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AMIDE BOND FORMATION OF SIALIC ACID IN OLIGOSACCHARIDE WITHOUT PROTECTING GROUP

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Abstract – With the aim of preparing glycan-linked antibody-drug conjugates, we investigated the feasibility of amide bond formation at the carboxylic acid of sialic acid in a protection-free oligosaccharide. The use of a phosphonium salt-based reagent, PyBOP, gave the desired compound. Bio-orthogonal functional groups were conjugated at the carboxylic acid site of sialic acid in glycans.

Antibody-drug conjugates (ADC), which are monoclonal antibodies attached to biologically active drugs via chemical linkers, have received much attention as next-generation therapeutic agents.[1] Many ADCs are believed to be internalized in cells, and cytotoxic agents are released from lysosomes in their active form. ADCs can expand the narrow therapeutic index (the ratio of the toxic dose to effective dose) of traditional chemotherapeutic agents. The linker connects the antibody and payload in the ADC, and plays important roles that include controlling the conjugation site, drug per antibody ratio, and influencing the rate and mechanism of drug release. The initial ADC was unsuccessful because the linker used (e.g., hydrazine linkage) was unstable in the blood circulation.[2] Later, cathepsin-cleavable linker Val-Cit (citrulline) was developed for a successful ADC in the clinic.[3]

Besides the cleavage mode of a drug, the development of a homogeneous ADC is important for expanding the therapeutic index. Current ADCs in the clinic use Lys and Cys residues for payload conjugation sites, which results in heterogeneity. Among various methods on the development of homogeneous ADC preparation,[4] the use of glycan as a conjugation site is a promising approach. The Fc region of monoclonal immunoglobulin G (IgG) carries a conserved *N*-linked glycan chain at Asn297 (Figure 1). Since the *N*-glycan is located far from the antigen-binding Fab region, modification of this glycan is expected not to affect the antigen-binding ability of the antibody and should be site-selective. Several methods for conjugation at the glycan site have been developed. For instance, carbonyl group generation by the oxidative cleavage of the 1,2-*cis* diol of fucose and sialic acid, and subsequent conjugation of payloads have been reported.[5,6] Modified galactoses and sialic acids are transferred to the

N-glycan site to afford a functional group for payload conjugation by monosaccharyl transferases such as galactosyl transferase and sialic acid transferase with relatively good substrate tolerance.[7,8] Recently, another approach using mutant endo-β–*N*-acetylglucosaminidases (ENGases) for glycan remodeling of the antibody has been reported.[9,10] Some ENGase mutants have transglycosylation activity with reduced hydrolytic activity. The typical procedure is as follows: i) the heterogeneous *N*-glycan is removed by ENGases from the chitobiose core of the heavy chain of native IgG; ii) a transition-mimic oxazoline glycan is added *en bloc* as a donor to give IgG with a homogeneous glycan. Based on this strategy, if a functional group for drug conjugation is added to the glycan donor, the payloads can be conjugated only at the glycan position (Scheme 1). Parsons et al. reported homogeneous ADC preparation though glycan modification and subsequent bioorthogonal reaction.[11] They used triazine-based 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)[12] **8** for the amidation of glycan isolated from egg yolk.[13] Although DMT-MM **8** is a well-known amide bond forming reagent that is suitable for use in aqueous reactions, we could not reproduce the reaction. Amide formation at the sialic acid derivatives of glycan without hydroxy group protection has been reported for sample preparation in MS,[14,15] but the substrates used were restricted to simple amines and excess amounts of reagents were used. Herein, we report a reliable method for amide bond formation in sialic acid in oligosaccharides without functional group protection for application in organic synthesis.

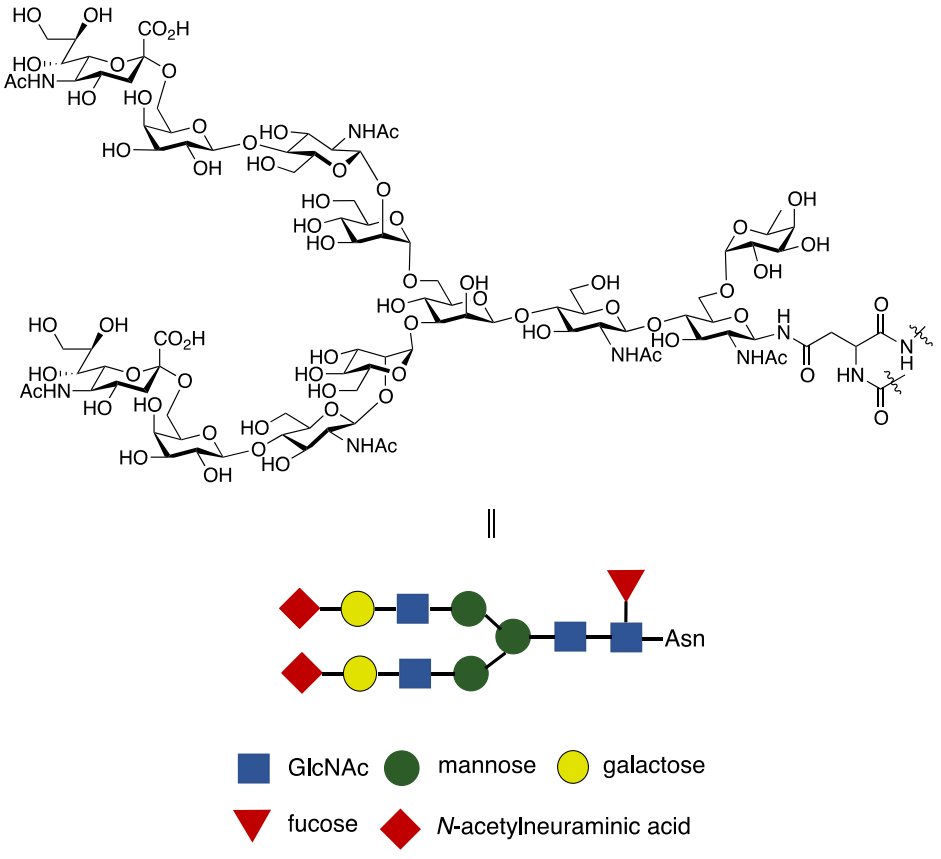
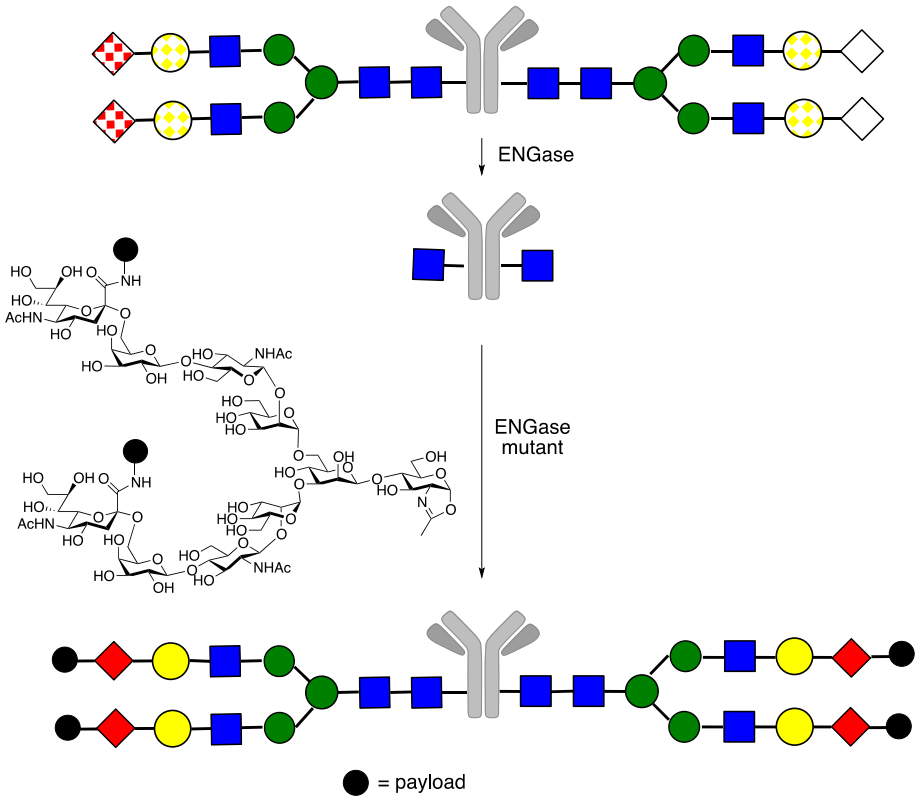


Figure 1. Structure of *N*-glycan in antibody



Scheme 1. Homogeneous ADC preparation via glycan-site conjugation. Dot-circles and squares indicate the heterogeneous portions.

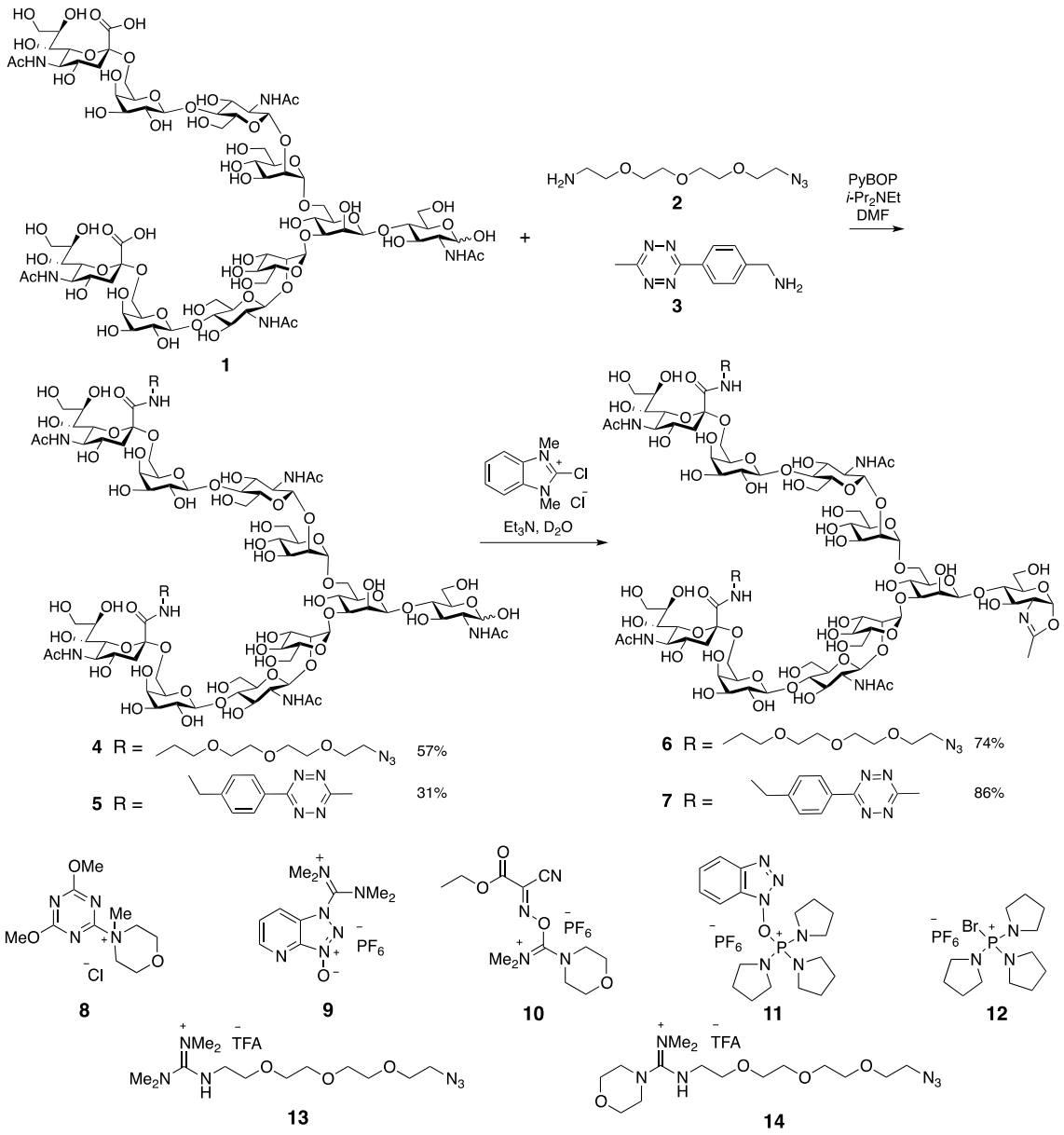
First, we attempted amide bond formation between glycan **1** and amine **2** by using DMT-MM, as reported by Parsons.[11] Unfortunately, HPLC analysis revealed that product **4** was not formed. In addition, the product was not obtained when 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate, (HATU: 5 equiv.) **9** or 1-cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate **10** (COMU: 5 equiv.) was used as the condensation reagents in dimethylformamide (DMF). The yield of **4** did not improve exceed over 5% even when the amounts of amine **2** and the condensation reagents were increased up to 20 equiv. Instead, adducts of amine **2** with **9** and **10** (**13** and **14**, respectively) were isolated as the major products. Formation of a guanidino group via the reaction between uronium/guanidinium-type condensation reagents and amines has been reported, especially when sterically hindered carboxylic acids are used as substrates.[16] In order to suppress the reaction between **2** and the condensation reagents, we attempted activation of the carboxylic acid by mixing the carboxylic acid and HATU/COMU for 2 h prior to the addition of the amine. Unfortunately, this approach, too, afforded the adducts (**13** and **14**) instead of the desired products.

Finally, product **4** was isolated in 57% yield when phosphonium ion reagent (benzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) **11** was employed. A similar phosphonium condensation reagent, bromotripyrrolidinophosphonium hexafluorophosphate (PyBrop) **12**, gave a much lower yield of **4** (5.8%), probably because bromide group was replaced by **2**. Next, we applied our system

to an amine with a tetrazine moiety **3** to give **5** in 31% yield. Similar to azide, the tetrazine group has been reported to react with alkenes via an inverse Diels-Alder reaction.[17] It is expected that payload conjugation through the tetrazine group is possible after glycan addition to the antibody.

Finally, oxazolines **6** and **7** were prepared from **4** and **5** in 74% and 86% yields as per Shoda's protocol[18] to be used as donors for ENGase mutant to give antibodies with a functional glycan. We found RP-HPLC column chromatography tolerant to basic conditions was effective for precise purification of the oxazolines after gel filtration.

In conclusion, we have developed a reliable method for amide bond formation in glycans without protection of hydroxy groups. Further investigation into homogeneous ADC preparation using a combination of modified glycan and ENGase is underway.



Scheme 2. Preparation of donors for ENGase mutant with payload conjugation site. Counter ions of compounds **13** and **14** are changed to TFA in HPLC purification.

EXPERIMENTAL

^{1}H-NMR spectra of compounds **4-7, 13** and **14** were recorded either with ECX 400 spectrometer (JEOL, 400 and 100 MHz, respectively) or with a 600 MHz NMR spectrometer (Bruker BioSpin) at ambient temperatures (23–24 °C). Chemical shifts (δ) are reported in ppm relative to remaining solvent peak $CDCl_3$ (δ = 7.26 ppm) for ^{1}H-NMR spectra. ^{13}C-NMR chemical shifts (δ) are reported in ppm relative to $\underline{C}DCl_3$ (δ = 77.00 ppm). ^{1}H- and ^{13}C-NMR chemical shifts were reported relative to the internal standard MeOH (as 3.34 ppm for ^{1}H-NMR, and 49.0 ppm for ^{13}C-NMR in D_2O. HPLC (Prominence, SHIMADZU, Kyoto, Japan) was used for purification of low-molecular weight compounds and analyses of time-course of enzymatic reaction. MALDI-TOF MS spectra were recorded in the positive ion mode on a KOMPACT MALDI IV tDE (SHIMADZU/KRATOS).

Amide bond formation: Preparation of compound **4**:

To a suspension of disialyloctasaccharide **1** (30 mg, 0.0148 mmol), 2-(2-(2-(2-azidoethoxy)-ethoxy)ethoxy)ethan-1-amine **2** (4 M in DMF, 37 μL, 0.148 mmol), and DIPEA (26 μL, 0.148 mmol) in DMF (333 μL), PyBOP (77 mg, 0.148 mmol) was added at 4 °C, and the mixture was stirred at 40 °C for 8 h. Analytical HPLC was performed using a C8 reverse phase column (Mightysil RP-8 GP, 4.6 × 250 mm, KANTO CHEMICAL Co., Inc.) with 0% MeCN for 2 min and a linear gradient of 0-100% MeCN in 0.1% aqueous TFA over 40 min (room temperature, flow rate of 1 mL/min, detection at 214 nm, 280 nm). After 8 h, the reaction mixture was directly purified by gel filtration column chromatography (G-25, GE Healthcare Life Sciences, eluent: H_2O), followed by then preparative HPLC (Mightysil RP-18 GP, 20 × 250 mm, KANTO CHEMICAL Co. Inc.) to afford the desired product **4** (20.4 mg, 57% yield). ^{1}H-NMR (D_2O) δ 5.18-5.17 (m, 1H), 5.10 (s, 1.2H), 4.91 (s, 1.2H), 4.57-4.55 (m, 2.6H), 4.42-4.40 (m, 2.6H), 4.41-4.29 (m, 2H), 4.23 (s, 1.2H), 4.16 (s, 1.2H), 4.08 (s, 1.2H), 2.69-2.65 (m, 2H), 2.02-2.00 (multiple s, 15H), 1.83 (t, J = 12.8 Hz, 1H), 1.81 (t, J = 12.8 Hz, 1H); ^{13}C-NMR (D_2O) δ 175.1, 174.8, 174.7, 169.2, 103.8, 103.8, 99.7, 99.5, 81.0, 80.9, 80.6, 80.4, 76.5, 74.5, 73.7, 72.6, 72.2, 71.2, 70.8, 69.8, 69.6, 69.3, 69.3, 68.6, 68.4, 67.9, 67.5, 67.4, 67.1, 63.1, 60.3, 54.7, 51.8, 50.3, 38.9, 38.4, 38.4, 38.3, 38.0, 37.8, 37.6, 22.6, 22.2; MS calcd for [$C_{92}H_{152}N_{13}O_{61}$+$Na^+$] 2442, found 2442.

Compound **5**: ^{1}H-NMR (D_2O) δ 8.38 (d, J = 7.8 Hz, 4H), 8.16 (bs, 0.5 H), 7.59 (d, J = 7.8 Hz, 4H), 5.17 (s, 0.6 H), 5.08 (s, 0.9H), 4.89 (bs, 0.8H), 4.53-4.47 (m, 5H), 4.37-4.35 (m, 2H), 4.21 (s, 0.9H), 4.14 (s, 0.9H), 4.07 (s, 0.9H), 3.04-3.03 (m, 6H), 2.67 (dt, J = 12.8 Hz, 4.6 Hz, 2H), 2.01-1.97 (multiple s, 15H), 1.84 (t, J = 12.8 Hz, 2H); ^{13}C-NMR (D_2O) δ 175.2, 174.9, 169.9, 167.5, 164.2, 142.6, 130.9, 128.9, 128.6, 103.9, 99.6, 81.1, 80.7, 80.5, 80.0, 76.4, 76.2, 74.5, 73.8, 73.7, 72.6, 72.3, 71.2, 70.8, 70.1, 69.6, 68.4, 67.9, 67.3, 63.1, 62.9, 62.0, 61.9, 61.6, 61.5, 60.3, 60.2, 54.7, 51.9, 43.4, 38.1, 22.5, 22.2, 20.3; MS calcd for [$C_{96}H_{143}N_{15}O_{55}$+$Na^+$] 2409, found 2409.

Preparation of oxazoline **6**:

To a solution of lactol **4** (5.0 mg, 0.00207 mmol) and triethylamine (20.2 μL, 0.145 mmol) in D_2O (590 μL), 2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride (CDMBI, 20.2 mg, 0.0930 mmol) was added at 4 °C, and the mixture was stirred for 2 h. The reaction progress was analyzed by ^{1}H NMR to confirm oxazoline formation and then purified with gel filtration column chromatography (G-25, GE Healthcare Life Sciences, eluent: 0.1% aqueous triethylamine). After lyophilization, the desired product **6** was obtained in 74% yield. Analytical and preparative HPLC was performed using a C18 reverse phase column (YMC-Triart C18; YMC Co., Inc. 4.6 × 150 mm) with 0% MeCN for 2 min and a linear gradient of 15-37.5% MeCN in 0.1% aqueous triethylamine over 45 min (room temperature, flow rate of 0.7 mL/min, detection at 214 nm, 254 nm). ^{1}H-NMR (D_2O) δ 6.06 (d, J = 7.3 Hz, 1H), 5.09 (s, 1H), 4.92 (s, 1H), 4.57-4.55 (m, 2H), 4.41-4.40 (m, 2H), 4.35 (m, 1H), 4.15-4.12 (m, 4H), 2.68-2.65 (m, 2H), 2.03 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H), 1.80 (t, J = 13.2 Hz, 2H), ^{13}C-NMR (D_2O) δ 181.6, 175.1, 174.8, 169.2, 168.7, 103.8, 101.5, 99.5, 81.0, 80,6, 78.1, 76.5, 76.1, 74.5, 73.6, 72.6, 72.2, 71.2, 71.0, 70.8, 70.4, 69.8, 69.7, 69.4, 69.3, 68.6, 68.5, 67.9, 67.4, 67.1, 63.2, 62.8, 61.8, 60.3, 54.7, 51.9, 50.3, 38.9, 38.4, 23.4, 22.6, 22.2, 20.2, 13.1, 12.5.

Oxazoline **7**: ^{1}H-NMR(D_2O) δ 8.41 (d, J = 8.2 Hz, 4H); 7.61 (d, J = 8.2 Hz, 4H), 6.07 (d, J = 8.2 Hz, 1H), 4.39-4.38 (m, 3H), 4.18-4.11 (m, 4H), 2.71-2.68 (m, 2H), 2.04-2.00 (multiple s, 15H), 1.91-1.83 (m, 2H).

Compound **13**: ^{1}H-NMR ($CDCl_3$) δ 8.28 (bs, 1H), 3.74-3.72 (m, 2H), 3.67-3.61 (m, 8H), 3.40 (m, 3H), 307 (m, 3H), 3.00-2.80 (m, 12H); ^{13}C-NMR ($CDCl_3$) δ 162.8, 70.6, 70.5, 70.4, 70.3, 70.0, 69.4, 50.6, 45.0, 39.9; calcd for [$C_{13}H_{29}N_6O_3$+H^+] found 318, found 318.

Compound **14**: ^{1}H-NMR ($CDCl_3$) δ 3.77-3.72 (m, 10H), 3.63-3.51 (m, 8H), 3.40-3.34 (m, 4H), 3.09 (m, 2H), 2.96 (s, 6H); ^{13}C-NMR ($CDCl_3$) δ 162.0, 70.2, 69.2, 69.1, 66.3, 66.2, 48.9, 48.6, 45.5, 45.2, 40.2, 40.0; calcd for [$C_{16}H_{32}N_6O_3$+H^+] 360, found 360.

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