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Synthesis and Evaluation of Three Azide-Modified Disaccharide Oxazolines as Enzyme Substrates for Single-Step Fc Glycan-Mediated Antibody-Drug Conjugation

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Abstract

Antibody-drug conjugates (ADCs) hold great promise for targeted cancer cell killing. Site-specific antibody-drug conjugation is highly desirable for synthesizing homogeneous ADCs with optimal safety profiles and high efficacy. We have recently reported that azide-functionalized disaccharide oxazolines of the Manβ1,4GlcNAc core were an efficient substrate of wild-type endoglycosidase Endo-S2 for Fc glycan remodeling and conjugation. In this paper, we report the synthesis and evaluation of new disaccharide oxazolines as enzyme substrates for examining the scope of the site-specific conjugation. Thus, azide-functionalized disaccharide oxazolines derived from Manβ1,4GlcNAc, Glcβ1,4GlcNAc, and Galβ1,4GlcNAc (LacNAc) were synthesized. Enzymatic evaluation revealed that wild-type Endo-S2 demonstrated highly relaxed substrate specificity and could accommodate all the three types of disaccharide derivatives for transglycosylation to provide site-specific azide-tagged antibodies, which were readily clicked with a payload to generate homogeneous ADCs. Moreover, we also found that Endo-S2 was able to accommodate drug-preloaded minimal disaccharide oxazolines as donor substrates for efficient glycan transfer,

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Supporting Information

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Figures S1–S8 and copies of NMR spectra (PDF)

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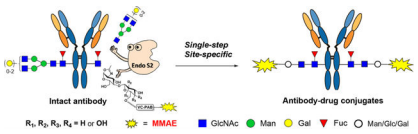
enabling a single-step and site-specific antibody-drug conjugation without the need of an antibody click reaction. The ability of Endo-S2 to accommodate simpler and more easily synthesized disaccharide oxazoline derivatives for Fc glycan remodeling further expanded the scope of this bioconjugation method for constructing homogeneous antibody-drug conjugates in a single-step manner. Finally, cell-based assays indicated that the synthetic homogeneous ADCs demonstrated potent targeted cancer cell killing.

Graphical Abstract



INTRODUCTION

Antibody–drug conjugates (ADCs) have emerged as a novel class of anticancer agents that combines the specificity of antibodies and the high potency of cytotoxic drugs.[1] So far, 12 ADCs have been approved by the US Food and Drug Administration (FDA) for the treatment of cancers,[2] with dozens more at various stages of preclinical and clinical development. The first-generation ADCs are generated through non-specific conjugation,[3] which usually results in heterogeneous mixtures with varied drug/antibody ratios (DARs) and different pharmacological properties. Recent studies have shown that site-specific ADCs could display improved pharmacokinetics, *in vivo* stability, and favorable safety profiles.[4–6] Recent development in the linker technology and conjugation strategies have shown promise for a new generation of ADCs with more homogeneous structures.[7,8] However, most strategies involve protein engineering of antibodies and subsequent site-selective conjugation, requiring extra steps of manipulations and purification.

The conserved *N*-glycans attached to the Fc domain of IgG antibodies at Asn-297 that are spatially distant from the antigen-binding region offer an attractive conjugation site for the development of homogeneous ADCs.[8–10] Significant progress has been made via the glycan-mediated chemoenzymatic bioconjugation of antibodies, which is enabled by the enzymatic transfer of azide- or other selectively modified sugars to antibodies via the catalysis of glycosyltransferases or endoglycosidase mutants.[11–18] However, most of the chemoenzymatic approaches for ADC synthesis require three key steps: the deglycosylation at the Fc glycosite, the enzymatic transfer of a tagged sugar moiety, and the click conjugation with a payload. Recently, we have reported that selectively azide-tagged Man-β1,4-GlcNAc disaccharide oxazolines can act as donor substrates of several endoglycosidases, including the Endo-S and Endo-S2 from *Streptococcus pyogenes*, for Fc glycan remodeling with simultaneous deglycosylation and glycosylation without product hydrolysis, providing an efficient one-pot strategy for the labeling and conjugation of intact antibodies.[18] The resulting azide-tagged antibodies can be efficiently converted to homogeneous ADCs with different antibody/drug ratios by subsequent click reactions.[18] To further examine the scope of this method, we report herein the synthesis and evaluation of selectively modified new disaccharide oxazolines, including the Glc-β1,4-GlcNAc and Gal-

β1,4-GlcNAc (LacNAc) disaccharides, as substrates for enzymatic Fc-glycan remodeling of antibodies. We found that wild-type Endo-S2 had a remarkable flexibility to accommodate the "unnatural core disaccharides" for transglycosylation to provide azide-functionalized antibodies (Figure 1). Moreover, we discovered that the wild-type Endo-S2 could perform a simultaneous deglycosylation and glycosylation of an antibody with the drug-loaded disaccharide oxazoline substrates to give homogeneous ADCs in a single step. The resulting ADCs showed high selectivity for the target cells as indicated in the cytotoxicity studies. This method opens a new avenue to producing homogeneous antibody-drug conjugates, which represents a truly single-step and site-specific bioconjugation of a payload to intact antibodies. During the preparation of this manuscript, Shi and co-workers have reported a nice and independent work on Endo-S2 catalyzed transfer of drug-modified Gal-β1,4-GlcNAc oxazolines to antibody to form antibody-drug conjugates, in which the drug and antibody are conjugated through an oxime linkage.[19]

RESULTS AND DISCUSSION

Our recent study has shown that wild-type Endo-S2 could accommodate selectively modified disaccharide oxazolines corresponding to the natural disaccharide (Manβ1,4GlcNAc) core for transglycosylation without product hydrolysis.[18] However, whether this enzyme could recognize and transfer unnatural core disaccharide structures to antibodies remains to be tested. We sought to test simpler disaccharide derivatives, such as Glcβ1,4GlcNAc and Galβ1,4GlcNAc (LacNAc) oxazolines, which are much easier to synthesize than the Manβ1,4GlcNAc core. To explore the substrate specificity of Endo-S2 and to identify simple functionalized disaccharide oxazoline substrates for antibody glycan remodeling, we designed and synthesized three selectively modified disaccharide cores (Manβ1,4GlcNAc, Glcβ1,4GlcNAc, and Galβ1,4GlcNAc) with an amine or azide functional group at the 6′ position for further derivatization (Scheme 1). First, global deprotection of the known compound (**4**)[18] afforded the Man-GlcNAc disaccharide (**5**) in almost quantitative yield. Under this condition, the azido group was simultaneously reduced to an amino group, which could be used for the introduction of functional groups. For the synthesis of the glucose-based disaccharide (Glc-GlcNAc), the benzoyl group in **7**[20] was converted to benzyl group in two steps, giving **8** in 87% overall yield. Next, regioselective opening of the benzylidene moiety-with BH_3·THF/Bu_2BOTf furnished compound **9** with a free OH at the C-6 position in excellent yield. After the conversion of the 2-azido group into the 2-acetamido group with AcSH,[21] an azido-tagged polyethylene chain was introduced at this position by treatment of **10** with NaH and $N_3(CH_2CH_2O)_3Ts$ to give **11** in 85% yield. Catalytic hydrogenation removed all the protecting groups and afforded free glycan **12** in excellent yield. To test the substrate specificity of Endo-S2, the amine group in **12** was transformed back to an azido group by the copper-catalyzed diazo transfer reaction,[22,23] giving **13** in 81% yield, which was further transformed to oxazoline **14** by treatment of DMC and Et_3N in water. Finally, the galactose-based disaccharide was constructed by coupling of **15**[24] and **16**[18] under standard glycosylation conditions. Selective protection of the C6-OH with the TBDPS group at the Gal residue followed by per-benzylation after de-acetylation afforded **18** in 68% overall yield, which was transformed to **19** with AcSH in 79% yield. Upon the deprotection of TBDPS, the azido-tagged polyethylene chain

was introduced to give **21**, which, after global deprotection, provided **22** quantitatively. Subsequent diazo transfer reaction and oxazoline formation gave **24** in excellent yield (Scheme 1).

To test if the Glc- or Gal-containing disaccharide oxazolines (**14** and **24**) could still be recognized by wild-type Endo-S2 for Fc glycan remodeling of antibodies, we tried the one-pot transglycosylation with trastuzumab as the model antibody (Scheme 2). We found that disaccharide oxazolines **14** and **24** both could act as donor substrates for Endo-S2 catalyzed transglycosylation, but the enzymatic reactions were slower than that of the Man-β1,4-GlcNAc oxazoline (**6**) corresponding to the natural disaccharide core (Supporting Information, Figure S1). This result suggests that replacing the "natural" β-D-Man moiety with β-D-Glc or β-D-Gal residue reduced their substrate activity toward Endo-S2 to some extent, but Endo-S2 could still recognize them as donor substrates. Thus, more enzyme and/or prolonged reaction time were required to drive the transglycosylation to completion. We also observed that the Man-β1,4-GlcNAc oxazoline (**6**) could slowly be hydrolyzed by Endo-S2, while the azide-tagged Glc- or Gal-containing disaccharide oxazolines (**14** and **24**) were more resistant to Endo-S2 catalyzed hydrolysis. Nevertheless, the Endo-S2 catalyzed transglycosylation was much faster than the hydrolysis of the Man-β1,4-GlcNAc oxazoline (**6**). Thus, when an excess amount (20 equiv) of the disaccharide oxazoline (**6**) was used, a complete conversion of the antibody to the azide-tagged antibody product could be achieved within 30 min with 0.1% (w/w) of the enzyme under the reaction conditions, while the complete conversion with the azide-tagged Glc- or Gal-containing disaccharide oxazolines (**14** and **24**) would need over 2 h in the presence of 0.3 and 0.5% enzyme, respectively (Scheme 2). The ability of Endo-S2 to accept the Glcβ1,4GlcNAc and LacNAc oxazolines for transglycosylation is significant, as synthesis of these disaccharide derivatives are more efficient and straightforward than the Manβ1,4GlcNAc derivative as we have previously reported.[18] The transglycosylation products, **26** and **27**, were purified by a protein A affinity column, and their identity was confirmed by LC-ESI-MS analysis (Figures S2–S4).

Encouraged by the excellent transglycosylation activities of the azido-tagged disaccharide oxazolines, we sought to pre-introduce the cytotoxic drug to the disaccharide oxazolines and to test the feasibility of a single-step glycan remodeling for constructing ADCs. To achieve this strategy, several factors need to be considered. First, the drug should tolerate the DMC/Et_3N treatment for sugar oxazoline formation. Second, it needs to be stable under alkaline conditions. Lastly, the drug-disaccharide oxazoline should have sufficient solubility in aqueous solution. We chose monomethyl auristatin E (MMAE) as the cytotoxic drug to test the strategy. Thus, compound **28**[17] bearing a cleavable dipeptide linker (valine-citrulline) was reacted with bis-NHS-PEG5 to give the NHS-activated payload **29**, which was further conjugated with the disaccharides via the amine-coupling reaction followed by oxazoline formation in one-pot, providing the drug-oxazoline conjugates 1–3 in good yields after RP-HPLC purification (Scheme 3).

With the drug-sugar oxazoline conjugates in hand, next we investigated the one-step synthesis of ADCs via the Endo-S2-catalyzed transglycosylation (Scheme 4). Notably, the oxazolines **1–3** could not be completely dissolved in aqueous buffer solution due to the hydrophobicity of MMAE and the cleavable linker; thus, 5% DMSO was added to

help dissolve the oxazolines in the reaction solution. It was found that the Man-derived drug-oxazoline conjugate (**1**) acted as an excellent substrate of wild-type Endo-S2, and the reaction reached completion within 1 h with a catalytic amount of enzyme (0.2%, enzyme to antibody, w/w) and 15 equiv of the sugar oxazoline donor (**1**). Once formed, the product was largely resistant to hydrolysis under the reaction conditions, thus offering a practical one-step method for the preparation of homogeneous ADCs with a precise control of DAR. As for Glc- and Gal-derived drug-oxazolines (**2** and **3**, respectively), a relatively larger amount of enzyme and oxazoline donors and longer reaction time were required to complete the transformation (Figure S5). In all the cases, the corresponding antibody-drug conjugates (**33**, **34**, and **35**) were obtained. To verify that the linker-payloads were specifically attached to the Fc domain, the ADCs (**33**, **34**, and **35**) were digested with protease IdeS, which cleaves the antibody to yield the monomeric Fc domain followed by LC-ESI-MS analysis (Figures S6–S8). The results showed high homogeneity of the products. The LC-ESI-MS analysis of the intact ADCs (**33–35**) showed that the shifts of molecular weight were consistent with the attached payloads (for the drug-conjugated Fc domain, calculated, M = 26,056; observed, 26,057, deconvoluted data), thus further confirming the structure of the products (Figure 2). The findings that Endo-S2 can perform simultaneous antibody deglycosylation and transfer of a payload-conjugated disaccharide oxazoline further expands the scope of the chemoenzymatic Fc glycan remodeling method for synthesizing homogeneous antibody-drug conjugates.

Finally, we tested the cytotoxicity of the synthetic ADCs (**33**, **34**, and **35**) in breast cancer cell lines SK-BR-3 and BT474 that have high levels of HER2 expression, and T47D that has low level expression of HER2 antigen (Figure 3). The trastuzumab-MMAE conjugate (**36**) (with a DAR of 2) that we have synthesized previously using a two-step approach[18] was used as a reference for comparison. We found that all these ADCs achieved significant killing of the high antigen-expressed cell lines (SK-BR-3 and BT474) with almost the same potency, as indicated by the EC_{50} values. As for the T47D line that expresses the low level of HER2 antigen, no apparent killing was observed under the same concentrations, indicating the high selectivity of the synthetic ADCs for the target cells. The anti-cancer potency of the ADCs (**33**, **34**, and **35**) synthesized by this disaccharide conjugation method was quite comparable to those ADCs produced through the full *N*-glycan conjugation method that we have reported previously.[17] For example, for the killing of the SK-BR-3 cell line, the present ADCs (**33**–**36**) gave an EC_{50} of 41–71 pM, while the ADCs made from the full-glycan conjugation method gave an EC_{50} of 88–162 pM. It should be noted that the full glycan conjugation ADCs have a drug-to-antibody ratio (DAR) of 4, while the disaccharide-conjugated ADCs presented here have a DAR of 2. The cell-based assay data indicate the comparable efficiency of the two types of ADCs. Further evaluation and comparison of these ADCs in animal models are in progress, and the results will be reported in due course.

CONCLUSIONS

A highly efficient method for a single-step and site-specific chemoenzymatic synthesis of homogeneous antibody-drug conjugates is established. The findings that Endo-S2 can

accept different disaccharide oxazolines, including the simpler and more easily synthesized cellobiose and *N*-acetyllactosamine derivatives for Fc glycan remodeling, further expands the scope of the chemoenzymatic method for antibody bioconjugation. In addition, the ability of Endo-S2 to perform deglycosylation of native antibodies and simultaneously transfer drug-preloaded disaccharide oxazolines enables a truly single-step protocol to construct homogeneous antibody-drug conjugates.

EXPERIMENTAL SECTION

General Procedure.

Chemicals, solvents, and reagents were purchased from TCI and Sigma-Aldrich and were used without further purifications unless specially noted. Silica gel (230–400 mesh) used for flash chromatography was purchased from SILICYCLE. The reactions were monitored by TLC on glass plates (Sigma-Aldrich), and spots were detected under UV-254 nm then charring with 5% (v/v) sulfuric acid in ethanol or cerium molybdate stain (CAM) followed by heating with a heat gun. NMR spectra were recorded on a Bruker-400 MHz spectrometer with $CDCl_3$ or D_2O as the solvent. Coupling constants (J) are reported in Hertz. The chemical shifts were assigned in ppm, and multiplicities are indicated by s (singlet), d (doublet), t (triplet), and m (multiplet). MALDI-TOF analysis was performed on a Bruker Autoflex Speed mass spectrometer with DHB ($MeCN/H_2O$ = 1:1) as the matrix (positive reflectron mode). HRMS was performed on an Exactive Plus Orbitrap mass spectrometer (Thermo Scientific) equipped with a C18 column. Preparative HPLC was performed with a Waters 600 HPLC instrument and Waters C18 columns (5.0 μm, 10 × 250 mm). The column was eluted with a gradient of $MeCN–H_2O$ containing 0.1% TFA or 0.1% FA at a flow rate of 4 mL/min. LC–MS analysis was performed on an Ultimate 3000 HPLC system coupled to an Exactive Plus Orbitrap mass spectrometer (Thermo Fischer Scientific) with C4 (whole antibody, gradient 5–95% aq MeCN containing 0.1% FA for 6 min, 0.4 mL/min) or C8 (IdeS digestion, gradient 25–35% aq MeCN containing 0.1% FA for 6 min, 0.4 mL/min) column. Deconvolution data was transformed by MagTran software.

6-O-2-[2-(2-Aminoethoxy)ethoxy]ethyl-β-D-mannopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (5).—A mixture of **4**[18] (15.0 mg, 0.014 mmol) and Pd/C (10 wt.% loading, 10 mg) in THF/H_2O (1.5 mL/0.5 mL) was added 3 M HCl (aq, 9 μL, 2 equiv) and then stirred under a H_2 atmosphere overnight. After LC–MS analysis showed the complete conversion to free amine, the reaction mixture was filtered through a Celite pad and then concentrated and directly purified by Sephadex LH-20 (H_2O) to give **5** (7.2 mg, 97%) as hydrochloride salt. ^{1}H NMR (400 MHz, D_2O) δ 5.07 (0.77H, m), 4.61−4.59 (1.31H, m), 3.96−3.95 (1.00H, m), 3.85−3.80 (0.81H, m), 3.78−3.76 (1.90H, m), 3.75−3.73 (1.04H, m), 3.69−3.67 (0.99H, m), 3.66−3.63 (3.34H, m), 3.63−3.56 (11.1H, m), 3.54−3.49 (1.90H, m), 3.48−3.43 (2.39H, m), 3.10 (2H, t, J = 4.6 Hz), 1.92 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 174.47, 174.17, 100.19, 94.81, 90.50, 79.54, 79.25, 74.68, 74.49, 72.64, 72.36, 70.44, 70.40, 69.92, 69.88, 69.73, 69.58, 69.40, 69.16, 66.58, 66.29, 63.81, 60.16, 60.03, 56.06, 53.63, 39.06, 22.13, 21.84; HRMS: $[M + H]^+$ calcd for $C_{20}H_{39}N_2O_{13}^+$, 515.2447; found, 515.2442.

Benzyl 2-O-benzyl-4,6-O-benzylidene-3-O-p-methoxybenzyl-β-D-glucopyranosyl-(1 → 4)-2-azido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (8).—To a solution of **7**[20] (290 mg, 0.31 mmol) in MeOH (2.0 mL) was added sodium methoxide to maintain a pH of 10; the solution was heated to 50 °C and stirred overnight. After the complete disappearance of the starting material, the solution was diluted with CH_2Cl_2, successively washed with H_2O and brine, and then concentrated to dryness. The residue was dissolved in dry *N*,*N*-dimethylformamide (2.0 mL) and cooled to 0 °C; sodium hydride (24 mg) and benzyl bromide (100 μL) were added successively, and the mixture was allowed to warm to room temperature. After the completion of the reaction as indicated by TLC, MeOH was added to quench the excess sodium hydride. The reaction was diluted with CH_2Cl_2 and successively washed with H_2O and brine. The organic layer was dried over anhydrous Na_2SO_4. After concentration, the residue was purified by flash column chromatography (hexanes/EtOAc = 10:1−5:1) to afford **8** (248 mg, 87% for 2 steps) as a white solid. R_f = 0.45 (hexanes/EtOAc = 5:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53−7.51, 7.45−7.26 (27H, m, Ar-*H*), 6.85−6.83 (2H, m, Ar-*H*), 5.52 (1H, s, PhC*H*), 4.97−4.93 (2H, m, PhCH_2), 4.88−4.83 (2H, m, PhCH_2), 4.78−4.68 (4H, m, PhCH_2), 4.63 (1H, d, *J* = 12.1 Hz, PhCH_2), 4.56 (1H, d, *J* = 7.8 Hz), 4.43 (1H, d, *J* = 12.1 Hz, PhCH_2), 4.31 (1H, d, *J* = 8.1 Hz), 4.21 (1H, dd, *J* = 5.0 Hz, *J* = 10.5 Hz), 4.06 (1H, t, *J* = 9.3 Hz), 3.88 (1H, dd, *J* = 3.7 Hz, *J* = 11.1 Hz), 3.82 (3H, s, OCH_3), 3.70−3.58 (3H, m), 3.54−3.45 (2H, m), 3.40−3.33 (2H, m), 3.30−3.26 (1H, m), 3.22−3.15 (1H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.26, 138.37, 138.31, 138.05, 137.42, 136.89, 130.62, 129.70, 129.00, 128.48, 128.45, 128.32, 128.27, 128.25, 128.07, 127.97, 127.95, 127.82, 127.73, 127.68, 126.07, 113.75, 102.79, 101.14, 100.38, 82.54, 81.78, 81.33, 80.83, 76.46, 75.51, 75.25, 75.18, 74.75, 73.30, 70.79, 68.71, 67.62, 65.85, 65.73, 55.29; MALDI-TOF: $[M + Na]^+$ calcd for $C_{55}H_{57}N_3NaO_{11}^+$, 958.39; found, 958.05.

Benzyl 2,4-di-O-benzyl-3-O-p-methoxybenzyl-β-D-glucopyranosyl-(1 → 4)-2-azido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (9).—To a solution of **8** (110 mg, 0.118 mmol) in BH_3·THF (1 M, 2.0 mL) was added a solution of Bu_2BOTf in CH_2Cl_2 (1 M, 200 μL) under an argon atmosphere at 0 °C, and the mixture was stirred at 0 °C for 40 min when TLC indicated the completion of the reaction. Et_3N (150 μL) was added to the mixture followed by careful addition of MeOH (500 μL). The mixture was co-evaporated with MeOH for three times, and the residue was purified by silica gel flash chromatography (hexanes/EtOAc = 6:1−2:1) to afford **9** (100 mg, 91%) as a white solid. R_f = 0.30 (hexanes/EtOAc = 3:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45−7.32, 7.25−7.23 (27H, m, Ar-*H*), 6.88−6.86 (2H, m, Ar-*H*), 5.00−4.96 (2H, m, PhCH_2), 4.88−4.84 (3H, m, PhCH_2), 4.83−4.78 (3H, m, PhCH_2), 4.73 (1H, d, *J* = 12.0 Hz, PhCH_2), 4.67−4.62 (2H, m), 4.51−4.48 (2H, m), 4.34 (1H, d, *J* = 8.1 Hz), 4.02 (1H, t, *J* = 9.4 Hz), 3.87 (1H, dd, *J* = 3.7 Hz, *J* = 11.1 Hz), 3.83 (3H, s, OCH_3), 3.73−3.66 (2H, m), 3.60−3.52 (2H, m), 3.48 (1H, t, *J* = 9.1 Hz), 3.41−3.31 (4H, m), 3.22−3.17 (1H, m), 1.68 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.28, 138.42, 138.36, 138.13, 137.96, 136.91, 130.65, 129.56, 128.51, 128.42, 127.99, 127.91, 127.89, 127.81, 127.74, 127.71, 127.41, 113.87, 102.51, 100.41, 84.48, 82.86, 81.35, 77.97, 76.46, 75.50, 75.19, 75.16, 75.08, 75.04, 74.87, 73.40, 70.81, 67.60, 65.91, 61.84, 55.32; MALDI-TOF: $[M + Na]^+$ calcd for $C_{55}H_{59}N_3NaO_{11}^+$, 960.40; found, 959.99.

Benzyl 2,4-di-O-benzyl-3-O-p-methoxybenzyl-β-D-glucopyranosyl-(1 → 4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (10).—A solution of **9** (35.0 mg, 0.037 mmol) in a mixture of AcSH/pyridine/$CHCl_3$ (0.6 mL/0.4 mL/0.6 mL) was stirred at 50 °C for 15 h. After the completion of the reaction as monitored by TLC, the resulting mixture was concentrated and the residue was subjected to flash chromatography on silica gel (hexanes/Acetone = 4:1−2:1) to afford **10** (31.6 mg, 89%) as a white solid. R_f = 0.20 (hexanes/acetone = 3:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40−7.28, 7.23−7.21 (27H, m, Ar-*H*), 6.86−6.84 (2H, m, Ar-*H*), 5.65 (1H, d, *J* = 7.6 Hz), 5.00 (1H, d, *J* = 7.6 Hz), 4.94−4.92 (2H, m), 4.89−4.87 (1H, m), 4.86 (1H, m), 4.84−4.75 (3H, m), 4.67−4.58 (4H, m), 4.53−4.46 (2H, m), 4.19 (1H, t, *J* = 9.0 Hz), 3.89 (1H, t, *J* = 8.6 Hz), 3.87 (1H, dd, *J* = 3.9 Hz, *J* = 11.0 Hz), 3.82 (3H, s, OCH_3), 3.75−3.69 (2H, m), 3.59−3.54 (2H, m), 3.48−3.34 (4H, m), 3.23−3.20 (1H, m), 1.85 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.47, 159.23, 138.83, 138.37, 138.12, 138.08, 137.58, 130.68, 129.51, 128.47, 128.44, 128.41, 128.40, 127.98, 127.90, 127.87, 127.81, 127.78, 127.70, 127.51, 113.83, 102.63, 98.91, 84.41, 82.81, 77.95, 77.76, 77.34, 75.43, 74.99, 74.85, 74.28, 73.32, 70.86, 68.11, 61.96, 56.87, 55.29, 23.61; MALDI-TOF: $[M + Na]^+$ calcd for $C_{57}H_{63}NNaO_{12}^+$, 976.42; found, 976.25.

Benzyl 2,4-di-O-benzyl-3-O-p-methoxybenzyl-6-O-2-[2(2-azidoethoxy)ethoxy]ethyl-β-D-glucopyranosyl-(1 → 4)-2acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (11).—To a solution of **10** (23.0 mg, 0.024 mmol) and the tosylate linker[25] (23.8 mg, 0.073 mmol) in anhydrous DMF (0.6 mL) was added 60% sodium hydride (5.0 mg, 0.125 mmol) at 0 °C. After stirring for 0.5 h at 0 °C then 6 h at room temperature, MeOH (50 μL) and AcOH (10 μL) were added at 0 °C, and the reaction mixture was concentrated to dryness. The residue was purified by column chromatography on silica-gel (hexanes/acetone = 5:1 ~ 3:2) to give **11** (22.7 mg, 85%) as a white solid. R_f = 0.30 (hexanes/Acetone = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39−7.26 7.22−7.20 (27H, m, Ar-*H*), 6.85−6.83 (2H, m, Ar-*H*), 5.67 (1H, d, NH, *J* = 7.8 Hz), 4.94−4.82 (6H, m), 4.79−4.74 (2H, m), 4.68−4.63 (2H, m), 4.61−4.56 (2H, m), 4.51−4.45 (2H, m), 4.05−4.03 (2H, m), 3.88 (1H, dd, *J* = 4.1 Hz, *J* = 10.7 Hz), 3.81 (3H, s, OCH_3), 3.77−3.65 (3H, m), 3.61−3.48 (14H, m), 3.39 (1H, t, *J* = 8.1 Hz), 3.34−3.28 (3H, m), 1.82 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.14, 159.19, 139.09, 138.43, 138.40, 138.17, 137.66, 130.77, 129.49, 128.43, 128.41, 128.40, 128.33, 128.26, 128.13, 128.06, 127.96, 127.88, 127.85, 127.80, 127.74, 127.69, 127.66, 127.63, 127.47, 113.80, 102.82, 99.13, 84.50, 82.76, 77.86, 77.73, 76.92, 75.39, 75.06, 75.02, 74.99, 74.81, 73.61, 73.25, 70.87, 70.69, 70.59, 70.55, 69.93, 69.85, 68.38, 55.50, 55.29, 50.60, 23.50; MALDI-TOF MS: $[M + Na]^+$ calcd for $C_{63}H_{74}N_4NaO_{14}^+$, 1133.51; found, 1133.27.

6-O-2-[2-(2-Aminoethoxy)ethoxy]ethyl-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (12).—A mixture of **11** (20.0 mg, 0.018 mmol) and Pd/C (10 wt.% loading, 10 mg) in THF/H_2O (1.5 mL/0.5 mL) was added 3 M HCl (aq, 12 μL, 2 equiv) and then stirred under a H_2 atmosphere overnight. After LC–MS analysis showed the complete deprotection and conversion of the azide to free amine, the reaction mixture was filtered through a Celite pad. The filtrate was concentrated, and the residue was purified by Sephadex LH-20 (H_2O) to give **12** (9.2 mg, 93%) as hydrochloride

salt. ^{1}H NMR (400 MHz, D_2O) δ 5.09 (0.52H, m), 4.62 (0.43H, d, J = 7.9 Hz), 4.43−4.39 (1.04H, m), 4.26−4.22 (0.22H, m), 4.04−4.01 (0.26H, m), 3.89−3.82 (1.40H, m), 3.80−3.73 (3.73H, m), 3.67−3.55 (13.8H, m), 3.55−3.51 (2.34H, m), 3.43−3.38 (1.57H, m), 3.36−3.31 (1.26H, m), 3.24−3.18 (1.31H, m), 3.11 (1.18, t, J = 4.9 Hz), 1.94 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 174.53, 174.29, 102.59, 94.78, 90.51, 79.45, 79.11, 75.35, 74.69, 74.48, 73.05, 72.42, 70.10, 70.02, 69.63, 69.58, 69.46, 69.20, 69.15, 66.56, 60.34, 59.99, 59.85, 56.22, 53.74, 39.11, 22.16, 21.87; HRMS: $[M + H]^+$ calcd for $C_{20}H_{39}N_2O_{13}^+$, 515.2447; found, 515.2440.

6-O-2-[2-(2-Azidoethoxy)ethoxy]ethyl-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (13).—To a solution of **12** (9.0 mg, 0.016 mmol) in H_2O (1.0 mL) was added a freshly prepared solution of TfN_3[26] in CH_2Cl_2 (0.5 mL, ~0.16 mmol) containing K_2CO_3 (6.8 mg) and $CuSO_4$ (0.8 mg) at 0 °C. Then MeOH was added to make the solution homogenous, and the mixture was stirred at room temperature for 36 h. The reaction mixture was filtered, and the filtrate was concentrated to dryness. The residue was purified on a Sephadex LH-20. Fractions containing the product were pooled and lyophilized and then further purified by preparative RP-HPLC (gradient, 5–15% aq MeCN containing 0.1% FA for 30 min; flow rate, 4 mL/min) to give **13** (7.2 mg, 81%) as a white solid after lyophilization. ^{1}H NMR (400 MHz, D_2O) δ 5.09 (0.58H, d, J = 2.5 Hz), 4.61 (0.47H, d, J = 7.7 Hz), 4.42−4.40 (0.97H, m), 3.88−3.83 (1.23H, m), 3.80−3.75 (2.89H, m), 3.75−3.73 (1.01H, m), 3.65−3.56 (12.9H, m), 3.53−3.49 (1.75H, m), 3.43−3.38 (2.83H, m), 3.36−3.31 (1.18H, m), 3.24−3.19 (1.20H, m), 1.94 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 174.51, 174.27, 102.61, 94.79, 90.48, 79.57, 79.30, 75.34, 74.70, 74.50, 73.05, 72.40, 70.11, 69.58, 69.53, 69.49, 69.19, 69.14, 60.05, 59.90, 56.20, 53.74, 50.10, 22.14, 21.84; HRMS: $[M + H]^+$ calcd for $C_{20}H_{37}N_4O_{13}^+$, 541.2352; found, 541.2344.

2-Methyl-{6-O-2-[2-(2-azidoethoxy)ethoxy]ethyl-β-D-glucopyranosyl-(1 → 4)-1,2-dideoxy-α-D-glucopyrano}-[2,1-d]2-oxazoline (14).—To a solution of **13** (3.3 mg, 6.1 μmol) in H_2O (150 μL) were added Et_3N (30 mol equiv) and 2-chloro1,3-dimethylimidazolinium chloride (DMC, 20 mol equiv) at 0 °C. The reaction mixture was kept at this temperature for 8 h then purified by gel filtration on a Sephadex G-10 column that was eluted with 0.1% aq Et_3N to afford **14** (3.0 mg, 94%) as a white solid after lyophilization with aq. NaOH (0.05 mol equiv, to keep the product at a basic condition). ^{1}H NMR (400 MHz, D_2O) δ 5.96 (1H, d, J = 7.3 Hz), 4.35 (1H, d, J = 7.9 Hz), 4.26−4.23 (1H, m), 4.08−4.06 (1H, m), 3.78−3.75 (1H, m), 3.70 (1H, dd, J = 2.3 Hz, J = 12.4 Hz), 3.64−3.57 (12H, m), 3.54−3.52 (1H, m), 3.47−3.43 (1H, m), 3.40−3.37 (2H, m), 3.34−3.29 (2H, m), 3.18−3.14 (1H, m), 2.88−2.86 (1H, m), 1.94 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 168.29, 104.14, 99.72, 78.35, 75.35, 74.74, 72.98, 70.79, 70.22, 69.58, 69.48, 69.18, 65.12, 61.52, 50.07, 12.85; HRMS: $[M + H]^+$ calcd for $C_{20}H_{35}N_4O_{12}^+$, 523.2246; found, 523.2233.

Benzyl 2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl-(1 → 4)-2-azido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (17).—A mixture of trichloroacetimidate donor **15**[27] (810 mg, 1.65 mmol), acceptor **16**[18] (570 mg, 1.2 mmol), and activated 4 Å molecular sieves (1.5 g) in anhydrous CH_2Cl_2 (15.0 mL) was stirred at room temperature under an argon atmosphere for 1.5 h. It was then cooled to −40 °C, and TMSOTf (27 μL, 0.15 mmol)

was added. After stirring at −40 °C for 50 min, the mixture was quenched with triethylamine (50 μL). The mixture was filtered, and the filtrate was concentrated in *vacuo*. The residue was purified by flash silica gel chromatography (hexanes/EtOAc = 10:1−3:2) to give **17** (832 mg, 86%) as a white foam. R_f = 0.30 (hexanes/EtOAc = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46−7.30 (15H, m, Ar-*H*), 5.29 (1H, d, *J* = 3.3 Hz), 5.13 (1H, dd, *J* = 8.0 Hz, *J* = 10.4 Hz), 4.99−4.93 (2H, m), 4.85 (1H, dd, *J* = 3.5 Hz, *J* = 10.4 Hz), 4.81−4.74 (2H, m), 4.70 (1H, d, *J* = 12.0 Hz, PhC*H*), 4.60 (1H, d, *J* = 8.0 Hz), 4.50 (1H, d, *J* = 12.0 Hz, PhC*H*), 4.30 (1H, d, *J* = 8.1 Hz), 4.07−4.01 (2H, m), 3.87 (1H, dd, *J* = 6.0 Hz, *J* = 11.1 Hz), 3.79−3.72 (2H, m), 3.59 (1H, t, *J* = 7.0 Hz), 3.50 (1H, dd, *J* = 8.3 Hz, *J* = 9.8 Hz), 3.37 (1H, t, *J* = 9.1 Hz), 3.32−3.30 (1H, m), 2.13 (3H, s), 2.01 (3H, s), 2.00 (3H, s), 1.99 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.19, 170.07, 169.18, 138.21, 137.73, 136.76, 128.63, 128.49, 128.23, 128.13, 128.04, 128.00, 127.95, 127.93, 127.72, 100.51, 100.07, 80.95, 76.05, 75.14, 74.85, 73.70, 70.95, 70.93, 70.52, 69.58, 67.44, 66.80, 65.78, 60.60, 20.78, 20.66, 20.64, 20.59; MALDI-TOF: $[M + Na]^+$ calcd for $C_{41}H_{47}N_3NaO_{14}^+$, 828.29; found, 828.05.

Benzyl 2,3,4-Tri-O-benzyl-6-o-(tert-butyldiphenylsilyl)-β-D-galactopyranosyl-(1 → 4)-2-azido-3,6-di-O-benzyl-2deoxy-β-D-glucopyranoside (18).—To a solution of **17** (340 mg, 0.422 mmol) in MeOH (4.0 mL) was added sodium methoxide until pH = 10, the solution was heated to 50 °C and stirred overnight. After the complete disappearance of the starting material, the reaction mixture was diluted with CH_2Cl_2 and successively washed with H_2O and brine. The organic layer was dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness to give the crude deacylated intermediate. The residue was then dissolved in dry *N*,*N*-dimethylformamide (3.0 mL); imidazole (144 mg, 2.11 mmol) and *tert*-butyl(chloro)diphenylsilane (312 μL, 1.2 mmol) were added successively; and the resulting mixture was stirred at room temperature until the completion of the reaction as indicated by TLC. The reaction mixture was diluted with CH_2Cl_2 and successively washed with H_2O and brine. The organic layer was dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness. Then, the residue was dissolved in dry *N*,*N*-dimethylformamide (3.0 mL) and cooled to 0 °C, sodium hydride (135 mg, 3.38 mmol) and benzyl bromide (300 μL, 2.53 mmol) were added successively, and the mixture was slowly warmed to room temperature. After the completion of the reaction as monitored by TLC, MeOH was added to quench the excess sodium hydride. The reaction mixture was diluted with CH_2Cl_2 and successively washed with H_2O and brine. The organic layer was dried over anhydrous Na_2SO_4 and filtered. The filtrated was concentrated, and the residue was purified by flash column chromatography on silica gel (hexanes/EtOAc = 15:1−8:1) to give **18** (328 mg, 68% for 3 steps) as a colorless syrup. R_f = 0.40 (hexanes/EtOAc = 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59−7.55, 7.46−7.25 (40H, m, Ar-*H*), 5.12 (1H, d, *J* = 11.4 Hz), 5.00 (1H, d, *J* = 10.0 Hz), 4.94 (1H, d, *J* = 12.1 Hz), 4.86−4.76 (4H, m), 4.71−4.62 (3H, m), 4.60−4.58 (2H, m), 4.44−4.37 (2H, m), 4.28 (1H, d, *J* = 8.1 Hz), 4.07 (1H, d, *J* = 2.3 Hz), 3.96 (1H, t, *J* = 9.2 Hz), 3.89−3.84 (2H, m), 3.79 (1H, t, *J* = 9.5 Hz), 3.73−3.70 (1H, m), 3.65 (1H, dd, *J* = 9.5 Hz, *J* = 5.0 Hz), 3.48−3.42 (2H, m), 3.33−3.23 (3H, m), 1.08 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.27, 138.88, 138.68, 138.31, 138.01, 136.99, 135.57, 134.86, 133.25, 133.19, 129.85, 129.73, 128.54, 128.48, 128.37, 128.29, 128.17, 128.10, 128.04, 127.97, 127.92, 127.88, 127.81, 127.77, 127.71, 127.64, 127.58, 127.51, 127.38, 127.21, 102.75, 100.45, 82.44, 81.38, 80.22, 76.11, 75.50, 75.40,

75.32, 74.79, 74.33, 73.76, 73.26, 72.84, 70.76, 67.85, 65.67, 61.16, 27.02, 26.96, 26.62, 19.23; MALDI-TOF: $[M + Na]^+$ calcd for $C_{70}H_{75}N_3NaO_{10}Si^+$, 1168.51; found, 1168.22.

Benzyl 2,3,4-Tri-O-benzyl-6-O-(tert-butyldiphenylsilyl)-β-D-galactopyranosyl-(1 → 4)-2-acetamido-3,6-di-O-benzyl2-deoxy-β-D-glucopyranoside (19).—A solution of **18** (300 mg, 0.262 mmol) in a mixture of AcSH/pyridine/$CHCl_3$ (0.8 mL/0.6 mL/0.8 mL) was stirred at 50 °C for 14 h. After the completion of the reaction as monitored by TLC, the resulting mixture was concentrated and the residue was subjected to flash chromatography on silica gel (hexanes/EtOAc = 4:1−1:1) to afford **19** (240 mg, 79%) as a colorless syrup. R_f = 0.30 (hexanes/EtOAc = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60−7.56, 7.44−7.23, 7.20−7.18 (40H, m, Ar-*H*), 5.78 (1H, d, *J* = 7.7 Hz), 5.08 (1H, d, *J* = 11.3 Hz), 4.97 (1H, d, *J* = 6.8 Hz), 4.89 (1H, d, *J* = 11.9 Hz), 4.86−4.77 (5H, m), 4.63−4.51 (4H, m), 4.42−4.39 (2H, m), 4.06−4.02 (2H, m), 3.94 (1H, t, *J* = 7.5 Hz), 3.89−3.76 (4H, m), 3.68−3.64 (2H, m), 3.55−3.49 (1H, m), 3.45 (1H, dd, *J* = 9.8 Hz, *J* = 2.8 Hz), 3.30−3.26 (1H, m), 1.81 (3H, s), 1.07 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.12, 139.12, 138.70, 138.62, 138.50, 138.37, 137.68, 135.53, 133.14, 129.87, 129.75, 128.44, 128.32, 128.30, 128.22, 128.12, 128.10, 127.99, 127.89, 127.82, 127.79, 127.76, 127.68, 127.66, 127.56, 127.53, 127.37, 127.19, 103.13, 99.06, 82.26, 80.08, 77.13, 76.66, 75.32, 75.24, 74.65, 74.31, 73.84, 73.68, 73.16, 72.89, 70.74, 68.73, 61.38, 55.02, 26.95, 23.47, 19.19; MALDI-TOF: $[M + Na]^+$ calcd for $C_{72}H_{79}NNaO_{11}Si^+$, 1184.53; found, 1184.07.

Benzyl 2,3,4-Tri-O-benzyl-β-D-galactopyranosyl-(1 → 4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (20).—To a solution of **19** (215 mg, 0.185 mmol) in THF (2.0 mL) was added TBAF (1 M in THF, 900 μL), and the mixture was stirred at 40 °C for 2 h. After the completion of the reaction as monitored by TLC, the resulting mixture was concentrated and the residue was subjected to flash chromatography on silica gel (hexanes/EtOAc = 5:1−1:1) to afford **20** (140 mg, 82%) as a colorless syrup. R_f = 0.25 (hexanes/acetone = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38−7.24 (30H, m, Ar-*H*), 5.70 (1H, d, *J* = 7.8 Hz), 4.99−4.93 (3H, m), 4.90 (1H, d, *J* = 12.1 Hz), 4.85−4.73 (4H, m), 4.64−4.54 (4H, m), 4.44−4.41 (2H, m), 4.13 (1H, t, *J* = 8.2 Hz), 3.96 (1H, t, *J* = 8.0 Hz), 3.88−3.78 (3H, m), 3.74−3.73 (1H, m), 3.69−3.62 (2H, m), 3.55−3.49 (1H, m), 3.45−3.36 (2H, m), 3.26−3.23 (1H, m), 1.85 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.42, 138.67, 138.62, 138.49, 138.39, 138.29, 137.61, 128.47, 128.38, 128.36, 128.32, 128.25, 128.19, 128.10, 127.92, 127.77, 127.74, 127.57, 127.54, 127.50, 103.19, 98.95, 82.48, 79.92, 77.48, 75.34, 75.13, 75.00, 74.41, 74.38, 73.71, 73.17, 73.11, 70.72, 68.64, 62.00, 55.71, 23.57; MALDI-TOF: $[M + Na]^+$ calcd for $C_{56}H_{61}NNaO_{11}^+$, 946.41; found, 946.04.

Benzyl 2,3,4-Tri-O-benzyl-6-O-2-[2-(2-azidoethoxy)ethoxy]ethyl-β-D-galactopyranosyl-(1 → 4)-2-acetamido3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (21).—To a solution of **20** (120 mg, 0.13 mmol) and the tosylate linker[25] (128 mg, 0.39 mmol) in anhydrous DMF (2.5 mL) was added 60% sodium hydride (26 mg, 0.65 mmol) at 0 °C. After stirring for 0.5 h at 0 °C then 6 h at room temperature, the reaction was diluted with CH_2Cl_2, successively washed with H_2O and brine and dried over anhydrous Na_2SO_4. The mixture was filtered, and the filtrate was concentrated. The residue was purified by column chromatography on silica-gel (hexanes/acetone = 6:1−2:1)

to give **21** (120 mg, 85%) as a colorless syrup. R_f = 0.30 (hexanes/Acetone = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37−7.23 (30H, m, Ar-*H*), 5.78 (1H, d, *J* = 7.7 Hz), 4.99 (1H, d, *J* = 11.5 Hz), 4.96−4.89 (3H, m), 4.87−4.80 (2H, m), 4.76−4.74 (2H, m), 4.64−4.54 (4H, m), 4.47−4.41 (2H, m), 4.07 (1H, t, *J* = 8.0 Hz), 4.00 (1H, t, *J* = 7.5 Hz), 3.93 (1H, d, *J* = 2.5 Hz), 3.88−3.84 (1H, m), 3.81−3.77 (2H, m), 3.69−3.60 (8H, m), 3.57−3.51 (4H, m), 3.48−3.40 (4H, m), 3.35 (2H, t, *J* = 5.0 Hz), 1.86 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.12, 138.98, 138.88, 138.66, 138.50, 138.34, 137.68, 128.39, 128.34, 128.31, 128.21, 128.16, 127.99, 127.90, 127.84, 127.69, 127.59, 127.55, 127.49, 127.46, 103.15, 99.12, 82.26, 79.92, 75.34, 75.22, 74.62, 73.76, 73.60, 73.13, 72.72, 70.70, 70.61, 70.55, 70.39, 70.04, 69.05, 68.72, 55.12, 50.65, 23.53; MALDI-TOF: $[M + Na]^+$ calcd for $C_{62}H_{72}N_4NaO_{13}^+$, 1103.50; found, 1103.10.

6-O-2-[2-(2-Aminoethoxy)ethoxy]ethyl-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (22).—To a mixture of **21** (75.0 mg, 0.069 mmol) and Pd/C (10 wt.% loading, 40 mg) in THF/H_2O (4.5 mL/1.5 mL) was added 3 M HCl (aq, 46 μL, 2 equiv), and then the mixture was stirred under a H_2 atmosphere overnight. After ESI-MS indicated the completion deprotection and conversion of the azide to amine, the reaction mixture was filtered through a Celite pad. The filtrate was concentrated, and the residue was purified by Sephadex LH-20 (H_2O) to give **22** (37.2 mg, 97%) as hydrochloride salt. ^{1}H NMR (400 MHz, D_2O) δ 5.10 (0.56H, m), 4.62 (0.42H, d, *J* = 7.6 Hz), 4.37 (1.05H, d, *J* = 7.9 Hz), 3.90−3.82 (2.22H, m), 3.81−3.76 (3.46H, m), 3.74−3.70 (0.71H, m), 3.65−3.55 (15.61H, m), 3.47−3.42 (1.28H, m), 3.00−2.98 (1.61H, m), 1.94 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 174.51, 174.26, 102.98, 94.83, 90.51, 79.40, 79.07, 74.69, 73.33, 72.48, 72.40, 71.67, 70.80, 70.09, 70.00, 69.87, 69.62, 69.55, 69.49, 69.42, 69.21, 68.67, 67.95, 67.91, 60.35, 60.08, 59.93, 56.12, 53.70, 39.30, 22.18, 21.89; HRMS: $[M + H]^+$ calcd for $C_{20}H_{39}N_2O_{13}^+$, 515.2447; found, 515.2440.

6-O-2-[2-(2-Azidoethoxy)ethoxy]ethyl-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (23).—To a solution of **22** (9.0 mg, 0.016 mmol) in H_2O (1.0 mL) was added a freshly prepared solution of TfN_3[26] in CH_2Cl_2 (0.5 mL, ~0.16 mmol) containing K_2CO_3 (6.8 mg) and $CuSO_4$ (0.8 mg) at 0 °C, and then MeOH was added to make the solution homogenous. The mixture was stirred at room temperature for 36 h, and then the reaction mixture was filtered. The filtrate was concentrated to dryness, and the residue was purified on a Sephadex LH-20 column by elution with H_2O. Fractions containing the product were pooled and lyophilized and then further purified by preparative RP-HPLC (gradient, 5–15% aq MeCN containing 0.1% FA for 30 min; flow rate, 4 mL/min) to give **23** (7.5 mg, 85%) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 5.09 (0.64H, m), 4.61 (0.43H, d, *J* = 7.6 Hz), 4.37 (1.01H, d, *J* = 7.8 Hz), 3.88−3.82 (2.28H, m), 3.79−3.75 (3.59H, m), 3.74−3.70 (0.62H, m), 3.67−3.54 (15.67H, m), 3.51−3.50 (0.41H, m), 3.47−3.38 (2.97H, m), 3.12−3.09 (0.23H, m), 1.94 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 174.50, 174.25, 102.98, 94.82, 90.47, 79.44, 79.17, 74.72, 73.36, 72.46, 72.40, 70.79, 70.12, 69.94, 69.57, 69.55, 69.49, 69.18, 68.64, 60.11, 59.96, 56.12, 53.71, 50.11, 22.14, 21.84; HRMS: $[M + H]^+$ calcd for $C_{20}H_{37}N_4O_{13}^+$, 541.2352; found, 541.2336.

2-Methyl-{6-O-2-[2-(2-azidoethoxy)ethoxy]ethyl-β-D-galactopyranosyl-(1 → 4)-1,2-dideoxy-α-D-glucopyrano}-[2,1-d]-2-oxazoline (24).—To a solution of compound **23** (5.3 mg, 9.8 μmol) in H_2O (200 μL) were added Et_3N (30 mol equiv) and 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 20 mol equiv) at 0 °C. The reaction mixture was kept at this temperature for 7 h then purified by gel filtration on a Sephadex G-10 column that was eluted with 0.1% aq Et_3N to afford **24** (4.9 mg, 96%) as a white solid after lyophilization with aqueous NaOH (0.05 mol equiv). ^{1}H NMR (400 MHz, D_2O) δ 5.98 (1H, d, J = 7.3 Hz), 4.31 (1H, d, J = 7.8 Hz), 4.29−4.27 (1H, m), 3.80 (1H, d, J = 3.1 Hz), 3.74−3.69 (2H, m), 3.67−3.57 (14H, m), 3.56−3.50 (2H, m), 3.42−3.38 (3H, m), 3.36−3.32 (1H, m), 1.96 (3H, s); ^{13}C NMR (100 MHz, D_2O) δ 168.22, 104.68, 99.86, 78.52, 73.50, 72.49, 71.02, 70.79, 70.23, 70.07, 69.57, 69.55, 69.51, 69.37, 69.20, 68.85, 65.29, 61.67, 50.12, 12.90; HRMS: $[M + H]^+$ calcd for $C_{20}H_{35}N_4O_{12}^+$, 523.2246; found, 523.2236.

Compound (25).—To a solution of commercial trastuzumab (100 μg) in a PBS buffer (4 μL, 150 mM, pH = 7.0) was added oxazoline **6**[18] (14.0 μg, 20 equiv per reaction site) and wild-type Endo-S2 (0.1 μg). The reaction was incubated at 28 °C and monitored by LC–MS aliquots. Within 30 min, LC–MS analysis indicated the disappearance of the starting material and the formation of the desired product 25 with complete transglycosylation at both reaction sites. LC–MS analysis: for whole antibody (*m*/*z*), calculated, M = 146,909 Da; found 146,912 (deconvoluted data); for Fc domain released by IdeS digestion (*m*/*z*), calculated, M = 24,656 Da; found 24,656 (deconvoluted data).

Compound (26).—To a solution of commercial trastuzumab (100 μg) in a PBS buffer (4 μL, 150 mM, pH = 7.0) was added oxazoline **14** (14.0 μg, 20 equiv per reaction site) and wild-type Endo-S2 (0.3 μg). The reaction was incubated at 28 °C and monitored by LC–MS aliquots. Within 2 h, LC–MS analysis indicated the disappearance of the starting material and the formation of the desired product **26** with complete transglycosylation at both reaction sites. LC–MS analysis: for whole antibody (*m*/*z*), calculated, M = 146,909 Da; found 146,911 (deconvoluted data); for Fc domain released by IdeS digestion (*m*/*z*), calculated, M = 24,656 Da; found 24,656 (deconvoluted data).

Compound (27).—To a solution of commercial trastuzumab (100 μg) in a PBS buffer (4 μL, 150 mM, pH = 7.0) was added oxazoline **24** (14.0 μg, 20 equiv per reaction site) and wild-type Endo-S2 (0.5 μg). The reaction was incubated at 28 °C and monitored by LC–MS aliquots. Within 2 h, LC–MS analysis indicated the disappearance of the starting material and the formation of the desired product **27** with complete transglycosylation at both reaction sites. LC–MS analysis: for whole antibody (*m*/*z*), calculated, M = 146,909 Da; found 146,912 (deconvoluted data); for Fc domain released by IdeS digestion (*m*/*z*), calculated, M = 24,656 Da; found 24,657 (deconvoluted data).

Compound (29).—To a solution of bis-PEG5-NHS (6.0 mg, 11.3 μmol) in anhydrous DMSO (100 μL) was added **28**[17] (3.2 mg, 2.8 μmol) in anhydrous DMSO (100 μL) in 5 portions every 10 min, and Et_3N was added to keep the pH = 8.5. After the completion of the reaction as monitored by LC–MS, 10% TFA (aq) was added (60 μL), and the reaction mixture was directly purified by preparative-HPLC (gradient, 30–70% aq MeCN containing

0.1% TFA for 40 min, 4 mL/min) to give **29** (3.6 mg, 82%) as a white foam. RP-HPLC retention time, t_R = 22.5 min (gradient, 20–70% aq MeCN containing 0.1% FA for 30 min; flow rate, 0.4 mL/min). ESI-MS $[M + H]^+$ calcd for $C_{76}H_{122}N_{11}O_{22}^+$, 1540.88; found, 1541.29; $[M + Na]^+$ calcd for $C_{76}H_{121}N_{11}NaO_{22}^+$, 1562.86; found, 1563.28.

Compound (1).—To a solution of **5** (1.4 mg, 2.55 μmol) and **29** (2.1 mg, 1.36 μmol) in anhydrous DMSO (40 μL) was added Et_3N (0.6 μL) to adjust pH = 8.5. The mixture was kept at room temperature until the complete consumption of **29** to give the crude product **30** in DMSO that was directly used in next step without further purification. RP-HPLC retention time for **30**, t_R = 16.9 min (gradient, 20–70% aq MeCN containing 0.1% FA for 30 min; flow rate, 0.4 mL/min). HRMS: $[M + H]^+$ calcd for $C_{92}H_{155}N_{12}O_{32}^+$, 1941.0898; found, 1941.0847. To the residue obtained in the first step was added H_2O (80 μL) and Et_3N (40 mol equiv); the mixture was cooled to 0 °C; and 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 30 mol equiv) was added. After 12 h at 0 °C, the reaction was purified by preparative-HPLC (gradient, 25–60% aq MeCN containing 0.1% $NH_3{\cdot}H_2O$ for 40 min, 4 mL/min) to give oxazoline **1** (1.9 mg, 73% for 2 steps) as a white foam. HRMS: $[M + H]^+$ calcd for $C_{92}H_{153}N_{12}O_{31}^+$, 1922.0759; found, 1922.0703.

Compound (2).—To a solution of **12** (1.4 mg, 2.60 μmol) and **29** (2.0 mg, 1.30 μmol) in anhydrous DMSO (40 μL) was added Et_3N (0.6 μL) to adjust pH = 8.5. The mixture was kept at room temperature until the complete consumption of **29** to give the crude product (**31**), which was used in the next step without further purification. RP-HPLC retention time for **31**, t_R = 17.1 min (gradient, 20–70% aq MeCN containing 0.1% FA for 30 min; flow rate, 0.4 mL/min). HRMS: $[M + H]^+$ calcd for $C_{92}H_{155}N_{12}O_{32}^+$, 1941.0898; found, 1941.0851. To the residue obtained in the first step was added H_2O (80 μL) and Et_3N (40 mol equiv). The mixture was cooled to 0 °C, and 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 30 mol equiv) was added. After 12 h at 0 °C, the reaction was purified by preparative-HPLC (gradient, 25–60% aq MeCN containing 0.1% $NH_3{\cdot}H_2O$ for 40 min, 4 mL/min) to give oxazoline **2** (2.0 mg, 80% for 2 steps) as a white foam. HRMS: $[M + H]^+$ calcd for $C_{92}H_{153}N_{12}O_{31}^+$, 1922.0759; found, 1922.0800.

Compound (3).—To a solution of **22** (2.3 mg, 4.14 μmol) and **29** (3.2 mg, 2.07 μmol) in anhydrous DMSO (60 μL) was added Et_3N (0.8 μL) to adjust pH = 8.5. The mixture was kept at room temperature until the complete consumption of **29** to give the crude product (**32**), which was used directly for the oxazoline formation without further purification. RP-HPLC retention time for **32**, t_R = 17.1 min (gradient, 20–70% aq MeCN containing 0.1% FA for 30 min; flow rate, 0.4 mL/min). HRMS: $[M + H]^+$ calcd for $C_{92}H_{155}N_{12}O_{32}^+$, 1941.0898; found, 1941.0836. To the residue obtained in the first step was added H_2O (100 μL) and Et_3N (40 mol equiv), the mixture was cooled to 0 °C and 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 30 mol equiv) was added. After 12 h at 0 °C, the reaction mixture was subjected to preparative-HPLC (gradient, 25–60% aq MeCN containing 0.1% $NH_3{\cdot}H_2O$ for 40 min, 4 mL/min) to give oxazoline **3** (2.8 mg, 70% for 2 steps) as a white foam. HRMS: $[M + H]^+$ calcd for $C_{92}H_{153}N_{12}O_{31}^+$, 1922.0759; found, 1922.0731.

Compound (33).—To a solution of commercial trastuzumab (500 μg) in a PBS buffer (25 μL, 150 mM, pH = 7.0) containing 5% of DMSO was added oxazoline **1** (200 μg, 15 equiv per reaction site) and wild-type Endo-S2 (1.0 μg). The reaction was incubated at 28 °C and monitored by LC–MS aliquots. After 1 h, LC–MS analysis indicated the complete transglycosylation. The reaction mixture was diluted with 50 mM phosphate buffer (3.0 mL, pH = 7.2) and filtered by 0.22 μm syringe filter (remove most of the hydrophobic linker-payload) before subjecting to protein A column to give the antibody-drug conjugate **33** (470 μg, 95%). LC–MS analysis: for whole ADC (*m*/*z*), calculated, M = 149,709 Da; found 149,709 (deconvoluted data); for drug-conjugated Fc monomer released by IdeS digestion (*m*/*z*), calculated, M = 26,056 Da; found 26,057 (deconvoluted data).

Compound (34).—To a solution of commercial trastuzumab (500 μg) in 150 mM PBS buffer containing 5% of DMSO (25 μL, pH = 7.0) was added oxazoline **2** (250 μg, 20 equiv per reaction site) and wild-type Endo-S2 (10 μg). The reaction was incubated at 28 °C and monitored by LC–MS aliquots. After 1 h, another portion of oxazoline **2** (60 μg, 5 equiv per reaction site) was added to push the reaction. Within 1.5 h, LC–MS analysis indicated the complete transglycosylation. The reaction mixture was diluted with 50 mM phosphate buffer (3.0 mL, pH = 7.2) and filtered by a 0.22 μm syringe filter (remove most of the hydrophobic linker-payload) before subjecting to a protein A column to give the antibody-drug conjugate **34** (460 μg, 93%). LC–MS analysis: for whole ADC (m/z), calculated, M = 149,709 Da; found 149,708 (deconvoluted data); for drug-conjugated Fc monomer released by IdeS digestion (*m*/*z*), calculated, M = 26,056 Da; found 26,057 (deconvoluted data).

Compound (35).—To a solution of commercial trastuzumab (500 μg) in a PBS buffer (25 μL, 150 mM, pH = 7.0) containing 5% of DMSO was added oxazoline **3** (250 μg, 20 equiv per reaction site) and wild-type Endo-S2 (5.0 μg). The reaction was incubated at 28 °C and monitored by LC–MS aliquots. After 1 h, another portion of oxazoline **3** (60 μg, 5 equiv per reaction site) was added to push the reaction. Within 2 h, LC–MS analysis indicated the complete transglycosylation. The reaction mixture was diluted with 50 mM phosphate buffer (3.0 mL, pH = 7.2) and filtered by 0.22 μm syringe filter (remove most of the hydrophobic linker-payload) before subjecting to a protein A column to give the antibody-drug conjugate **35** (430 μg, 86%). LC–MS analysis: for whole ADC (m/z), calculated, M = 149,709 Da; found 149,710 (deconvoluted data); for drug-conjugated Fc monomer released by IdeS digestion (*m*/*z*), calculated, M = 26,056 Da; found 26,057 (deconvoluted data).

Cell Lines and Culture Conditions.

SK-BR-3 cells (ATCC HTB-30) were maintained in suspension in McCoy's 5a Medium (ATCC 30–2007) containing 10% fetal bovine serum (FBS, not heated), 100 U/mL penicillin, and 100 μg/mL streptomycin in T-75 flasks (CELLTREAT). BT474 cells (ATCC HTB-20) were maintained in suspension in Hybri-Care Medium (ATCC 46-X) containing 10% fetal bovine serum (FBS, pre-heated), 100 U/mL penicillin, and 100 μg/mL streptomycin in T-75 flasks (CELLTREAT). T47D cells (ATCC HTB-133) were maintained in suspension in RPMI-1640 Medium (ATCC 30–2001) containing 10% fetal bovine serum (FBS, pre-heated), 4 mg/L insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin in T-75 flasks (CELLTREAT).

Cytotoxicity Assays.

For SK-BR-3 and T47D cell lines, the cells were planted into 96-well plates (cell number: 10,000 cells per well), and the plates were incubated for 24 h at 37 °C with 5% CO_2. The ADC samples were diluted by 3-fold serial dilution with the corresponding medium from 5000 to 0.085 ng/mL (11 concentrations) and then added to the wells in triplicate (150 μL per well) for every single concentration. The cells were cultured at 37 °C with 5% CO_2 for 3 days before the removal of the medium and addition of Cell Counting Kit-8 (Sigma). The absorbance of formazan released by viable cells was measured at 450 nm using a spectrophotometer after incubation at 37 °C with 5% CO_2 for 2–3 h, and the background absorption was deducted by 550 nm absorbance. Finally, the cell viability curve and EC_{50} values were calculated by GraphPad Prism software. For the BT474 cell line, the cells were planted into 96-well plates with 4000 cells per well. The plates were incubated for 24 h at 37 °C with 5% CO_2. The ADC samples were diluted by 3-fold serial dilution with the corresponding medium from 5000 to 0.085 ng/mL (11 concentrations) and then added to the wells in triplicate (200 μL per well) for every single concentration. The cells were cultured at 37 °C with 5% CO_2 for 6 days before the removal of the medium and addition of Cell Counting Kit-8 (Sigma). The absorbance of formazan released by viable cells was measured at 450 nm using a spectrophotometer after incubation at 37 °C with 5% CO_2 for 2–3 h, and the background absorption was deducted by 550 nm absorbance. Finally, the cell viability curve and EC_{50} values were calculated by GraphPad Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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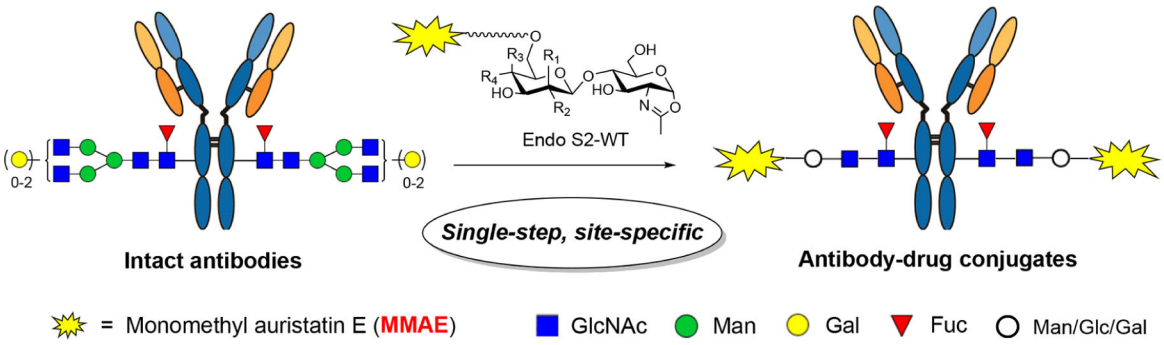


Figure 1.
Design of the single-step synthesis of ADCs from intact antibodies.

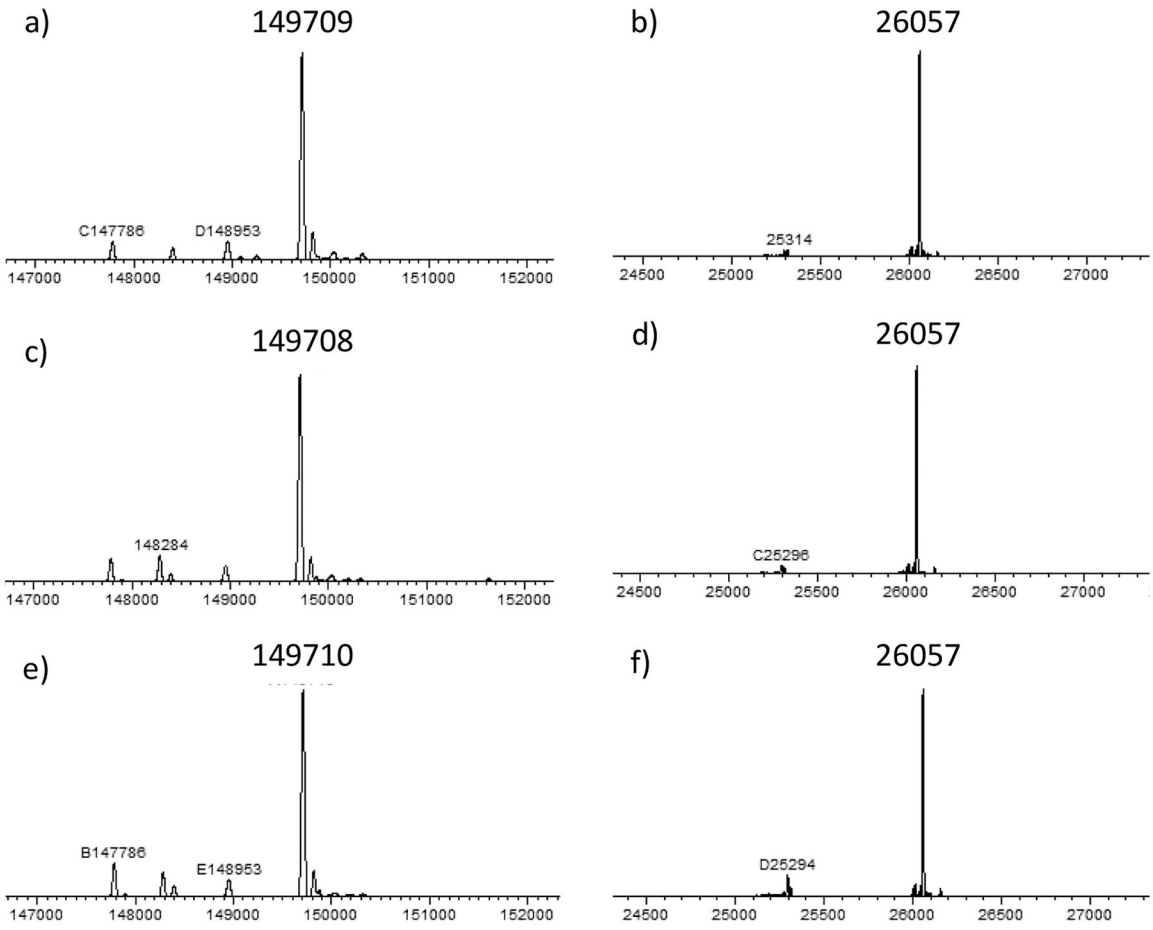
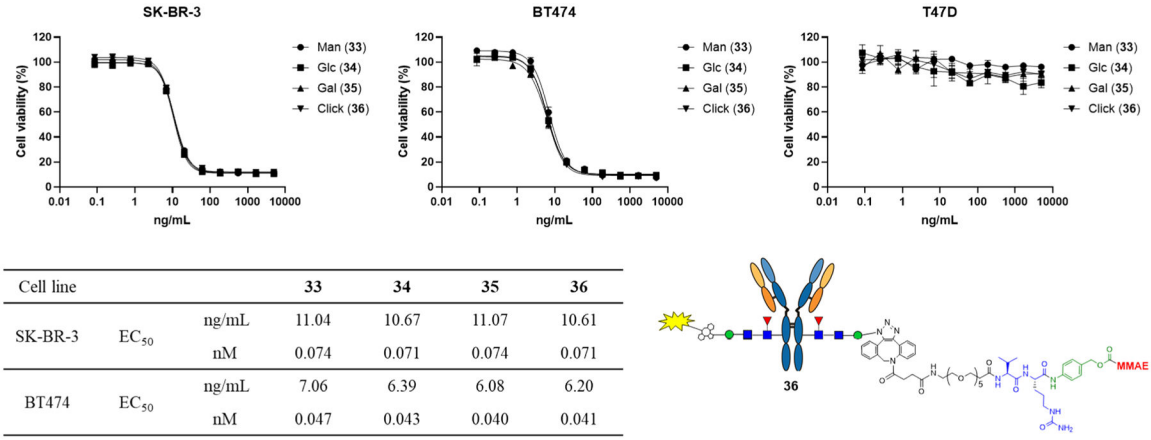


Figure 2.

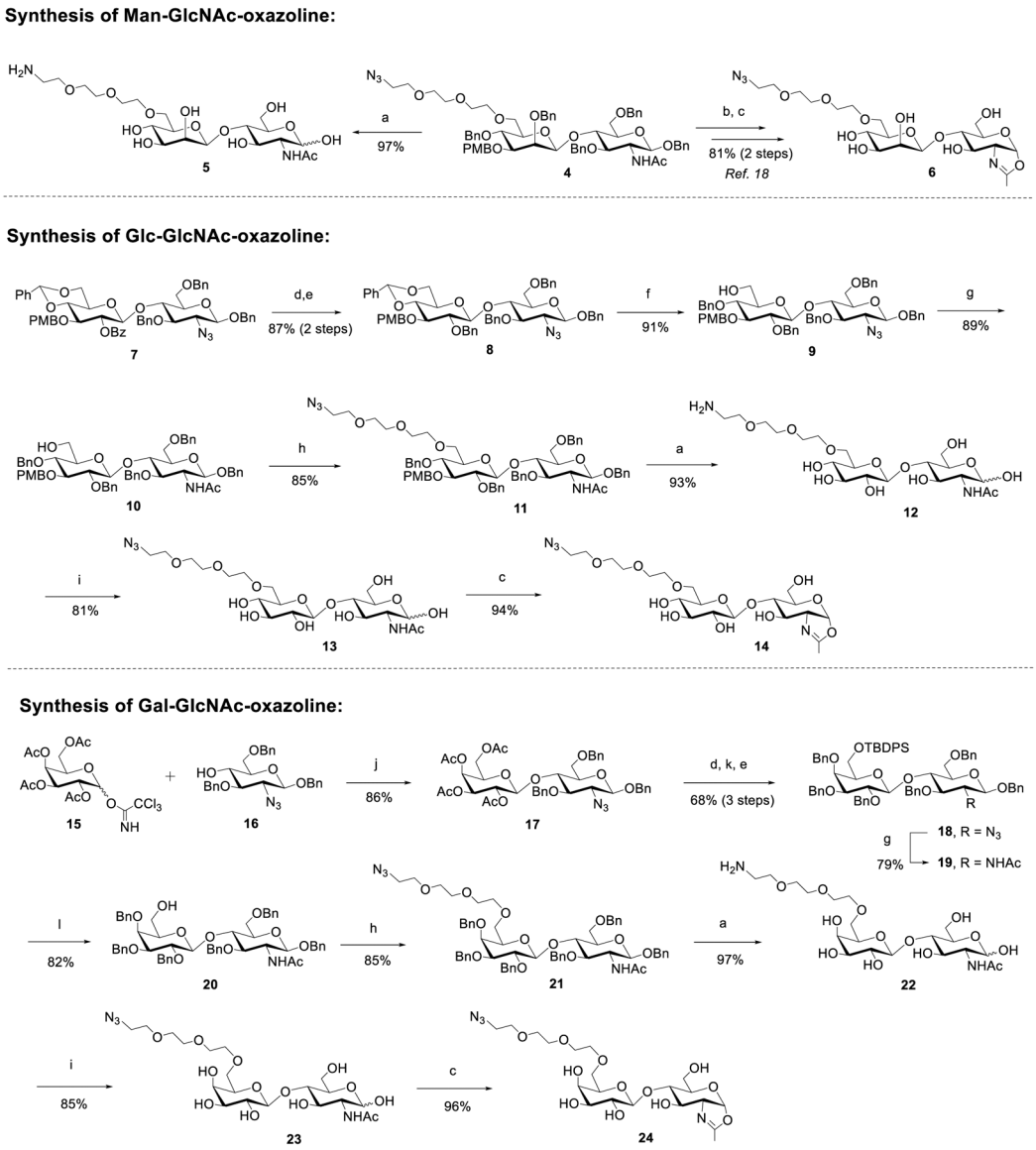
LC-ESI-MS analysis of the intact ADCs (**33**–**35**) and the Fc domains released by IdeS treatment (deconvoluted data). (a) Intact ADC **33**; (b) Fc domain of **33**; (c) intact ADC **34**; (d) Fc domain of **34**; (e) intact ADC **35**; (f) Fc domain of **35**.



Cell line			33	34	35	36
SK-BR-3	EC_{50}	ng/mL	11.04	10.67	11.07	10.61
		nM	0.074	0.071	0.074	0.071
BT474	EC_{50}	ng/mL	7.06	6.39	6.08	6.20
		nM	0.047	0.043	0.040	0.041

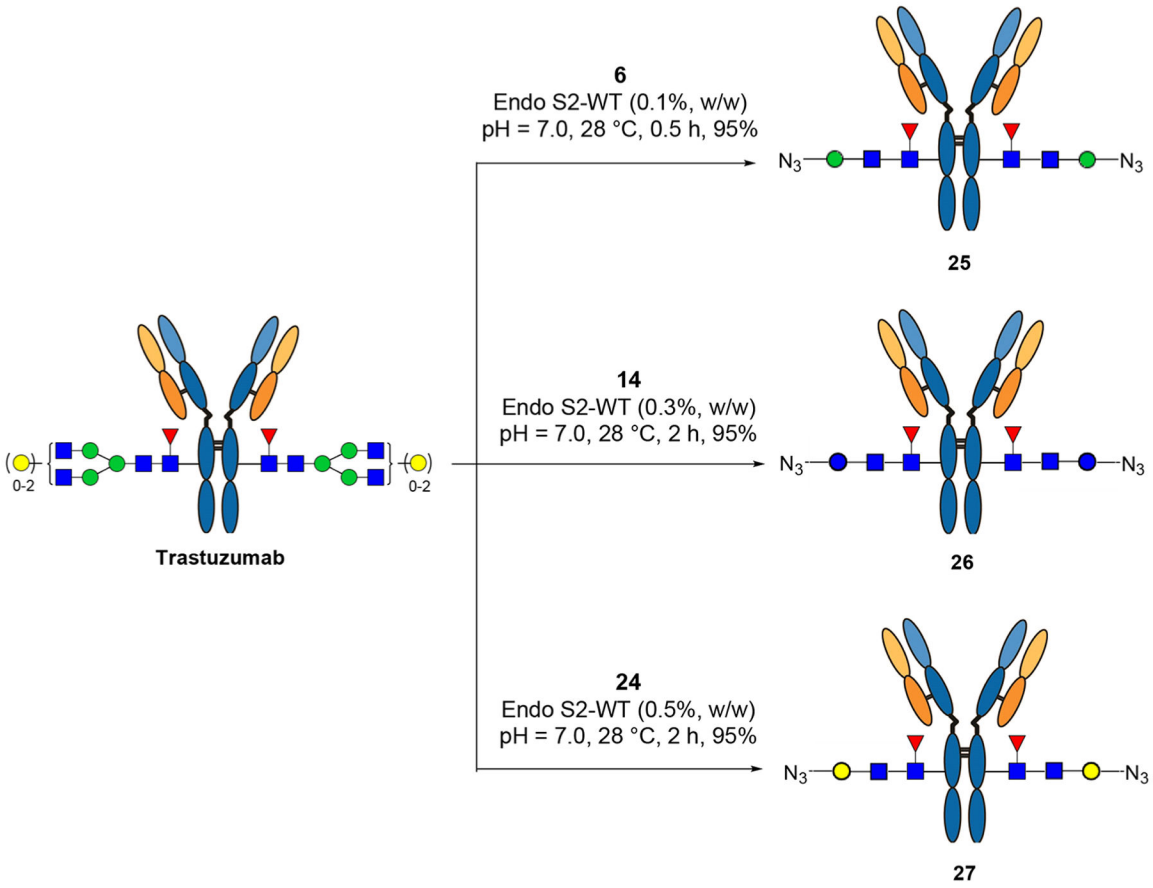
Figure 3.

Cytotoxicity assays with breast cancer cell line SK-BR-3, BT474 (HER2 high-expression) and T47D (HER2 low-expression). All assays were performed in triplicate.



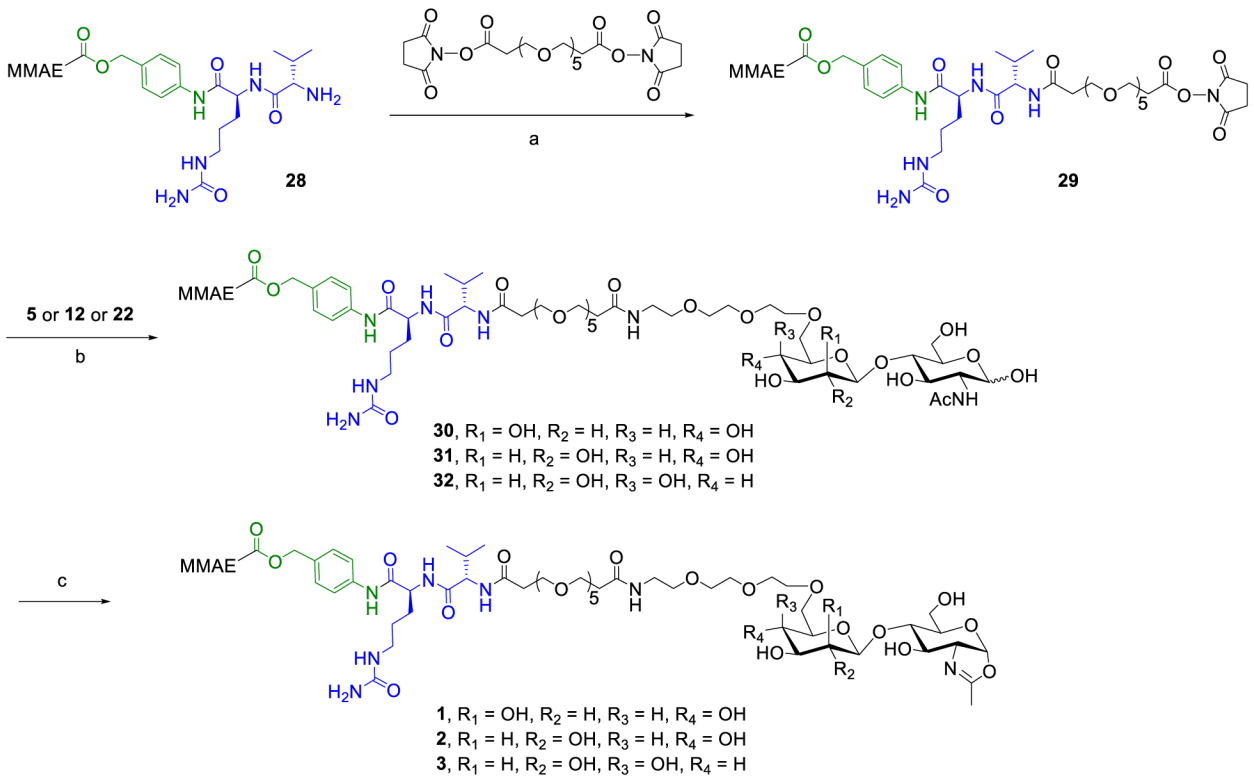
Scheme 1. Chemical Synthesis of Azido-Tagged Disaccharide Oxazolines[a]

[a]Reagents and conditions: (a) Pd/C, H_2, HCl (aq), THF/H_2O, RT; (b) $NaBrO_3$, $Na_2S_2O_4$, EtOAc/H_2O, RT; (c) DMC, Et_3N, H_2O, 0 °C; (d) CH_3ONa, CH_3OH, 50 °C; (e) BnBr, NaH, DMF, 0 °C ~ RT; (f) BH_3·THF, Bu_2BOTf, CH_2Cl_2, 0 °C; (g) AcSH, pyridine/$CHCl_3$, 50 °C; (h) $N_3(CH_2CH_2O)_3Ts$, NaH, DMF, 0 °C ~ RT; (i) TfN_3, K_2CO_3, $CuSO_4$, CH_2Cl_2/MeOH/H_2O, RT; (j) TMSOTf, 4 Å MS, CH_2Cl_2, −40 °C; (k) TBDPSCl, imidazole, DMF, RT; (l) TBAF, THF, 40 °C.



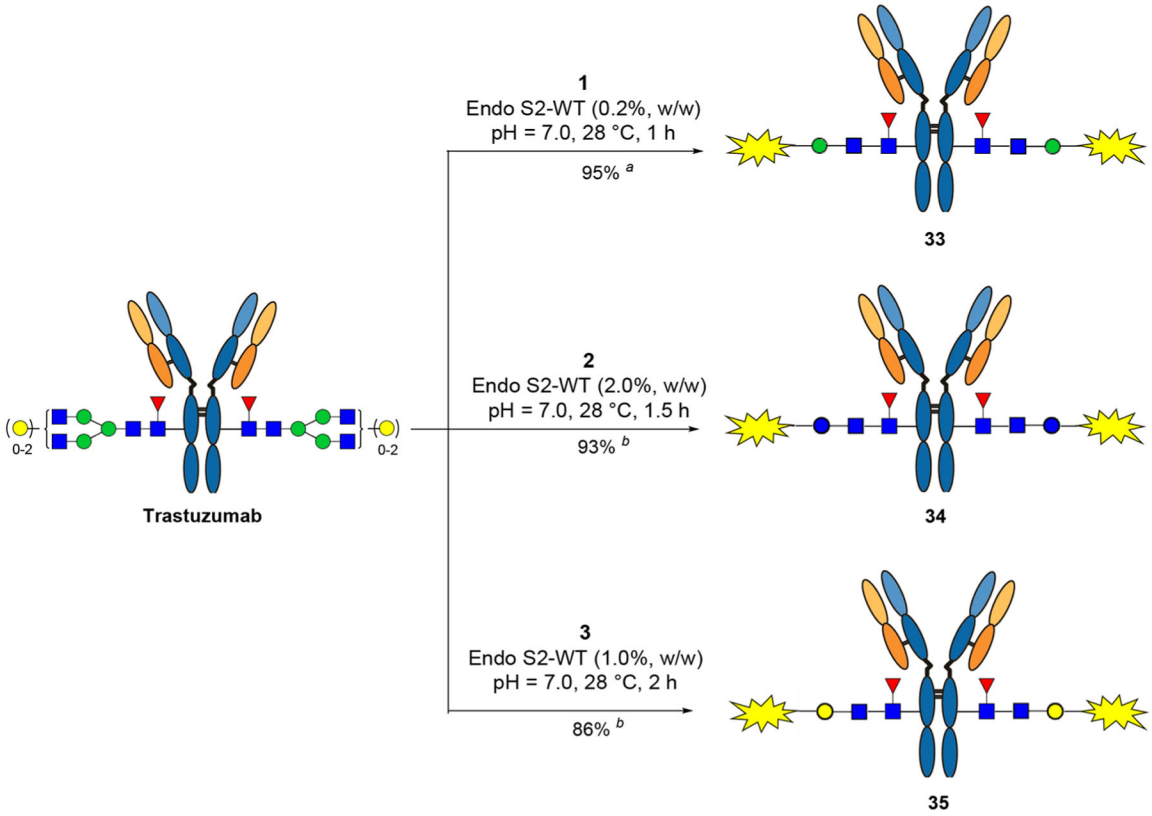
Scheme 2. Transglycosylation of Azido-Tagged Disaccharide Oxazolines[a]

[a]The reactions were performed in PBS buffer with an antibody concentration of 20 mg/mL, and 20 equiv of oxazoline was used (for each reaction site); the yields were based on the LC-MS peak intensity.



Scheme 3. Synthesis of Drug-Oxazoline Conjugates (1–3)[a]

[a]Reagents and conditions: (a) Et_3N, DMSO, RT, 82%; (b) Et_3N, DMSO, RT; (c) DMC, Et_3N, H_2O/DMSO, 0 °C, **1**, 73% for 2 steps, **2**, 80% for 2 steps, **3**, 70% for 2 steps.



Scheme 4. Single-Step Transglycosylation of Drug-Oxazoline Conjugates to Make ADCs (33–35)[a]

[a]The reactions were performed in a PBS buffer with 5% DMSO, and the antibody concentration was 20 mg/mL (isolated yields after protein A purification). For each reaction site: (a) 15 equiv of oxazoline was used; (b) 25 equiv of oxazoline was added in two portions.