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Tight binding ligand approach to oligosaccharide-grafted protein

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Abstract—A novel type of artificial glycoprotein was developed, by using dihydrofolate reductase (DHFR) and methotrexate (MTX) as a protein–ligand pair. Various oligosaccharides linked to MTX were shown to bind tightly with DHFR and afforded oligosaccharide-grafted protein, which could be isolated easily by lectin beads.

Oligosaccharide parts of glycoproteins play important roles in a variety of biological events.[1] In particular, the functions of asparagine (Asn)-linked oligosaccharides in glycoprotein quality control process is attracting recent attention.[2] Efforts to correlate the functions of glycan chains with their structures are often hampered by the heterogeneity of glycoproteins.[3] Glycoproteins usually exist as mixtures of various 'glycoforms' that differ in the structures of oligosaccharides. In order to understand their functions precisely, it is desired that glycoproteins having homogeneous and structurally defined oligosaccharide are available. Since the isolation of homogeneous glycoprotein is difficult, various approaches to prepare artificial glycoproteins have been developed.[4] These include (1) modification of natural glycoproteins using glycosidase and/or glycosyltransferase,[5] (2) ligation of synthetic glycopeptide with expressed protein,[6] and (3) introducing synthetic sugar derivatives using chemoselective ligation.[7] Fully chemical synthesis of large glycopeptide has met with substantial success.[8] We wish report herein a complementary approach to carbohydrate-grafted protein, which utilizes oligosaccharide–ligand conjugates.[9]

We focused our attention to the combination of dihydrofolate reductase (DHFR)[10] and methotrexate (MTX, **1**) (Fig. 1).[11] DHFR is relatively small protein (MW ~ 18 kDa), which is essential in karyokinasis and proliferation of cells. MTX is a strong inhibitor of DHFR having $K_D < 1$ nM.[12] Since DHFR inhibition is considered promising in cancer chemotherapy,[13] the mode of DHFR–MTX binding has been analyzed in detail.[14] To our interest, the inhibitory activity of MTX is insensitive to the modification of the γ-carboxy

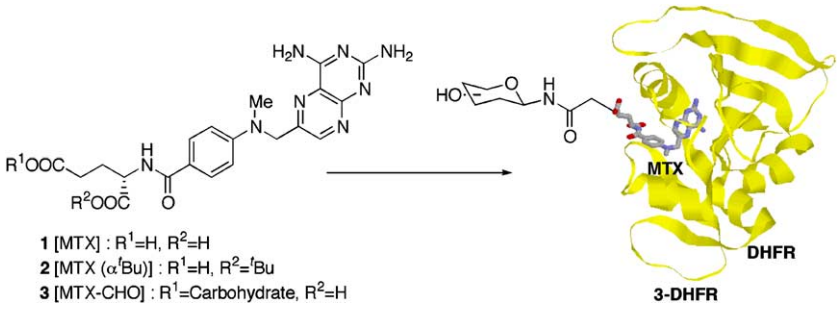

Figure 1. The MTX–DHFR complexes.

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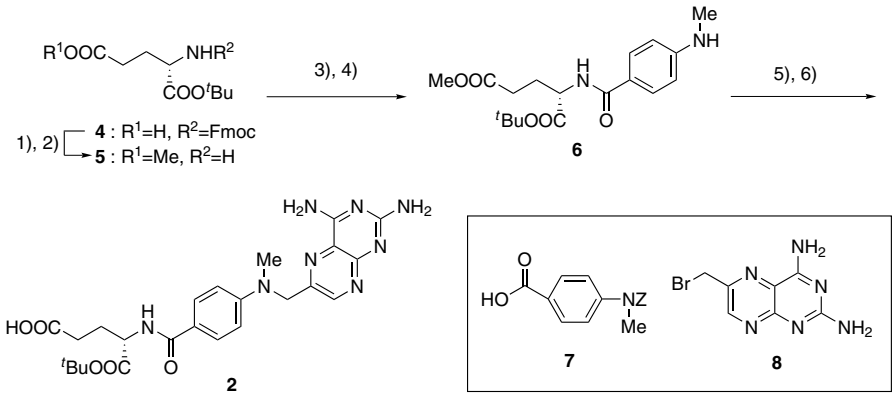
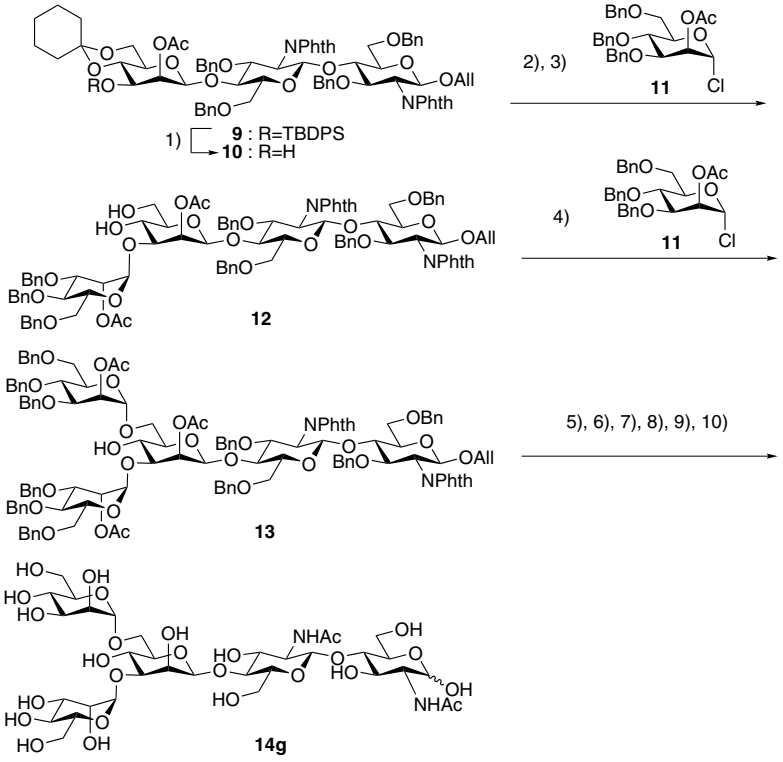
group.[15] Therefore, we expected that the oligosaccharide–MTX conjugate **3** bind tightly with DHFR to provide artificial glycoprotein.

Preparation of oligosaccharide–MTX hybrid **3** was conducted by linking oligosaccharide unit with the MTX component. This convergent approach would be flexible to provide various oligosaccharide-grafted DHFR. The MTX unit **2**[16] was synthesized as depicted in Scheme 1. L-Glutamate derivative **5** was prepared from **4** and condensed with **7**. Subsequent deprotection of the benzyloxycarbonyl (Z) group afforded **6**, which in turn was coupled with the pteridine unit **8**[17] and saponification of γ-Me ester gave MTX(α^{t}Bu) **2**.

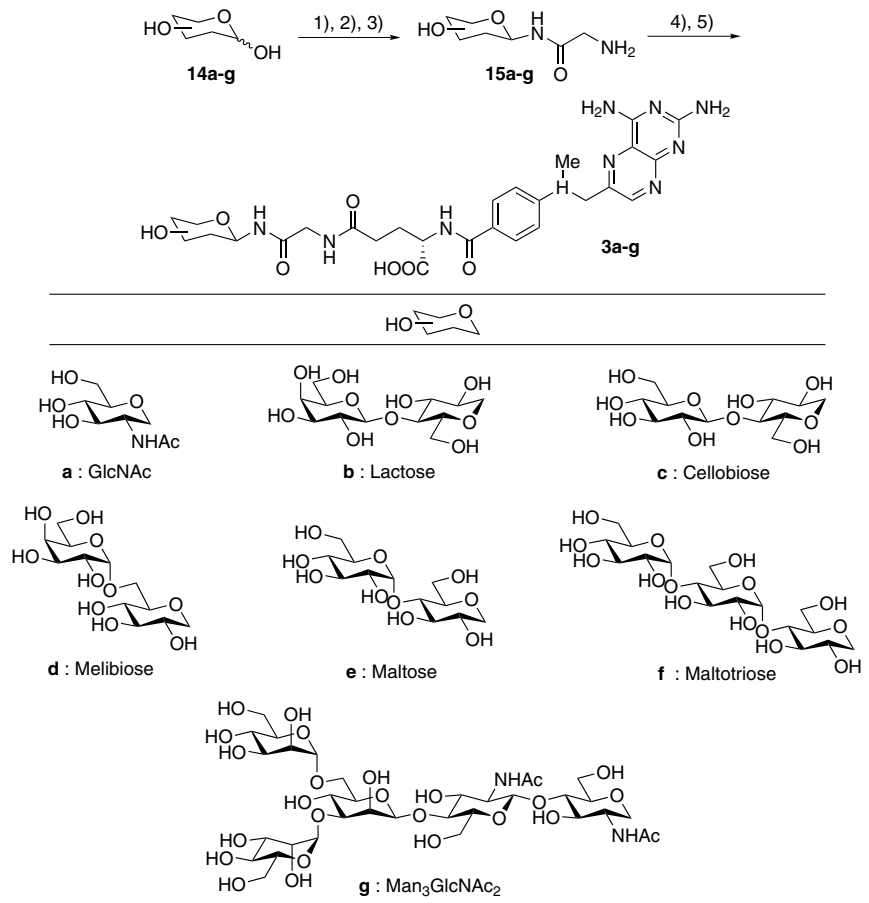
Pentasaccharide **14g**, which corresponds to the core region of asparagine-linked glycoprotein, was chosen as the prototypical oligosaccharide and synthesized as shown in Scheme 2, based on the previously reported procedure[18] with slight modification (Scheme 2). Removal of TBDPS group from **9**[18] was achieved under high-pressure conditions.[19] Stepwise glycosylations for **10** and **12** using **11** as glycosyl donor afforded pentasaccharide **13** that was deprotected to give **14g**.



Scheme 1. Synthesis of MTX unit **2**. Reagents and conditions: (1) $CsCO_3$, MeI, DMF; (2) piperidine, DMF, 87%, two steps; (3) **7**, WSCI·HCl, HOBt, DMF; (4) H_2, $Pd(OH)_2$/C, MeOH, 82%, two steps; (5) **8**, Me_2NAc, 50 °C, then Et_3N; (6) $Ba(OH)_2$, EtOH–H_2O (1:1), 95%, two steps.



Scheme 2. Synthesis of pentasaccharide **14g**. Reagents and conditions: (1) HF·pyr–DMF (1:10), 1 GPa, 30 °C, 89%; (2) **11**, AgOTf, MS4A, toluene, $ClCH_2CH_2Cl$, 87%; (3) *p*-TsOH·H_2O, CH_3CN, 87%; (4) **11**, AgOTf, MS4A, toluene, $ClCH_2CH_2Cl$, 72%; (5) $H_2NCH_2CH_2NH_2$–*n*-BuOH (1:6), 80 °C; (6) pyr–Ac_2O (2:1), DMAP; (7) 1 M NaOMe, MeOH, 50 °C, 89%, three steps; (8) $[Ir(COD)(PMePh_2)_2]PF_6$, H_2, THF; (9) HgO, $HgCl_2$, acetone–H_2O (10:1); (10) H_2, $Pd(OH)_2$/C, MeOH, 85%, three steps.



Scheme 3. Synthesis of bifunctional ligands **3a**–**g**. Reagents and conditions: (1) satd aq NH_4HCO_3, 40 °C; (2) Fmoc-Gly-Cl, $NaHCO_3$, dioxane–H_2O (1:1); (3) 20% piperidine, DMF, ~93%, three steps; (4) **2**, TBTU, DMF, −18 to 0 °C; (5) CF_3COOH, ~72%, two steps.

Bifunctional ligands **3a**–**g** were prepared as depicted in Scheme 3. Commercially available saccharides **14a**–**f** and aforesaid pentasaccharide **14g** were converted to corresponding glycosylamines[20] and acylated with Fmoc-Gly-Cl.[21] Deprotection of Fmoc gave glycine-linked saccharides **15a**–**g**. Subsequent condensation with MTX(α^{t}Bu) **2** was effected by TBTU and acidic cleavage of ^{t}Bu ester provided sugar–MTX hybrids **3a**–**g**.

In order to assess the affinity to DHFR, inhibitory activities of **3a**–**g** were measured. For this purpose, crude preparation of DHFR (*E. coli*) expressed in a cell-free protein synthesis system derived from wheat embryos (PROTEIOS™)[22] were employed. The inhibition assays were performed according to the reported procedure.[23] As summarized in Figure 2, inhibitory activities of sugar–MTX conjugates (**3a**–**g**) were comparable with that of unsubstituted MTX (**1**), suggesting the formation of tight binding complex. As expected, MTX(α^{t}Bu) **2** had substantially reduced avidity.

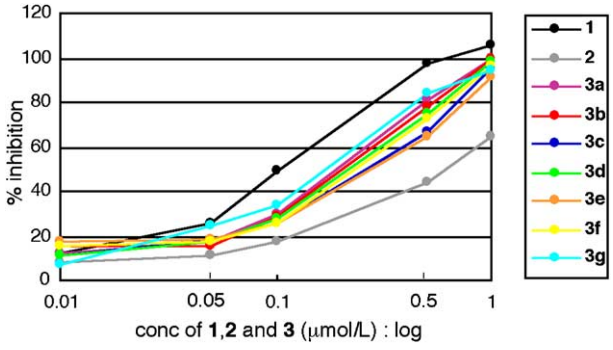


Figure 2. DHFR inhibition by MTX–sugar. Dihydrofolate (FH2) solution [FH2; 0.104 g/L, 2-mercaptoethanol; 6.06 mL/L in buffer A (0.05 M Tris·HCl, pH 7.5)] (130 μL), MTX derivatives [0.01–1 μmol/L in buffer A] (20 μL), and NADPH/DHFR [NADPH; 0.308 g/L, DHFR expression mixture; 38.5 mL/L in buffer A] (50 μL) were added to each well of the 96-well titer-plate. 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.

Further evidence of the formation of the tight binding complex between **3** and DHFR was obtained from lectin binding experiment (Fig. 3), which also facilitated the isolation of the conjugates. Thus, DHFR preparation mixture (lane 1) was incubated with pentasaccharide–MTX **3g** (lane 2), in a plastic tube equipped with membrane filter (Millipore: Ultrafree-MC, 0.22 μm), to form the **3g**–DHFR conjugate. Subsequently, it was captured by treatment with ConA-Aga and separated by filtration. At this stage, DHFR vanished from flow through and from washing fractions (lanes 3 and 4), implying that **3g**–DHFR was retained on ConA-Aga.

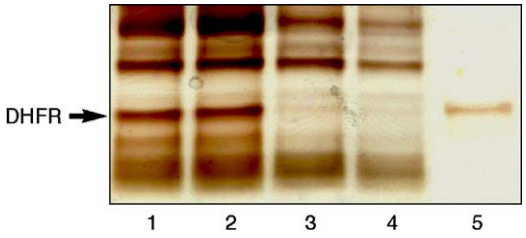


Figure 3. Lectin binding experiment of pentasaccharide containing DHFR: lane 1, DHFR prep. mix. in 0.05 M Tris·HCl (pH 7.5) (buffer A); lane 2, DHFR prep. mix.+**3g** in buffer A; lane 3, DHFR prep. mix.+**3g**+ConA-Aga (flow through fraction); lane 4, washings; lane 5, elution fraction (eluent; 0.1 M methyl mannoside in buffer A).

The conjugate was then eluted with 0.1 M α-Me-Man (lane 5). Similarly, DHFR conjugates carrying **3a** and **3b** were isolated using WGA-Aga and RCA120-Aga, respectively.

That the conjugate consists of an equimolar amount of DHFR and **3g** was confirmed in a following manner. Firstly, **3g**–DHFR complex containing eluent (Fig. 3, lane 5) was separated from the excess ligand by ultrafiltration (Millipore: Centricon YM3). The amount of DHFR was determined to be 1.37 μM by BCA protein assay. This value was in agreement with the amount of the ligand **3g** (1.35 μM) that was determined by the absorbance of the MTX chromophore at 304 nm (Fig. 4).

In summary, a novel type of glycoprotein mimetic was created by employing specific binding of MTX and DHFR. In principle, it has no limitation in oligosaccharide structure that can be introduced. Since conversion of complex glycan chain to corresponding glycosylamine is well established,[24] it is expected that various types of sugar–MTX conjugates can be prepared and converted to artificial glycoproteins, which will be useful molecular probes to analyze interaction with lectins and enzymes. Further studies are in progress along this line and will be reported in due course.

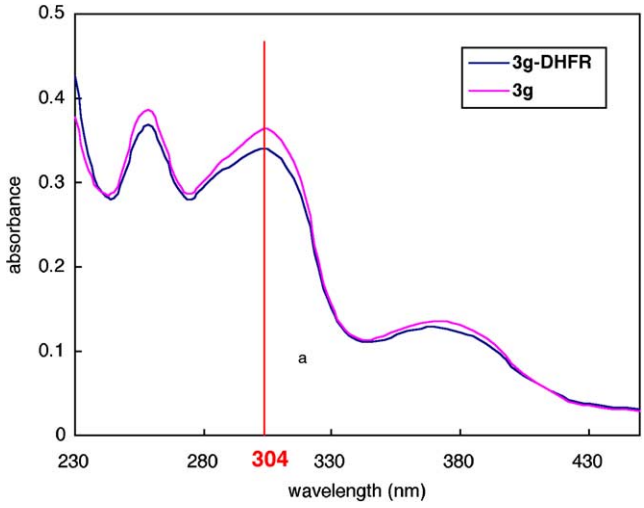


Figure 4. UV–visible spectra of **3g**–DHFR and **3g**. Compound **3g** (24 μM) and DHFR (24 μM) in 0.05 M Tris·HCl (pH 7.5).

Acknowledgements

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