:2); ^{1}H NMR (600 MHz, D_2O) δ 1.78 (s, 3H), 1.88–1.99 (m, 1H), 1.92 (s, 3H), 2.06–2.14 (m, 1H), 2.21–2.37 (m, 2H), 3.07 (s, 3H), 3.28 (t, J = 9.3 Hz, 1H), 3.41–3.98 (m, 74H), 4.10 (br s, 2H), 4.23 (br s, 1H), 4.45 (d, J = 8.2 Hz, 1H, H-1^{GlcNAc}), 4.63 (br s, 1H, H-1^{Man}), 4.73 (br s, 1H, H-1^{Man}), 4.86 (d, J = 9.9 Hz, 1H, H-1^{GlcNAc}), 4.90 (2br s, 2H, 2× H-1^{Man}), 4.92 (br s, 1H, H-1^{Man}), 5.01 (br s, 1H, H-1^{Man}), 5.12 (d, J = 3.8 Hz, 1H, H-1^{Glc}), 5.18 (br s, 1H,

H-1^{Man}), 5.20 (br s, 1H, H-1^{Man}), 5.27 (br s, 1H, H-1^{Man}), 6.75 (d, J = 8.2 Hz, 2H), 7.55 (d, J = 8.2 Hz, 2H), 8.46 (br s, 1H); MS (MALDI-TOF) calcd for $C_{98}H_{152}N_{12}O_{65}Na$ $(M+Na)^+$ m/z: 2559.9, found: 2559.6.

Glc1Man8(B)GlcNAc2-MTX (**2b**): TLC, R_f 0.60 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.80 (s, 3H), 1.87–2.00 (m, 1H), 1.93 (s, 3H), 2.05–2.15 (m, 1H), 2.22–2.37 (m, 2H), 2.99 (s, 3H), 3.27 (t, J = 9.3 Hz, 1H), 3.42–4.02 (m, 69H), 4.11 (br s, 1H), 4.23 (br s, 1H), 4.44 (d, J = 8.1 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.74 (br s, 1H, H-1^{Man}), 4.87 (d, J = 9.5 Hz, 1H, H-1^{GlcNAc}), 4.91 (2br s, 2H, 2× H-1^{Man}), 4.96 (br s, 1H, H-1^{Man}), 5.02 (br s, 1H, H-1^{Man}), 5.13 (d, J = 3.7 Hz, 1H, H-1^{Glc}), 5.18 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}), 6.57 (d, J = 8.2 Hz, 2H), 7.44 (d, J = 8.2 Hz, 2H), 8.38 (br s, 1H); MS (MALDI-TOF) calcd for $C_{92}H_{142}N_{12}O_{60}Na$ $(M+Na)^+$ m/z: 2397.8, found: 2398.0.

Glc1Man8(C)GlcNAc2-MTX (**2c**): TLC, R_f 0.60 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.79 (s, 3H), 1.85-2.00 (m, 1H), 1.93 (s, 3H), 2.03–2.15 (m, 1H), 2.20–2.37 (m, 2H), 3.02 (s, 3H), 3.27 (t, J = 9.5 Hz, 1H), 3.42–4.02 (m, 69H), 4.10 (br s, 1H), 4.24 (br s, 1H), 4.44 (d, J = 7.2 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.77 (br s, 1H, H-1^{Man}), 4.87 (d, J = 9.5 Hz, 1H, H-1^{GlcNAc}), 4.91 (2br s, 2H, 2× H-1^{Man}), 4.93 (br s, 1H, H-1^{Man}), 5.13 (d, J = 3.7 Hz, 1H, H-1^{Glc}), 5.18 (br s, 1H, H-1^{Man}), 5.21 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}), 6.68 (d, J = 8.1 Hz, 2H), 7.51 (d, J = 8.1 Hz, 2H), 8.43 (br s, 1H); MS (MALDI-TOF) calcd for $C_{92}H_{142}N_{12}O_{60}Na$ $(M+Na)^+$ m/z: 2397.8, found: 2398.2.

Glc1Man7GlcNAc2-MTX (**2d**): TLC, R_f 0.65 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.79 (s, 3H), 1.86–2.00 (m, 1H), 1.93 (s, 3H), 2.03–2.15 (m, 1H), 2.20–2.37 (m, 2H), 3.08 (s, 3H), 3.27 (t, J = 9.4 Hz, 1H), 3.42–4.01 (m, 63H), 4.10 (s, 1H), 4.25 (br s, 1H), 4.44 (d, J = 7.1 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.78 (br s, 1H, H-1^{Man}), 4.87 (d, J = 9.5 Hz, 1H, H-1^{GlcNAc}), 4.90 (2br s, 2H, 2× H-1^{Man}), 4.96 (br s, 1H, H-1^{Man}), 5.12 (d, J = 3.6 Hz, 1H, H-1^{Glc}), 5.17 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}), 6.79 (d, J = 8.4 Hz, 2H), 7.58 (d, J = 8.4 Hz, 2H), 8.49 (br s, 1H); MS (MALDI-TOF) calcd for $C_{86}H_{132}N_{12}O_{55}Na$ $(M+Na)^+$ m/z: 2235.8, found: 2235.7.

Man9GlcNAc2-MTX (**2e**): TLC, R_f 0.47 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.82 (s, 3H), 1.90–2.02 (m, 1H), 1.94 (s, 3H), 2.06–2.16 (m, 1H), 2.23–2.38 (m, 2H), 3.07 (s, 3H), 3.48–3.98 (m, 69H), 4.04 (s, 1H), 4.11 (br s, 1H), 4.26 (m, 1H), 4.46 (d, J = 7.8 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.75 (br s, 1H, H-1^{Man}), 4.88 (d, J = 9.3 Hz, 1H, H-1^{GlcNAc}), 4.93 (2br s, 2H, 2× H-1^{Man}), 4.94 (br s, 1H, H-1^{Man}), 5.02 (br s, 1H, H-1^{Man}), 5.19 (br s, 1H, H-1^{Man}), 5.21 (br s, 1H, H-1^{Man}), 5.28 (br s, 1H, H-1^{Man}), 6.77 (d, J = 8.9 Hz, 2H), 7.57 (d, J = 8.9 Hz, 2H), 8.47 (br s, 1H); MS (MALDI-TOF) calcd for $C_{92}H_{142}N_{12}O_{60}Na$ $(M+Na)^+$ m/z: 2397.8, found: 2397.4.

Man8(B)GlcNAc2-MTX (**2f**): TLC, R_f 0.67 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.80 (s, 3H), 1.87–2.01 (m, 1H), 1.94 (s, 3H), 2.07–2.17 (m, 1H), 2.24–2.38 (m, 2H), 3.06 (s, 3H), 3.40–3.99 (m, 63H), 4.03 (s, 1H), 4.11 (s, 1H), 4.27 (br s, 1H), 4.45 (d, J = 7.2 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.75 (br s, 1H, H-1^{Man}), 4.88 (d, J = 9.3 Hz, 1H, H-1^{GlcNAc}), 4.93 (2br s, 2H, 2× H-1^{Man}), 4.97 (br s, 1H, H-1^{Man}), 5.03 (br s, 1H, H-1^{Man}), 5.19 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}), 6.76 (d, J = 8.3 Hz, 2H), 7.57 (d, J = 8.3 Hz, 2H), 8.47 (br s, 1H); MS (MALDI-TOF) calcd for $C_{86}H_{132}N_{12}O_{55}Na$ $(M+Na)^+$ m/z: 2235.8, found: 2235.3.

Man8(C)GlcNAc2-MTX (**2g**): TLC, R_f 0.66 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.79 (s, 3H), 1.86–2.01 (m, 1H), 1.94 (s, 3H), 2.03–2.17 (m, 1H), 2.22–2.37 (m, 2H), 3.09 (s, 3H), 3.43–4.03 (m, 64H), 4.11 (s, 1H), 4.27 (br s, 1H), 4.47 (d, J = 7.1 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.78 (br s, 1H, H-1^{Man}), 4.88 (d, J = 9.0 Hz, 1H, H-1^{GlcNAc}), 4.93 (3br s, 3H, 3× H-1^{Man}), 5.19 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}), 5.29 (br s, 1H, H-1^{Man}), 6.80 (d, J = 8.6 Hz, 2H), 7.59 (d, J = 8.6 Hz, 2H), 8.50 (br s, 1H); MS (MALDI-TOF) calcd for $C_{86}H_{132}N_{12}O_{55}Na$ $(M+Na)^+$ m/z: 2235.8, found: 2235.7.

Man7GlcNAc2-MTX (**2h**): TLC, R_f 0.70 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.77 (s, 3H), 1.87–2.00 (m, 1H), 1.92 (s, 3H), 2.03–2.15 (m, 1H), 2.22–2.37 (m, 2H), 3.05 (s, 3H), 3.37–3.96 (m, 57H), 4.00 (s, 1H), 4.09 (s, 1H), 4.24 (br s, 1H), 4.44 (d, J = 7.2 Hz, 1H, H-1^{GlcNAc}), 4.62 (br s, 1H, H-1^{Man}), 4.76 (br s, 1H, H-1^{Man}), 4.86 (d, J = 9.8 Hz, 1H, H-1^{GlcNAc}), 4.90 (2br s, 2H, 2× H-1^{Man}), 4.95 (br s, 1H, H-1^{Man}), 5.16 (br s, 1H, H-1^{Man}), 5.20 (br s, 1H, H-1^{Man}), 6.76 (d, J = 8.2 Hz, 2H), 7.55 (d, J = 8.2 Hz, 2H), 8.46 (br s, 1H); MS (MALDI-TOF) calcd for $C_{80}H_{122}N_{12}O_{50}Na$ $(M+Na)^+$ m/z: 2073.7, found: 2073.4.

Man8(B)GlcNAc1-MTX (**2i**): TLC, R_f 0.71 (water/*i*-PrOH, 1:2); ^1H NMR (400 MHz, D_2O) δ 1.80 (s, 3H), 1.87–2.02 (m, 1H), 2.06–2.17 (m, 1H), 2.23–2.38 (m, 2H), 3.11 (s, 3H), 3.49–4.02 (m, 58H), 4.12 (s, 1H), 4.26 (br s, 1H), 4.62 (br s, 1H, H-1^{Man}), 4.74 (br s, 1H, H-1^{Man}), 4.89 (d, J = 8.8 Hz, 1H, H-1^{GlcNAc}), 4.93 (2br s, 2H, 2× H-1^{Man}), 4.96 (br s, 1H, H-1^{Man}), 5.02 (br s, 1H, H-1^{Man}), 5.19 (br s, 1H, H-1^{Man}), 5.22 (br s, 1H, H-1^{Man}), 6.73 (d, J = 7.8 Hz, 2H), 7.55 (d, J = 7.8 Hz, 2H), 8.46 (br s, 1H); MS (MALDI-TOF) calcd for $C_{79}H_{119}N_{11}O_{50}Na$ $(M+Na)^+$ m/z: 2032.7, found: 2233.0.

4.6. Generation of *Escherichia coli* DHFR

A 1087 bp *Bam*HI–*Hind*III fragment, corresponding to the complete coding sequence of the *E. coli* DHFR, of pEU-DHFR plasmid was inserted into the *E. coli* expression plasmid pET-42a (Novagen) and named pET-42-DHFR to express GST-DHFR. GST-DHFR was expressed and purified from *E. coli* essentially according to the manufacturer's instructions. The GST-tag of the fusion protein was cleaved and purified

by Factor Xa cleavage capture kit (Novagen) to obtain *E. coli* DHFR. The protein concentration was estimated by UV absorption at 280 nm (ε_M = 27,960, calculated as reported).[24]

4.7. Enzymatic glucose trimming reaction of 3a

The reaction mixture contained, in a total volume of 100 μL, 10 mM $CaCl_2$, soluble rat liver glc'ase II, 4 mM Tris–HCl buffer, pH 8.0, 0.6 mM deoxymannojirimycin, and substrate, **3a** (prepared by treating **2a** with equimolar amount of *E. coli* DHFR and following pre-incubation at 37 °C for 10 min) (100 μM). After 0.25–24 h at 37 °C, the reactions were stopped by heating at 100 °C for 5 min. The mixtures were separated by HPLC [using a TSK-GEL Amide-80 (4.6 mm ∅ × 25 cm) column with 3% AcOH–Et_3N (pH 7.3)/CH_3CN mixed solvent (35:65–50:50, linear gradient for 50 min) at 40 °C, 1 mL/min] and detected by absorption at 304 nm.

4.8. DHFR inhibition assay by glycan–MTX (2a–i)

Dihydrofolic acid (FH2) solution [FH2; 0.234 mM, 2-mercaptoethanol; 86.4 mM in buffer A (0.05 M Tris–HCl, pH 7.5)] (130 μL), MTX derivatives (**2a**–**i** and MTX) [0.01–5 μM in buffer A] (20 μL), and NADPH/DHFR [NADPH; 0.370 mM, DHFR; 0.4 μM in buffer A] (50 μL) were added to each well of the 96-well titerplate. The absorbance was read in the microplate reader at room temperature at wavelength of 340 nm, using the kinetic mode with a reading interval of 20 s for 18 min. Each percentage inhibition was calculated from slope of the absorbance change.

4.9. Lectin binding experiment of 3e

Compound **2e** (1.36 nmol) was treated with *E. coli* DHFR (1.36 nmol) in 0.05 M Tris–HCl (pH 7.5) (250 μL). After being incubated at 37 °C for 10 min (SDS–PAGE, lane 1) in a plastic tube equipped with membrane filter (Millipore: Ultrafree-MC, 0.22 μm) to form **3e**, it was captured by treatment with ConA-Aga (Seikagaku Co.) (12.3 mg/mL gel; 500 μL) and separated by spin-down filtration (SDS–PAGE, lane 2). The gel was washed with 0.05 M Tris–HCl (pH 7.5) (500 μL) and separated by spin-down filtration (SDS–PAGE, lane 3). The gel binding **3e** was eluted with elution buffer (1 M methyl α-D-mannoside and 1 M methyl α-D-glucoside in 0.05 M Tris–HCl (pH 7.5), 500 μL) (SDS–PAGE, lane 4).

4.10. Synthesis of Glc1Man9GlcNAc2-IAc (9a)

Compound **1a** (20 mg, 9.8 μmol) was dissolved in saturated aqueous NH_4HCO_3 (5 mL) and stirred at 40 °C for 48 h then the mixture was concentrated and lyophilized with H_2O (3×) to give **5a**. To a cold (0 °C) solution of **5a** in DMF (0.6 mL) were added $NaHCO_3$ (20 mg, 0.24 mmol) and iodoacetic anhydride (25 mg, 0.071 mmol). After being stirred at 0 °C for 20 min, H_2O (0.6 mL) was added and purified by gel filtration [Amersham: Superdex 30 (26 mm ∅ × 60 cm), H_2O, 2 mL/min] to give 15.9 mg (74%, two steps) of **9a**. ^1H NMR (400 MHz, D_2O) δ 1.90 (s, 3H), 1.93 (s, 3H), 3.26 (t, J = 9.4 Hz, 1H), 3.40–4.02 (m, 71H), 4.10 (br s, 2H), 4.47 (d, J = 7.0 Hz, 1H, H-1^{GlcNAc}), 4.63 (br s, 1H, H-1 Man), 4.73 (br s, 1H, H-1^{Man}), 4.90 (2br s, 2H, 2× H-1^{Man}2), 4.92 (br s, 1H, H-1^{Man}), 4.93 (d, J = 8.9 Hz, 1H, H-1^{GlcNAc}), 5.00 (s, 1H, H-1^{Man}), 5.12 (d, J = 4.1 Hz, 1H, H-1^{Glc}), 5.17 (br s, 1H, H-1^{Man}), 5.20 (br s, 1H, H-1^{Man}), 5.27 (br s, 1H, H-1^{Man}); MS (MALDI-TOF) calcd for $C_{78}H_{130}N_3O_{61}Na$ $(M+Na)^+$ m/z: 2234.6, found: 2234.8.

4.11. Preparation of Glc1Man9GlcNAc2-DHFR (10)

To a solution of *E. coli* DHFR (1.13 mg, 69.3 nmol) in 10 mM $(NH_4)_2CO_3$/AcOH (pH 7.5) (1 mL) were added **9a** (1.9 mg, 840 nmol) and 8 M guanidine (0.35 mL). After being stirred at 25 °C for 6 h, the mixture was dialyzed overnight against 10 mM $(NH_4)_2CO_3$/AcOH (pH 7.5), 0.05% Triton X-100 at 4 °C. The resulting mixture was diluted with 20 mL of 10 mM $(NH_4)_2CO_3$/AcOH (pH 8.0), 0.05% Triton X-100, and 0.05% β-cyclodextrin, and purified by ConA-Aga (Seikagaku Co.) (12.3 mg/mL gel; 6 mL) affinity gel [wash; 10 mM $(NH_4)_2CO_3$/AcOH (pH 7.5) (3× 5 mL), elution; 0.2 M methyl α-D-mannoside in 10 mM $(NH_4)_2CO_3$/AcOH (pH 7.5) (3× 5 mL)]. The Glc1Man9GlcNAc2-DHFR (**10**) containing eluent was concentrated, buffer exchanged (0.05 M Tris–HCl (pH 7.5)), and separated from the excess methyl α-D-mannoside by ultrafiltration (Millipore: Centricon YM-3) to give 23% of **10** (0.15 mg/mL, 2 mL).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.04.001.

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