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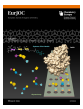
A Clickable Bioorthogonal Sydnone-Aglycone for the Facile Preparation of a Core 1 *O*-Glycan-Array



Zoeisha S. Chinoy,[a, b] Kelley W. Moremen,[c, d] and Frédéric Friscourt*[a, b]

Protein-*O*-glycosylation has been shown to be essential for many biological processes. However, determining the exact relationship between *O*-glycan structures and their biological activity remains challenging. Here we report that, unlike azides, sydnones can be incorporated as an aglycon into core 1 *O*-glycans early-on in their synthesis since it is compatible with carbohydrate chemistry and enzymatic glycosylations, allowing us to generate a small library of sydnone-containing core 1 *O*-glycans by chemoenzymatic synthesis. The sydnone-aglycon was then employed for the facile preparation of an *O*-glycan array, via bioorthogonal strain-promoted sydnone-alkyne cycloaddition click reaction, and in turn was utilized for the high-throughput screening of *O*-glycan-lectin interactions. This sydnone-aglycon, particularly adapted for *O*-glycomics, is a valuable chemical tool that complements the limited technologies available for investigating *O*-glycan structure-activity relationships.

Introduction

Glycosylation is a predominant post-translational modification on cell-membrane and secreted proteins, with more than half of all human proteins being glycosylated.[1] It is now evident that the glycan-part of glycoproteins is not a mere decoration but is directly involved in many physiological and pathological events, including cell adhesion, host–pathogen interactions, and cancer progression.[2] All eukaryotic glycoproteins can be classified as either *N*- or *O*-linked, whether the glycan is covalently connected to an asparagine residue, or a serine/threonine amino acid.[3] In contrast to *N*-glycans, our knowledge of *O*-glycan structure-activity relationship is currently limited, largely because of a lack of effective technologies for *O*-glycomics, including their synthetic preparation as well as their systematic biological study both *in vitro* and *in vivo*.[4]

Glycan-microarrays have recently revolutionized our knowledge of functional glycomics by allowing high-throughput study of glycan-protein recognition.[5] Glycan-arrays are generated by covalently coupling complex carbohydrates to an activated solid surface, using a functional group priorly introduced at the reducing end of the glycan, often by exploiting reductive amination strategies, which leads to ring opening of this reducing end sugar. However, any structural modifications of the reducing monosaccharide can have significant effects on the biological function or recognition of the glycan, as compared to the intact structure.[6] To overcome this limitation, a novel linkering strategy based on oxime ligation, which exploits a methyl *N*,*O*-hydroxylamine, was recently developed that allows the introduction of an anomeric linker on glycans while preserving the ring structure of the reducing sugar.[7] Importantly, this oxime-linkering approach generates preferentially a β-glycoside at the reducing end, a positive outcome for *N*-glycan structures but less so for the preparation of core 1 *O*-glycans, which are α-linked at their reducing sugars. Accordingly, while various glycan-arrays have been developed in recent years, they often lack *O*-glycan structures.[8]

In mammals, mucin-type *O*-glycans, also known as core 1 *O*-glycans, which represent the largest family of *O*-glycans, are all biosynthesized from the Tn antigen, an *N*-acetylgalactosamine (GalNAc) monosaccharide α-linked to the hydroxyl group of a serine or threonine residue on glycoproteins.[9] While most β-glycosides can easily be prepared in a stereo-controlled manner by exploiting participating neighboring protecting groups, notably ester functionalities, the synthesis of oligosaccharides containing α-glycosidic bonds remains challenging (Figure 1A).[10] The most common glycosylation approach to generate preferentially α-linkage relies on combining the introduction of a non-participating functional group on C-2 of the hexose donor and the use of α-orienting solvents such as

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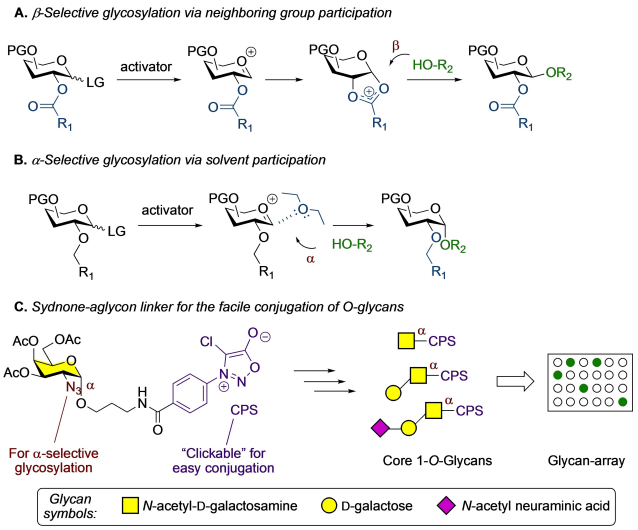


Figure 1. A-B: Stereo-control of chemical glycosylations. **C**: Chemical design for the chemoenzymatic preparation of core 1 *O*-glycans and their conjugation by strain-promoted sydnone-alkyne cycloaddition (SPSAC). PG: Protecting Group; CPS: 4-Chloro-3-PhenylSydnone.

diethyl ether (Figure 1B).[11] In the case of GalNAc, an azide is often employed as the non-participating chemical precursor of the *N*-acetyl functionality (later generated via azide reduction and acetylation). This implies that an azido-linker aglycon, often used as a clickable moiety for late functionalization of glycans via copper-catalyzed alkyne-azide cycloaddition,[12] cannot be employed for *O*-glycomics without having to rely on multiple protecting group manipulations.[13]

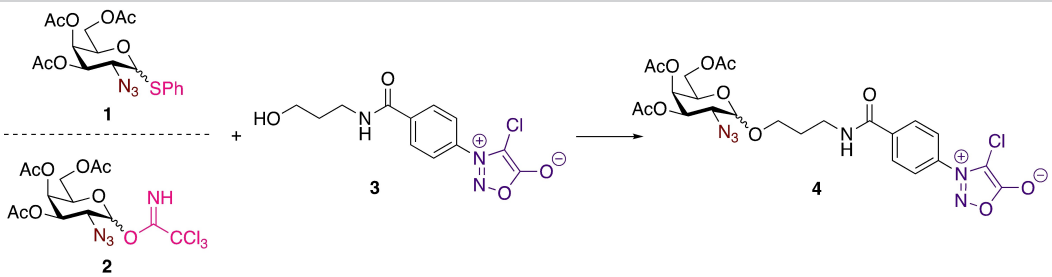
We have recently shown that sydnones, a unique class of mesoionic 1,3-dipoles, are not only capable of fast metal-free strain-promoted cycloadditions with cyclooctynes,[14] but are also highly stable under reducing conditions, making them chemically orthogonal to azides.[15] Accordingly, we envisaged that a sydnone-linker could be installed early-on as the aglycon of *O*-glycans for future bioconjugation or array printing, while allowing us to keep the azide functionality at the C-2 of galactose to favor α-glycosylation (Figure 1C). Here, we report the chemical synthesis of a GalNAc-α-linked to a sydnone-containing linker and its efficient enzymatic extension to more complex mucin-type *O*-glycans using recombinant glycosyltransferases. The sydnone-modified mucin-type *O*-glycans were then attached to a microtiter plate using strain-promoted sydnone-alkyne cycloaddition (SPSAC) and the resulting *O*-glycan-array was exploited to study the recognition of various glycan-binding proteins.

Results and Discussion

To explore the possibility of generating a small library of mucin-type *O*-glycans having a sydnone-containing aglycon, we set out to chemically prepare GalNAc-α1-*O*-linker-sydnone from 2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-D-galactose donors **1**–**2** and sydnone-modified 3-propanol **3** (See Supporting Information for their synthetic preparation). *N*-iodosuccinimide (NIS)/trifluoromethanesulfonic acid (TfOH)-mediated glycosylation of thiophenol donor **1** with acceptor **3** in dichloromethane at room temperature led to the formation of the desired galactose-sydnone **4**, however in very low yield of 6% and as a mixture of anomers, surprisingly in favor of the β-glycoside (Table 1, entry 1).

To improve the glycosylation reactivity, we decided to investigate the use of other donors. Accordingly, glycosylation of trichloroacetimidate **2** with acceptor **3** in the presence of trimethylsilyl triflate (TMSOTf) in dichloromethane at room temperature generated 2-azido-galactose-sydnone **4** with an improved yield of 46% but still in favor of the β-anomer (entry 2). Since ethers are the most common α-directing solvents, we performed the glycosylation reaction between **2** and **3** in a mixture of dichloromethane and diethyl ether (1/2), which significantly improved the anomeric selectivity of the glycosylation in favor of the α-anomer (α/β, 1.5/1), however with a slight decrease in yield due to solubility issue (entry 3).

Table 1. Chemical glycosylation for the formation of 2-azido-galactose-sydnone **4**.



Entry	Donor **1**	Donor **2**	Activator	Solvent	Yield [%]	Selectivity[a] α/β
1	1 equiv	–	NIS/TfOH	CH_2Cl_2	6	1/1.5
2	–	1 equiv	TMSOTf	CH_2Cl_2	46	1/1.5
3	–	1 equiv	TMSOTf	Et_2O/CH_2Cl_2 (2/1)	40	1.5/1
4	–	2 equiv	TMSOTf	dioxane	53	1.5/1
5	–	2 equiv	TfOH	dioxane	71	1.5/1

[a] Determined by ^{1}H-NMR.

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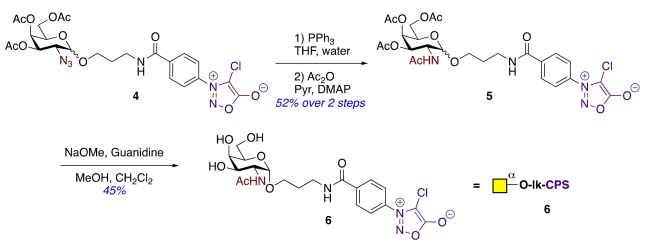
To improve the reaction solubility while maintaining the solvent participating effect, the glycosylation reaction between trichloroimidate donor **2** and acceptor **3** was then performed in pure dioxane, in the presence of TMSOTf, giving galactose **4** in a slightly improved 53% yield while preserving the good α-selectivity (entry 4). Finally, switching activation of the glycosyl donor **2**, from TMSOTf to TfOH, led to formation of the desired 2-azido-galactose-sydnone **4** in good yield (71%), while still favoring formation of the desired α-anomer (α/β, 1.5/1, entry 5).

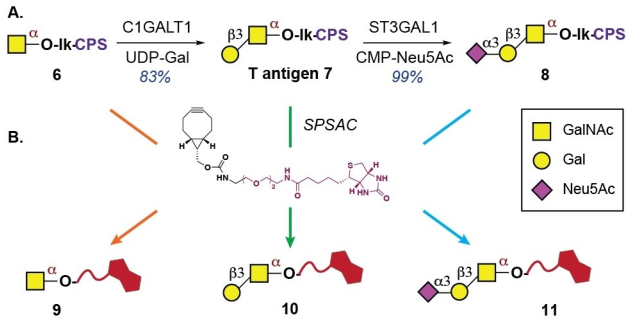
The azide functionality of galactose **4** was then subjected to reduction in the presence of triphenylphosphine and the corresponding amine was acetylated with acetic anhydride to generate the 3,4,6-tri-*O*-acetyl-GalNAc-1-*O*-linkered sydnone **5** in 52% yield over two steps (Scheme 1). Finally, removal of the *O*-acetyl protecting groups using sodium methoxide in the presence of guanidine[16] and purification by reverse-phase high performance liquid chromatography (HPLC) led to the isolation of the desired GalNAc-α1-*O*-linkered sydnone **6** as pure α-anomer in 45% yield. To note, the presence of the sydnone moiety, which strongly absorbs in the UV spectrum, significantly facilitated the detection and consequently the purification of the unprotected sugar GalNAc **6** by HPLC.

To increase the structural diversity of the mucin-type *O*-glycans prepared, we decided to investigate whether enzymatic glycosylations could be performed on our synthetic GalNAc-α1-*O*-linker-sydnone **6**, since chemoenzymatic oligosaccharide synthesis has been shown to be particularly efficient for the generation of highly complex glycan structures in recent years.[8b,17]

Following the *O*-glycans biosynthetic pathway, we first extended monosaccharide GalNAc-α1-*O*-linkered sydnone **6** into the T antigen **7** (also known as core 1 *O*-glycan: Galβ1,3-GalNAc-α1) using the β-1,3-galactose transferase C1GALT1 (T-synthase) in the presence of uridine-5′-diphosphogalactose (UDP-Gal), calf intestine alkaline phosphatase (CIAP) and $MnCl_2$ in excellent 83% yield. α-2,3-Sialylation of the T antigen **7** at the galactose residue was then efficiently achieved by incubating it with recombinant human sialyltransferase ST3GAL1-(selective for *O*-glycans) in the presence of cytidine-5′-monophospho-*N*-acetyl-neuraminic acid (CMP-Neu5Ac) and CIAP to give the desired sialylated trisaccharide **8** in 99% yield (Scheme 2A). Collectively, this data demonstrates that a sydnone moiety can be incorporated into glycans very early-on in the chemoenzymatic oligosaccharide synthesis strategy as it is not only compatible with classical carbohydrate chemistry,



Scheme 1. Chemical preparation of GalNAc-α1-*O*-linker-sydnone **6**.



Scheme 2. A: Enzymatic extension of the Tn antigen **6** to give core-1 *O*-glycan **7** and sialoside **8**. **B**: Biotinylation of **6**–**8** by SPSAC using the cyclooctyne BCN-biotin conjugate.

but also does not interfere with enzymatic glycosylation when introduced as an aglycon.

Biotinylation of glycans is an exceptionally versatile and valuable tool to probe the influence of glycoconjugates *in vitro* as well as in living systems, by exploiting the strong affinity of avidin for biotin and consequently, can be exploited for the preparation of glycan-arrays. Accordingly, mucin-type *O*-glycans **6**–**8** were biotinylated by reacting their sydnone moiety with the cyclooctyne BCN-biotin conjugate under strain-promoted sydnone-alkyne cycloaddition conditions (Scheme 2B). Briefly, sydnone-modified glycans **6**–**8** were incubated in water with BCN-biotin conjugate at 37 °C for 3 h and the resulting biotinylated sugars **9**–**11** were purified by P-2 size exclusion column chromatography.

To examine the lectin binding specificities of mucin-type *O*-glycan epitopes against other glycan structures, notably having different linkage-specificities, biotinylated GalNAc-β1 **12**, Gal-β1,4-GlcNAc-β1 **13**, Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1 **14** and Neu5Ac-α2,6-Gal-β1,4-GlcNAc-β1 **15**, which are common terminal epitopes of *N*-glycans, were also synthesized following similar strategy (for synthetic details, see SI). The collection of biotinylated glycans **9**–**15** were then immobilized on microtiter plates coated with NeutrAvidin, and the resulting glycan-array was probed with commercially available fluorescently-labeled lectins, including *Peanut* agglutinin (PNA), *Ricinus Communis* agglutinin (RCA), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* lectin II (MAL-II) (Figure 2).

The selective binding pattern of PNA for the T antigen **10** is in good agreement with the known specificity of PNA for Gal-β1,3-GalNAc,[18] validating that the pyrazole formed by the SPSAC reaction does not interfere with lectin binding and consequently that our glycan-array is suitable for probing glycan-protein interactions. RCA, which is known to have a broad specificity for galactoses,[18] bound effectively to Gal-terminating sugars **10** and **13**, but also to both α- and β-GalNAc terminating glycans **9** and **12**, indicating that the nature of the functional group present on the C-2 carbon of Gal is not critical for RCA recognition. In addition, RCA seemed to also strongly recognize α-2,6-sialylated glycan **15**, a result important to note for future application of RCA as a potential probe for Gal recognition.[18–19] Following its identified binding pattern,[18] SNA

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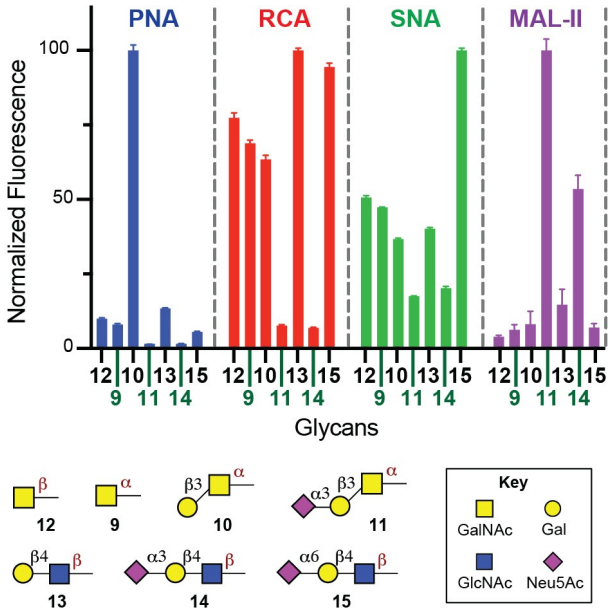

Figure 2. Binding profiles of PNA, RCA, SNA and MAL-II lectins towards glycans **9**–**15**. The array was incubated with FITC-labeled lectins for 1 h at 27 °C, washed 4 times, followed by fluorescence measurements (λ_{exc} = 485 nm/λ_{em} = 535 nm). Error bars are indicative of the standard deviation of three individual experiments.

preferentially recognized the α-2,6-sialoside **15**, albeit with a non-negligeable binding to Gal- and GalNAc terminating sugars **9**, **10**, **12** and **13**. Finally, MAL-II, which is often utilized for probing the presence of α-2,3-sialic acids,[18] exhibited significant biding to α-2,3-sialosides **11** and **14** but with a clear preference for the *O*-glycan structure. Taken together, these results demonstrate that this novel sydnone-linkering strategy can be exploited for the facile preparation of an *O*-glycan-array, an invaluable tool for deciphering carbohydrate-protein interactions at the molecular level.[8b,20]

Conclusion

In summary, we showed that unlike other 1,3-dipoles, sydnone-modified aglycon represents a new class of tagging reagent ideal for *O*-glycomics. In particular, we demonstrated that sydnones are not only compatible with carbohydrate chemistry, withstanding chemical glycosylation, acetylation, reduction and ester hydrolysis conditions, but also do not interfere with enzymatic glycosylation reactions. Consequently, a sydnone-aglycon could be conveniently installed early-on at the monosaccharide stage of GalNAc-α1, before converting it into more complex mucin-type *O*-glycan structures by enzymatic oligosaccharide synthesis. Attractive features of having a sydnone-tag include fast and facile modification with multiple cyclooctyne probes under metal-free strain-promoted sydnone-alkyne cycloaddition (SPSAC). While the prepared sydnone-tagged *O*-glycans can be fluorescently labeled or conjugated to proteins by SPSAC,[21] we employed SPSAC to biotinylate them in order to conveniently create highly underrepresented *O*-glycan-array, ideal for probing carbohydrate-protein interactions in high-throughput fashion.

Experimental Section

Materials and Methods

All solvents were of reagent grade and used as received. Chemicals and reagents were used as commercially supplied without any further purification unless otherwise stated. All solvents for chemical glycosylations were anhydrous and purchased from Sigma-Aldrich. Molecular sieves (4 Å) were flame activated *in vacuo* prior to use. Uridine 5′-diphosphogalactose (UDP-Gal) was purchased from Carbosynth Limited. Alkaline Phosphatase from calf intestine (CIAP) was purchased from Calbiochem EMD Millipore. All enzymatic reactions were performed in aqueous buffered system.

All nuclear magnetic resonance (NMR) spectra were acquired on a 300 MHz or 400 MHz Bruker spectrometer operating at 25 °C. Chemical shifts are reported in parts per million (ppm) relative to the NMR solvent as the internal standard. NMR data is presented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet and/or multiple resonances), coupling constant in Hertz (Hz), integration. All NMR signals were assigned on the basis of ^{1}H NMR, COSY and HSQC experiments. High-resolution mass spectra were recorded on a Thermo Exactive orbitrap (ESI) mass spectrometer. Reverse-Phase HPLC was performed on an Agilent 1260 Infinity II system equipped with an automated injector, UV-detector, fraction-collector and Agilent ZORBAX Eclipse XDB-C18 Semiprep column (5 μm, 9.4× 250 mm). Column chromatography was performed on silica gel G60 (Silicycle, 60–200 μm, 60 Å). TLC-analysis was conducted on Silicagel 60 F_{254} (EMD Chemicals inc.) with detection by UV-absorption (254 nm) were applicable, and by spraying with 20% sulfuric acid in ethanol followed by charring at ~150 °C or by spraying with a solution of Hanessian's stain followed by charring at ~150 °C.

Trichloroacetimidate glycosylations Table 1 **(Entries 4 and 5)**. To a solution of acceptor **3** (1 eq) and donor **2** (2 eq) in 1,4-dioxane (~ 13 mM conc of acceptor), activated molecular sieves (4Å) were added and the setup left to stir for 45 min at room temperature, under an atmosphere of argon. The activator (0.4 eq of TMSOTf for Entry **4** and 0.25 eq of TfOH for Entry **5**) was added and the reaction mixture was left to stir at room temperature for 1 hr, after which it was diluted with DCM, filtered and the filtrate washed with a saturated solution of $NaHCO_3$ and water, dried over $MgSO_4$, filtered and the filtrate was concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (acetone:DCM, 3:20, v:v) to afford the sydnone-linkered azidogalactose **4** as an inseparable mixture of the two anomers (α and β). **Sydnone-linkered azidogalactose 4 (α:β = 3:2 mixture)** – ^{1}H-NMR (300 MHz; $CDCl_3$): δ 2.03–2.17 (m, 11H, 3 × $COCH_3$, L2–CH_2), 4.21–3.44 (m, 9H), 4.39 (d, *J* = 8.1 Hz, 0.6H, H-1β), 4.83 (dd, *J* = 10.8, 3.4 Hz, 0.6H), 5.00 (d, *J* = 3.7 Hz, 0.4H, H-1α), 5.37–5.32 (m, 1H), 5.40 (dd, *J* = 3.4, 1.1 Hz, 0.4H), 6.68 (t, *J* = 5.3 Hz, 0.6H), 7.11 (t, *J* = 5.6 Hz, 0.4H), 7.73 (dd, *J* = 12.8, 8.8 Hz, 2H), 8.07 (d, *J* = 8.3 Hz, 2H). ^{13}C NMR assigned from HSQC (75 MHz, $CDCl_3$): δ 20.59 (×3), 28.75, 38.21, 39.28, 57.98, 60.77, 61.20, 66.36, 66.57, 67.22, 68.72 (×2), 70.87, 71.09, 97.73, 102.03, 124.60 (×2), 128.68 (×2). HRMS ESI: $[M+Na]^+$ $C_{24}H_{27}ClN_6NaO_{11}^+$, calcd 633.1319, obsd 633.1321.

2-Deoxy-2-acetamido-3,4,6-tri-*O*-acetyl-3-(3-[4-Chloro-1,2,3-oxadiazol-3-ium-5-olate]benzamido)-propyl-α,β-D-galactopyranoside (5). To a cooled (0 °C) solution of sydnone-linkered azidogalactose **3** (134 mg, 0.22 mmol) in THF (1.8 mL), triphenylphosphine (86.6 mg,

0.33 mmol) was added and the reaction mixture left to stir for 5 mins, followed by the addition of water (10.8 μL). The reaction mixture was left to stir, while warming to room temperature, for 3.5 h. The reaction mixture was quenched with water, diluted with ethyl acetate, washed with brine (x3), dried over $MgSO_4$, filtered and the filtrate was concentrated *in vacuo*. The resulting residue was dissolved in pyridine (5 mL) and acetic anhydride (3 mL), followed by the addition of DMAP (0.4 mg), and the reaction left to stir for 5 h. The reaction mixture was diluted with ethyl acetate, washed with a saturated solution of $NaHCO_3$ and a solution of $CuSO_4$ (x3), dried over $MgSO_4$, filtered and the filtrate was concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (acetone:DCM, 2:3, v:v) to obtain sydnone-linkered peracetylated GalNAc **5** (71 mg, 52% over two steps) as a mixture of the two (α:β) anomers. ^{1}H-NMR (300 MHz; $CDCl_3$): δ 1.94–2.03 (m, 10H), 2.11–2.17 (m, 4H), 3.33 (d, J = 0.4 Hz, 0.4H), 3.47–3.63 (m, 1.5H), 3.76–3.92 (m, 2H), 4.02–4.30 (m, 3.6H), 4.52–4.64 (m, 1H), 4.90 (d, J = 3.6 Hz, 0.5H), 5.05 (dd, J = 11.3, 3.3 Hz, 0.4H), 5.16 (dd, J = 11.3, 3.2 Hz, 0.5H), 5.36 (dd, J = 7.8, 2.8 Hz, 1H), 6.10 (d, J = 8.2 Hz, 0.3H), 6.23 (d, J = 9.5 Hz, 0.4H), 6.84 (t, J = 4.1 Hz, 0.4H), 7.42 (t, J = 5.4 Hz, 0.3H), 7.75 (d, J = 8.3 Hz, 1.6H), 7.90–7.96 (m, 0.4H), 8.10 (d, J = 8.6 Hz, 0.9H), 8.25 (d, J = 8.6 Hz, 0.7H). ^{13}C NMR assigned from HSQC (75 MHz, $CDCl_3$): δ 20.69 (×3), 23.27, 29.29, 36.60, 37.03, 47.77, 50.35, 61.52, 64.96 (×2), 66.25, 66.68, 67.54, 68.40, 70.55, 70.55, 97.63, 101.92, 124.27, 128.57 (×2), 129.00. HRMS ESI: $[M+H]^+$ $C_{26}H_{32}ClN_4O_{12}^+$, calcd 627.1700, obsd 627.1688.

1-*O*-3-(3-[4-Chloro-1,2,3-oxadiazol-3-ium-5-olate]benzamido)-propyl-2-deoxy-2-acetamido-α-D-galactopyranoside (6). Sydnone-linkered peracetylated GalNAc **5** (71 mg, 0.11 mmol) was dissolved in 5 mL of the gudanidine-NaOMe stock solution (477 mg guanidine.HCl, 23 mg Na, 45 mL MeOH, 5 mL DCM) and left to stir at room temperature for 1.5 h. The reaction mixture was neutralized with AcOH and concentrated under reduced pressure. The residue was dissolved in water and passed through a C18 plug, followed by purification using RP-HPLC using a gradient of MeOH in water (0–86% over 21 min) to give the GalNAc-linker-sydnone **6** as the α anomer (25.6 mg, 45% yield). ^{1}H-NMR (300 MHz; D_2O): δ 1.98 (td, J = 6.2, 1.9 Hz, 2H, L2–CH_2), 2.04 (s, 3H, $NCOCH_3$), 3.53–3.60 (m, 3H, L3–CH_2, L1–C*H*), 3.71–3.95 (m, 6H, H-6a, H-6b, L1–C*H*H, H-3, H-4, H-5), 4.16 (dd, J = 10.9, 3.7 Hz, 1H, H-2), 4.90 (d, J = 3.7 Hz, 1H, H-1), 7.91 (d, J = 8.8 Hz, 2H, 2 × Ph–C*H*), 8.06 (d, J = 8.9 Hz, 2H, 2 × Ph–*CH*). ^{13}C NMR assigned from HSQC (75 MHz, D_2O): δ 21.88 ($NCOCH_3$), 28.11 (L2–C), 36.92 (L3–C), 49.81 (C-2), 61.2 (C-6), 65.28 (L1–C), 67.65 (C-3), 68.51 (C-4), 70.66 (C-5), 96.97 (C-1), 125.03 (×2), 128.68 (×2). HRMS ESI: $[M+Na]^+$ $C_{20}H_{25}ClN_4NaO_9^+$, calcd 523.1202, obsd 523.1034.

β-D-Galactopyranosyl-(1→3)-1-*O*-3-(3-[4-Chloro-1,2,3-oxadiazol-3-ium-5-olate]benzamido)-propyl-2-deoxy-2-acetamido-α-D-galactopyranoside (7). GalNAc-linker-sydnone **6** (2 mg, 3.99 μmol) and UDP-Gal (3.2 mg, 5.2 μmol) were dissolved in Tris buffer (450 μL, 50 mM, pH 7.5) containing BSA (0.1%) and $MnCl_2$ (20 μL, 20 mM). To this, CIAP (7.5 μL, 10 mU) and C1GalT1 (21 μL, 5.3 mU/μmol substrate) were added to achieve a 8 mM final concentration of acceptor. The resulting reaction mixture was incubated at 37 °C for 18 h. The reaction mixture was centrifuged and the supernatant subjected to gel filtration over Biogel P-2 (eluent 5% aq. *n*-butanol). Fractions containing product were combined and lyophilized to give Gal-GalNAc-linker-sydnone **7** (2.2 mg, 83% yield) as an amorphous white solid. ^{1}H-NMR (300 MHz; D_2O): δ 1.93–2.00 (m, 5H, L2–CH_2, $NCOCH_3$), 3.48–3.63 (m, 6H, B H-2, L1–C*H*, L3–CH_2, B H-3, B H-5), 3.66–3.82 (m, 5H, A H-6a, AH-6b, B H-6a, B H-6b, L1–C*H*H), 3.88 (d, J = 3.4 Hz, 1H, B H-4), 3.94–4.03 (m, 2H, A H-5, A H-3), 4.20 (d, J = 2.9 Hz, 1H, A H-4), 4.31 (dd, J = 11.1, 3.7 Hz, 1H, A H-2), 4.40 (d, J = 7.6 Hz, 1H, B H-1), 4.87 (d, J = 3.7 Hz, 1H, A H-1), 7.88 (d, J = 8.6 Hz, 2H, 2 × Ph–C*H*), 8.03 (d, J = 8.5 Hz, 2H, 2 × Ph–C*H*). ^{13}C NMR assigned from HSQC (75 MHz, D_2O): δ 21.55 ($NCOCH_3$), 28.00 (L2–C), 37.03 (L3–C), 48.63 (A C-2), 60.66 (×2, A & B C-6), 64.96 (L1–C), 68.40 (×2, A & B C-4), 70.55 (×2, A C-5, B C-2), 71.84 (B C-3), 74.85 (B C-5), 76.99 (A C-3), 97.19 (A C-1), 104.50 (B C-1), 125.13 (×2), 128.57 (×2). HRMS ESI: $[M+Na]^+$ $C_{26}H_{35}ClN_4NaO_{14}^+$, calcd 685.1731, obsd 685.1511.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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