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PAPER

Ionic-liquid-based MS probes for the chemo-enzymatic synthesis of oligosaccharides†

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A new *N*-benzenesulfonyl-based ionic-liquid mass spectroscopy label (I-Tag2) for covalent attachment to substrates has been prepared. I-Tag2 was used to monitor oligosaccharide elongation and serve as a purification handle. Starting from chemically synthesized I-Tag2-labelled *N*-acetyl glucosamine (Glc*N*Ac) **1**, I-Tag2-Lac*N*Ac (Galβ(1–4)Glc*N*Ac) **2** and I-Tag2-Lewis^X (Galβ(1–4)[Fucα(1–3)]Glc*N*Ac) **3**, which are oligosaccharides of biological relevance, were enzymatically prepared. The apparent kinetic parameters for the enzyme catalysed transformations with β-1,4-galactosyltransferase (β-1,4-GalT) and fucosyltransferase VI (FucT VI) were measured by LC-MS demonstrating the applicability and versatility of the new I-Tags in enzymatic transformations with glycosyltransferases.

Introduction

Protein- and lipid-bound oligosaccharides play important recognition roles in a diverse range of biological processes such as protein folding, cell–cell communication, bacterial adhesion, viral infection and masking of immunological epitopes.[1] As a consequence, carbohydrates have become important targets in the discovery of novel therapeutics.[2] Protein glycosylation is more abundant and structurally diverse than all other types of post-translational modification combined.[3–5] *O*-Glycosylation is highly dynamic and fluctuates in response to cellular stimuli through the action of cycling enzymes. Glycosyltransferases typically act by adding monosaccharide residues from mono- or diphosphate sugar nucleotides to growing oligosaccharide chains in a specific fashion, resulting in remarkably complex structures.[6] It has been estimated that mammalian cells require well over 100 different glycosyltransferases to biosynthesise all known oligosaccharide motifs.[1,6] Thus, these enzymes represent an important target in terms of gaining fundamental knowledge of glycan diversity and for the development of potent inhibitors that could lead to drug discovery.

Understanding the factors that control the biological interactions between these important proteins and the oligosaccharides involved at a molecular level remains to this day of prime importance. However, lack of efficient carbohydrate-based tools for study has hampered progress in this area of glycobiology research.

In order to visualise enzyme activity, a large variety of enzymatic assays have been developed over the years.[6–10] The majority of these assays are based on the use of expensive synthetic substrates that release a radioactive, coloured or fluorescent product upon reaction or induce a directly detectable change in solution such as a precipitation. Alternatively, enzyme reactions may also be assayed using external indicators which respond indirectly to product formation or substrate consumption. In any case, reactions are typically monitored by analytical instruments such as HPLC, GC, MS, NMR or IR spectrometers, and thus the sensitivity of the methods is limited to the type of analyte and detector employed for each technique.

Ionic liquids (ILs) have emerged as a popular new class of solvents in organic chemistry due to their unique physical and chemical properties.[11,12] ILs are particularly useful as new vehicles for the immobilisation of reagents as has been shown in a number of synthetic applications[13–16] including oligosaccharide synthesis[17–20] and more recently in enzymatic transformations.[21,22] Notably, ILs are also ideal as mass spectroscopy (MS) probes for fast analysis because of their greater spectral peak intensities and lower limits of detection.[23]

We have recently reported the synthesis of an inexpensive and versatile IL-based chemical label (I-Tag) for fast and sensitive enzyme monitoring by MS as an alternative to using expensive radioactive or fluorescence labelled carbohydrates.[18] We demonstrated the potential of using IL-labelled-glycans for the biological screening of glycosyltransferases in enzymatic reactions with bovine milk β-1,4-galactosyltransferase (β-1,4-GalT).[21] In our previous work, a trifunctional cross-linker was developed for orthogonal attachment to substrates (I-Tag1, Fig. 1). The linker contained an alkyne group for facile coupling to

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†Electronic supplementary information (ESI) available: NMR traces for all novel compounds. See DOI: 10.1039/c2ob25855b

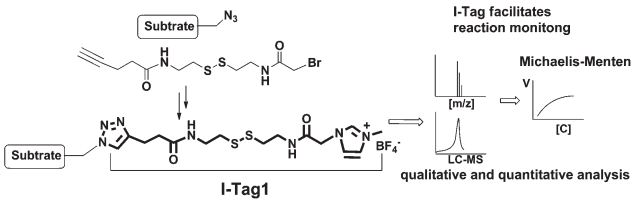


Fig. 1 Previously synthesised ionic liquid label I-Tag1.[21]

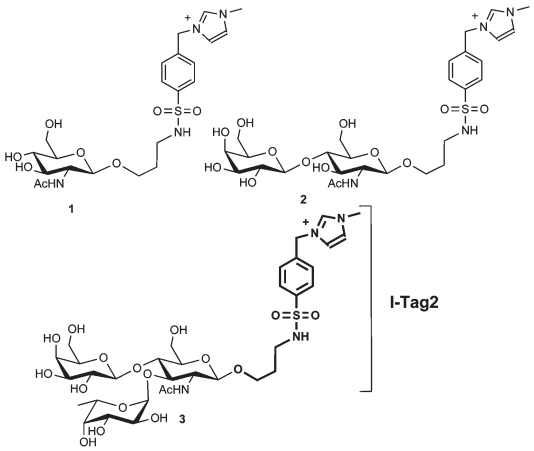


Fig. 2 I-Tag labelled oligosaccharides.

azide-containing sugar moieties, the ionic component for MS analysis and a disulfide bond for mild product release. Despite the versatility of the I-Tag, its use is limited by the chemical stability of the disulfide bond and moreover the IL-linker precursor needs to be synthesized in 4 steps from cystamine dihydrochloride.

In order to expand the scope of application of IL-based labels, we decided to design a novel I-Tag that was chemically more stable and simpler to prepare than the previously reported one.

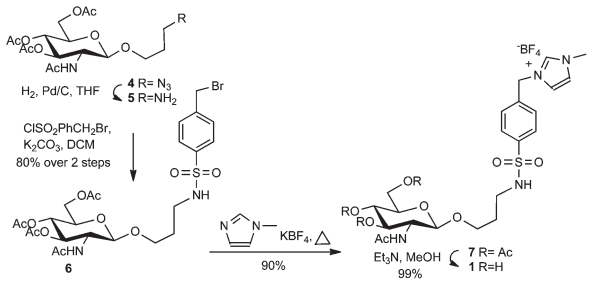
Herein, we describe the chemical synthesis of an *N*-benzenesulfonyl-based I-Tag (I-Tag2) for covalent attachment to glycan substrates. I-Tag2-Lac*N*Ac (Galβ(1–4)Glc*N*Ac) **2** and I-Tag2 Lewis^X (Galβ(1–4)[Fucα(1–3)]Glc*N*Ac) **3** were enzymatically prepared starting from chemically synthesized I-Tag2 *N*-acetyl glucosamine (Glc*N*Ac) **1** (Fig. 2).

Glycoconjugate compounds **2** and **3** are of particular interest, for instance *N*-acetyllactosamine (Lac*N*Ac) is a disaccharide that often appears as a structural element in many glycoconjugates of biological significance and is a substrate for several glycosyltransferases,[24,25] while Lewis^X is a tumour associated antigen.[26] The novel I-Tag was used to monitor reaction progress as well as a purification handle. The apparent kinetic parameters for the enzyme catalysed transformations with β-1,4-GalT and fucosyltransferase VI (FucT VI) were also measured by LC-MS.

Results and discussion

Synthesis

It was envisaged that an *N*-benzenesulfonyl-type linker will offer a more chemically stable I-Tag than the previously described disulfide-based I-Tag1 (Fig. 1), while concomitantly providing



Scheme 1 Synthesis of I-Tag2 labeled *N*-acetylglucosamine.

an additional UV handle as well as the ionic component. Furthermore, I-Tag2 is incorporated by a two-step procedure from easily accessible amino propyl-glycosides and commercially available 4-(bromomethyl)benzenesulfonyl chloride, which makes the I-Tagging process much simpler (Scheme 1).

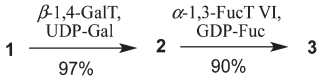
To that end, azido propyl *N*-acetylglucosamine **4** was prepared from commercial D-glucosamine in four steps.[21] The azido functionality was reduced to the corresponding amine by catalytic hydrogenolysis using Pd/C to yield **5** in quantitative yields. Reaction of **5** with 4-(bromomethyl)benzenesulfonyl chloride in the presence of K_2CO_3 gave halide containing precursor **6** in 80% yield after two steps. The introduction of the IL component was subsequently performed by reaction of **6** with 1-methylimidazole in the presence of KBF_4 to yield ionic-labelled substrate **7**. Since I-Tag-products are not soluble in non-polar solvents such as diethyl ether or hexanes,[18] product **7** was purified by simple biphasic extractions with diethyl ether to remove the non-I-Tag-materials (excess reagents, unreacted material) from the I-Tag-product and isolated in 90% yield. Removal of the ester groups from **7** using a mixture of triethylamine and methanol afforded **1** in 99% yield.

To demonstrate that the *N*-benzenesulfonyl-type ionic-liquid label (I-Tag2) in **1** was more stable than our previously synthesized disulphide-based I-Tag1, compound **1** was subjected to the reaction conditions used to cleave the IL component in I-Tag1.[21] Reaction of **1** with tris(2-carboxyethyl)phosphine hydrochloride in water for 24 h showed no decomposition of the starting material. Furthermore, compound **1** was also found to be stable under basic (Et_3N/MeOH) and acidic conditions (5% HCl in water).

Enzymatic glycosylations

With compound **1** in hand, our attention was focused on the enzymatic glycosylations by β-1,4-GalT and FucT VI (Scheme 2).

The transfer of galactose to I-Tag2-acceptor **1** was performed utilising an excess of uridine 5′-diphosphogalactose (UDP-Gal) as a glycosyl donor in the presence of bovine milk β-1,4-GalT as the catalyst following reported procedures.[27] The reaction was monitored by LC-MS (Scheme 2 and Fig. 3) and after 48 h Lac*N*Ac derivative **2** was purified by reverse phase column chromatography and isolated in 97% yield.[28] The structure of the resulting I-Tag-tethered disaccharide **2** was confirmed by ^{1}H- and ^{13}C-NMR and high-resolution MS analyses, demonstrating that I-Tag2 was tolerated by the enzyme.



Scheme 2 Enzymatic synthesis of I-Tag2-LacNAc **2** and I-Tag2 Lewis^X **3**.

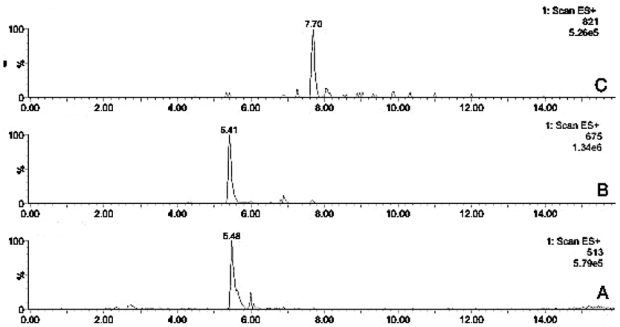


Fig. 3 LC-MS chromatogram of I-Tag2 compounds. (A) TIC trace for I-Tag2-GlcNAc **1** [M$^+$] 513. (B) TIC trace for I-Tag2-LacNAc **2** [M$^+$] 675. (C) TIC trace for I-Tag2-Lewis^X **3** [M$^+$] 821.

In order to obtain fucosylated I-Tag2-derivative **3**, compound **2** was treated with an excess of guanidine 5′-diphosphate-β-L-fucose (GDP-fucose) in the presence of recombinant human α-1,3-fucosyltransferase VI. Similarly, LC-MS was used to monitor reaction progress until all of the starting material was consumed. The crude reaction mixture was purified by reverse phase C-18 column chromatography to give **3** in an excellent yield of 90% after 48 h. The typical shift and coupling constant of the anomeric proton of the α-fucosyl linkage (H1″ δ 5.04 ppm, $J_{1''2''}$ 5.1 Hz) and the doublet corresponding to the signal for CH_3-6″ at δ 1.18 ppm ($J_{CH_3\text{-}6'',\ 5''}$ 6.8 Hz) confirmed its presence in compound **3**.

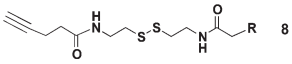
Enzyme kinetics

Based on the observation that compounds **1** and **2** are appropriate substrates for glycosyltransferases, apparent kinetic parameters for β-1,4-GalT and FucT VI were determined using previously described assays[27] (Table 1 and Fig. 4).

The formation of I-Tag2 disaccharide **2** was monitored by LC-MS, where I-Tag linker **8** was used as an internal standard.[29] The apparent K_m value for **1** was determined to be 2.1 ± 0.5 mM with a catalytic efficiency (V_{max}/K_m) of the enzyme of 0.002 min^{-1}. While the K_m value for compound **2** is quite similar to I-Tag1 bearing Glc*N*Ac (K_m 2.7 ± 0.5 mM),[21] the rate of transfer is over 10 times slower at the same enzyme concentration (15 μU) and that is reflected in the overall catalytic efficiency which is 10 times higher for Glc*N*Ac-ITag1 than for **2**. It is noteworthy that **2** bears a shorter linker between the anomeric position of the substrate and the ionic component (Table 1).

In the case of fucosyltransferase VI, following reported assay conditions[6] (Fig. 3), the depletion of **2** was monitored by LC-MS in this instance, with **8** used as an internal standard. The apparent K_m value for **2** was found to be 0.8 ± 0.2 mM with a catalytic efficiency (V_{max}/K_m) of 0.005 min^{-1}. It is apparent that this transferase responds similarly to β-1,4-GalT and tolerates well the introduction of I-Tag2 at the anomeric position of the lactosamine moiety.

Table 1 Apparent kinetic parameters for β-1,4-GalT and FucT VI with ITagged-substrates **1** and **2**

	β-1,4-GalT		
Substrate	K_m (mM)	V_{max} (mM min^{-1})	V_{max}/K_m (min^{-1})
GlcNAc-ITag1	2.7 ± 0.5	0.04 ± 0.002	0.02
1	2.1 ± 0.5	0.003 ± 0.0002	0.002
FucT VI			
2	0.8 ± 0.2	0.004 ± 0.0002	0.005
Internal Standard			

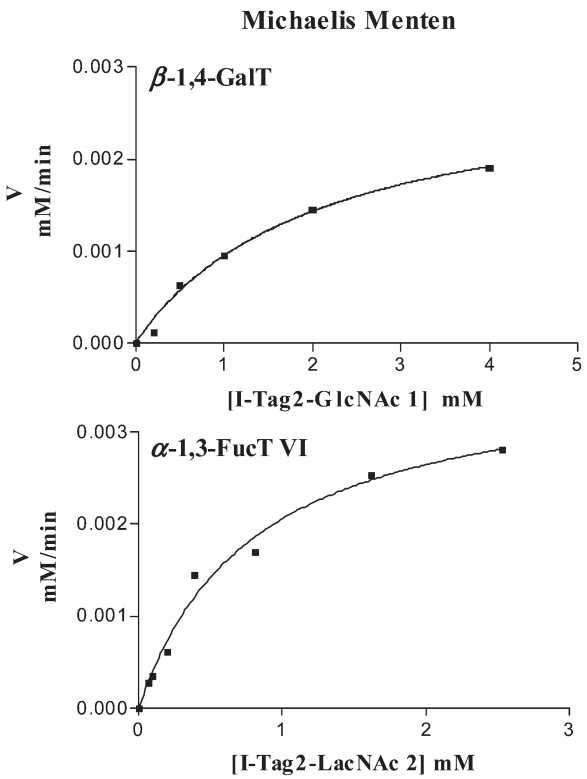


Fig. 4 Michaelis–Menten curves for the reaction of ITagged-substrates **1** and **2** with β-1,4-GalT and FucT VI, respectively.

Conclusions

In summary, we have developed an *N*-benzenesulfonyl-type ionic-liquid label (I-Tag2) that is compatible with enzymatic applications. The new I-Tag is chemically more stable and easier to prepare than our previously described I-Tag1. The new probes are readily accessed from commercial materials and contain both an MS probe as well as a UV moiety. These combined features make them ideal labels for fast and sensitive reaction monitoring by LC-MS without the need for expensive radioactive or fluorescence labelled substrates. Enzyme studies with β-1,4-GalT and

Fuct VI have proven that this methodology can be applied to qualitative and quantitative biological characterisation of glycosyltransferases. Furthermore, a chemo-enzymatic approach has been applied to prepare IL-labelled-glycan probes based on Lac*N*Ac and Lewis^X structures. The new glycan probes can potentially be used in further biological screenings since these oligosaccharides are substrates for a number of sugar modifying enzymes.[24–26]

We believe that this new class of IL-based MS probes will be very valuable for general and fast enzyme monitoring in a variety of biological systems, as well as a product purification handle.

Experimental

General methodology

Chemicals and β-1,4-galactosyltransferase (EC 2.4.1.22) were purchased from Aldrich and Fluka and used without further purification. A truncated human FucT VI form containing the amino acids 63–359 was fused to the interferon signal sequence and cloned into pREP9 vector (Invitrogen) as described in ref. 30. The construct was transfected into CHO-K1 cells generating a secretable FucT VI enzyme. For protein expression, cells were cultivated in an F-12 nutrient mixture medium (Gibco) supplemented with 10% FCS (Gibco) and penicillin (100 U)/streptomycin (100 μg mL^{-1}) (Sigma) at 37 °C and 5% CO_2. The medium was collected after 1 week of cell culture and filtrated (0.22 μm). FucT VI was then purified from the medium by affinity chromatography on a GDP-hexanolamine column as previously described.[31]

Preactivated molecular sieves kept in an oven at 150 °C were activated in a standard microwave (800 W) for 3 min (3 × 1 min) and cooled under vacuum. Dry solvents, where necessary, were obtained by distillation using standard procedures or by passage through a column of anhydrous alumina using equipment from Anhydrous Engineering (University of Bristol) based on the Grubbs' design. Reactions requiring anhydrous conditions were performed under an atmosphere of dry nitrogen; glassware, syringes and needles were either flame dried immediately prior to use or placed in an oven (150 °C) for at least 2 h and allowed to cool either in a desiccator or under an atmosphere of dry nitrogen; liquid reagents, solutions or solvents were added *via* a syringe or cannula through rubber septa; solid reagents were added *via* Schlenk type adapters. Reactions were monitored by TLC on Kieselgel 60 F254 (Merck). Detection was by examination under UV light (254 nm) and by charring with 10% sulfuric acid in ethanol. Flash chromatography was performed using silica gel [Merck, 230–400 mesh (40–63 μm)], the crude material was applied to the column as a solution in CH_2Cl_2 or by pre-adsorption onto silica, as appropriate. Extracts were concentrated under reduced pressure using both a Büchi rotary evaporator (bath temperatures up to 40 °C) at a pressure of either 15 mmHg (diaphragm pump) or 0.1 mmHg (oil pump), as appropriate, and a high vacuum line at room temperature. ^{1}H NMR and ^{13}C NMR spectra were measured in the solvent stated on Varian INOVA 400 or 500 instruments, respectively. Chemical shifts are quoted in parts per million from $SiMe_4$ and coupling constants (*J*) are given in hertz. Multiplicities are abbreviated as b (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or combinations thereof. Positive ion Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectra were recorded using an HP-MALDI instrument using a gentisic acid matrix. The LC/MS analysis of samples was performed on a Waters LC-MS system ionized by ESI equipped with a Micromass Quattro micro API Mass Spectrometer as a detector and additional Photodiode Array (PDA 2998) and Evaporative Light Scattering (ELSD 2424) detectors. The LC was run on a Luna C18, 4.6 mm × 250 mm analytical column. The capillary and sample cone voltages were 3300 and 35 V. The desolvation and source temperatures were 350 °C and 120 °C. The cone and desolvation gas flow rates were 50 and 600 L h^{-1}. Analysis was performed with MassLynx 4.1.

3-(4-(Bromomethyl)benzenesulfonamidyl) propyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside 6. A solution of **4** (157 mg, 0.37 mmol) in THF (5 mL) and Pd/C (150 mg, 10% w/w) was stirred at room temperature for 3 h under a H_2 atmosphere. The solvents were then concentrated under reduced pressure and the residue was purified by a short silica gel plug (DCM/MeOH/Et_3N: 5/5/0.1) to afford derivative **5**. Without further purification, a solution of 4-(bromomethyl)-benzenesulfonyl chloride (64 mg, 0.238 mmol) and anhydrous K_2CO_3 (33 mg, 0.24 mmol) in CH_2Cl_2 (1 mL) was added dropwise to a stirred solution of **5** in CH_2Cl_2 (80 mg, 0.20 mmol in 1 mL) at 0 °C. The mixture was then stirred for 30 min at 0 °C and another 20 min at room temperature. The crude mixture was then concentrated under reduced pressure and then purified by silica gel flash column chromatography (toluene/acetone, gradient from 8/2 to 7/3) to give **6** (100 mg, 80%). ^{1}H NMR ($CDCl_3$, 400 MHz, ppm): δ = 7.85 (d, 2H, *J* = 8.5 Hz, C*H*, Ph), 7.54 (d, 2H, *J* = 8.5 Hz, C*H*, Ph), 7.58 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*Ac), 5.47 (t, 1H, *J* = 6.5 Hz, N*H*SO_2), 5.21 (dd, 1H, $J_{3,4}$ = 9.5 Hz, $J_{3,2}$ = 10.5 Hz, H-3), 5.07 (t, $J_{4,3}$ = $J_{4,5}$ = 9.5 Hz, H-4), 4.64 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 4.50 (s, 2H, CH_2Br), 4.25 (dd, 1H, $J_{6a,5}$ = 4.5, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.15 (dd, 1H, $J_{6b,5}$ = 2.5, $J_{6b,6a}$ = 12.5 Hz, H-6b), 3.97–3.90 (m, 2H, H-2, OCH_2), 3.70 (ddd, 1H, $J_{5,6b}$ = 2.5 Hz, $J_{5,6b}$ = 4.5 Hz, $J_{5,4}$ = 9.5 Hz, H-5), 3.65–3.59 (m, 1H, OCH_2), 3.17–3.00 (m, 2H, CH_2NH), 2.09 (s, 3H, CH_3, *Ac*), 2.04 (s, 3H, CH_3, *Ac*), 2.03 (s, 3H, CH_3, *Ac*), 1.95 (s, 3H, CH_3, *Ac*), 1.82–1.70 (m, 2H, OCH$_2$CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz, ppm): δ = 171.0, 170.8, 170.7, 169.9 (CO, *Ac*), 142.4, 140.1 (C_q, Ph), 129.7, 127.4 (*C*H, Ph), 100.9 (C-1), 72.5 (C-3), 71.9 (C-5), 68.4 (C-4), 66.9 (O*C*H_2), 62.0 (C-6), 54.5 (C-2), 40.1 (*C*H_2NH), 31.5 (*C*H_2Br), 29.1 (OCH$_2$*C*H_2), 23.3 (*C*H_3, *Ac*), 20.8 (*C*H_3, *Ac*), 30.7 (*C*H_3, *Ac*), 20.6 (*C*H_3, *Ac*). MALDI-TOF (MNa^+) calcd: 659.1; found: 649.1; ESI-HRMS for $C_{24}H_{33}BrN_2NaO_{11}S$ (MNa^+) calcd: 659.0886; found: 659.0881. HPLC retention time = 5.48 min (5–75% acetonitrile/water (0.1% TFA) gradient over a 15 min period).

3-(4-(3-Methylimidazolium)methylbenzenesulfonamidyl) propyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside 7. A solution of **6** (95 mg, 0.15 mmol), 1-methyl imidazole (50 μL, 0.63 mmol) and potassium tetrafluoroborate (100 mg, 0.80 mmol) in CH_3CN (2 mL) were stirred at reflux for 18 h. The crude mixture was cooled to room temperature and then filtered. The filtrate was concentrated under reduced pressure and

dried under vacuum for 2 h. The dried residue was washed with Et_2O (3 × 5 mL) and dried under vacuum to give **7** (98 mg, 90%). ^{1}H NMR (CD_3CN, 500 MHz, ppm): δ = 8.74 (s, 1H, NC*H*N), 7.93 (d, 2H, J = 8.5 Hz, C*H*, Ph), 7.55 (d, 2H, J = 8.5 Hz, C*H*, Ph), 7.45 (t, 1H, J = 2.0 Hz, NCHC*H*N), 7.39 (t, 1H, J = 2.0 Hz, NC*H*CHN), 7.09 (d, 1H, $J_{NH,2}$ = 9.5 Hz, N*H*Ac), 6.56 (t, 1H, J = 6.5 Hz, N*H*SO_2), 5.45 (s, 2H, CH_2N), 5.09 (dd, 1H, $J_{3,4}$ = 9.5 Hz, $J_{3,2}$ = 10.5 Hz, H-3), 4.91 (t, $J_{4,3}$ = $J_{4,5}$ = 9.5 Hz, H-4), 4.71 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 4.20 (dd, 1H, $J_{6a,5}$ = 5.0, $J_{6a,6b}$ = 12.0 Hz, H-6a), 4.03 (dd, 1H, $J_{6b,5}$ = 2.5, $J_{6b,6a}$ = 12.0Hz, H-6b), 3.85–3.81 (m, 4H in which s at 3.84, 3H, NCH_3 H-2), 3.79–3.75 (m, 1H, OCH_2), 3.97–3.90 (m, 2H, H-2, OCH_2), 3.73 (ddd, 1H, $J_{5,6b}$ = 2.5 Hz, $J_{5,6b}$ = 5.0 Hz, $J_{5,4}$ = 9.5 Hz, H-5), 3.54 (ddd, 1H, J = 4.5, 8.0, 10.0 Hz, OCH_2), 2.95–2.84 (m, 2H, CH_2NH), 2.00 (s, 3H, CH_3, *Ac*), 1.96 (s, 3H, CH_3, *Ac*), 1.96 (s, 3H, CH_3, *Ac*), 1.73 (s, 3H, CH_3, *Ac*), 1.70–1.63 (m, 2H, OCH$_2$CH_2). ^{13}C NMR (CD_3CN, 125 MHz, ppm): δ = 171.3, 171.0, 170.9, 170.6 (CO, *Ac*), 142.2, 139.2 (C_q, Ph), 137.5 (N*C*HN), 130.1, 128.6 (*C*H, Ph), 125.0 (N*C*HCHN), 123.4 (NCH*C*HN), 101.6 (C-1), 73.8 (C-3), 72.3 (C-5), 69.7 (C-4), 67.5 (O*C*H_2), 62.9 (C-6), 54.5 (C-2), 52.9 (*C*H_2N), 40.6 (*C*H_2NH), 37.0 (N*C*H_3), 30.3 (OCH$_2$*C*H$_2$), 23.1, 20.9 (*C*H_3, *Ac*). MALDI-TOF (M^+) calcd: 639.2; found: 639.2; ESI-HRMS for $C_{28}H_{39}N_4O_{11}S$ (M^+) calcd: 639.2331; found: 639.2331.

3-(4-(3-Methylimidazolium)methylbenzenesulfonamidyl) propyl 2-acetamido-2-deoxy-β-D-glucopyranoside 1. A solution of **7** (96 mg, 0.13 mmol) in Et_3N/MeOH (1 mL/8 mL) was left stirring at room temperature for 18 h. Toluene was then added and the crude mixture was then concentrated under vacuum to give **1** (78 mg, 99%). ^{1}H NMR (D_2O, 500 MHz, ppm): δ = 7.87 (d, 2H, J = 8.5 Hz, C*H*, Ph), 7.56 (d, 2H, J = 8.5 Hz, C*H*, Ph), 7.47 (s, 1H, NCHC*H*N), 7.46 (s, 1H, NC*H*CHN), 5.49 (s, 2H, CH_2N), 4.42 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 3.89–3.82 (m, 5H in which s at 3.84, 3H, NCH_3, H-6a, OCH_2), 3.70 (dd, 1H, $J_{6b,5}$ = 5.0 Hz, $J_{6b,6a}$ = 12.5 Hz, H-6b), 3.61 (dd, 1H, $J_{2,1}$ = 8.5 Hz, $J_{2,3}$ = 10.0 Hz, H-2), 3.57–3.53 (m, 1H, OCH_2), 3.49 (t, 1H, $J_{3,2}$ = $J_{3,4}$ = 10.0 Hz, H-3), 3.42–3.36 (m, 2H, H-4, H-5), 2.95–2.87 (m, 2H, CH_2NH), 1.89 (s, 3H, CH_3, *Ac*), 1.69–1.63 (m, 2H, OCH$_2$CH_2). ^{13}C NMR (D_2O, 125 MHz, ppm): δ = 174.3 (CO, *Ac*), 139.4, 138.8 (C_q, Ph), 129.2, 127.5 (*C*H, Ph), 124.0 (N*C*HCHN), 123.4 (NCH*C*HN), 101.0 (C-1), 75.8 (C-5), 73.7 (C-3), 69.9 (C-4), 67.2 (O*C*H_2), 60.7 (C-6), 55.5 (C-2), 51.9 (*C*H_2N), 39.8 (*C*H_2NH), 35.8 (N*C*H_3), 29.0 (OCH$_2$*C*H$_2$), 22.0 (*C*H_3, *Ac*). MALDI-TOF (M^+) calcd: 513.2; found: 513.3; ESI-HRMS for $C_{22}H_{33}N_4O_{14}S$ (M^+) calcd: 513.2014; found: 513.2013. HPLC retention time = 5.48 min (5–75% acetonitrile/water (0.1% TFA) gradient over a 15 min period).

3-(4-(3-Methylimidazolium)methylbenzenesulfonamidyl) propyl β-D-galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranoside 2. Incubations were carried out in 1 mL MOPS buffer (50 mM, pH 8.2) containing $MnCl_2$ (20 mM) and bovine serum albumin (1 mg mL^{-1}). Acceptor **8** (4.0 mg), UDP-Gal (3 mg), and β-1,4-GalT (200 mU) were incubated at 37 °C for 48 h. Every 12 h, 1 mg of UDP-Gal was added to the incubation mixture to compensate for any hydrolysis of the donor. The reaction was monitored by LC-MS. At the completion of the reaction, the sample was purified on a reverse phase C-18 column (Ø × length; 1.3 × 2 in cm), gradient H_2O/MeOH (1/0–9/1) to afford **2** (5.1 mg, 97%). ^{1}H NMR (D_2O, 500 MHz, ppm): δ = 8.43 (s, 1H, NC*H*N), 7.89 (d, 2H, J = 8.8 Hz, C*H*, Ph), 7.57 (d, 2H, J = 8.8 Hz, C*H*, Ph), 7.48 (s, 1H, NCHC*H*N), 7.46 (s, 1H, NC*H*CHN), 5.50 (s, 2H, CH_2N), 4.44 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1′), 4.42 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 3.96 (dd, 1H, $J_{6b,5}$ = 2.3 Hz, $J_{6b,6a}$ = 12.3 Hz, H-6b), 3.90–3.61 (m, 3H, H-5, H-5′, H-3′), 3.88 (s, 3H, NCH_3), 3.86–3.60 (m, 1H, OCH_2), 3.79 (dd, 1H, $J_{6a,5}$ = 5.1 Hz, H-6a), 3.75–3.67 (m, 2H, H-6a′, H-6b′), 3.73 (m, 1H, H-4′), 3.66 (m, 1H, H-4), 3.65 (dd, 1H, $J_{2,3}$ = 10.0 Hz, H-2), 3.51 (t, 1H, $J_{3,2}$ = $J_{3,4}$ = 10.0 Hz, H-3), 3.48 (dd, 1H, $J_{2',3'}$ = 10.0 Hz, H-2′), 3.00–2.89 (m, 2H, CH_2NH), 1.88 (s, 3H, CH_3, *Ac*), 1.70–1.63 (m, 2H, OCH$_2$CH_2). ^{13}C NMR (D_2O, 125 MHz, ppm): δ = 174.3 (CO, *Ac*), 139.2–126.8 (C-Ph), 130.1 (N*C*HCHN), 131.8 (NCH*C*HN), 105.4 (C-1′), 103.4 (C-1), 81.0 (C-4), 77.9 (C-4′), 74.9 (C-2′), 78.0, 77.4, 75.11 (C-5, C-5′, C-3′), 73.6 (C-3), 69.8 (O*C*H_2), 63.8 (C-6′), 62.8 (C-6), 57.7 (C-2), 54.5 (*C*H_2N), 42.5 (*C*H_2NH), 38.5 (N*C*H_3), 24.9 (OCH$_2$*C*H$_2$), 24.6 (*C*H_3, *Ac*). MALDI-TOF (M^+) calcd: 675.2; found: 675.1; ESI-HRMS for $C_{28}H_{43}N_4O_{13}S$ (M^+) calcd: 675.2547; found: 675.2550. HPLC retention time = 5.41 min (5–75% acetonitrile/water (0.1% TFA) gradient over a 15 min period).

3-(4-(3-Methylimidazolium)methylbenzenesulfonamidyl) propyl α-D-fucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranoside 3. Incubations were carried out in 500 μL MOPS buffer (50 mM, pH 8.2) containing $MnCl_2$ (20 mM) and bovine serum albumin (1 mg mL^{-1}). Acceptor **2** (2.0 mg), GDP-Fuc (2 mg), and α-1,3-FucT VI (180 mU) were incubated at 37 °C for 48 h. Every 12 h, 1 mg of GDP-Fuc was added to the incubation mixture to compensate for any hydrolysis of the donor. The reaction was monitored by LC-MS. At the completion of the reaction, the sample was purified on a reverse phase C-18 column (Ø × length; 1.3 × 2 in cm), gradient H_2O/MeOH (1/0–9-1) to afford **2** (2.4 mg, 90%). ^{1}H NMR (D_2O, 500 MHz, ppm): δ = 7.93 (d, 2H, J = 8.4 Hz, C*H*, Ph), 7.60 (d, 2H, J = 8.4 Hz, C*H*, Ph), 7.51 (s, 1H, NCHC*H*N), 7.50 (s, 1H, NC*H*CHN), 5.53 (s, 2H, CH_2N), 5.10 (d, 1H, $J_{1'',2''}$ = 4.0 Hz, H-1″), 4.85–4.78 (m, 1H, H-5″), 4.49 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1′), 4.48 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1), 4.00 (dd, 1H, $J_{6b,5}$ = 2.3 Hz, $J_{6b,6a}$ = 12.0 Hz, H-6b), 3.90–3.56 (m, 3H, H-3″, H-4″, H-4), 3.94–3.65 (m, 3H, H-5, H-5′, H-3′), 3.92 (s, 3H, NCH_3), 3.90 (m, 1H, OCH_2), 3.87 (m, 1H, H-6a), 3.86–3.71 (m, 2H, H-6a′, H-6b′), 3.70 (m, 1H, H-2″), 3.60 (m, 1H, OCH_2), 3.65 (d, 1H, $J_{3',4'}$ < 2, H-4′), 3.86 (dd, 1H, $J_{2,3}$ = 10.0 Hz, H-2), 3.57 (m, 1H, Hz, H-3), 3.51 (dd, 1H, $J_{2,3}$ = 9.8 Hz, H-2′), 3.00–2.99 (m, 2H, CH_2NH), 1.91 (s, 3H, CH_3, *Ac*), 1.71–1.66 (m, 2H, OCH$_2$CH_2), 1.18 (d, 3H, $J_{Me\text{-}6'',5''}$ = 6.8, CH_3-6″). ^{13}C NMR (D_2O, 125 MHz, ppm): δ = 174.3 (CO, *Ac*), 147.8 (*C*H_3-6″), 139.2–126.8 (C-Ph), 131.8 (NCH*C*HN), 130.1 (N*C*HCHN), 101.2 (C-1″), 104.3 (C-1), 103.3 (C-1′), 75.0 (C-4′), 77.9 (C-3), 77.4, 74.4 (C-2″), 73.5 (C-2′), 72.1, 70.8, 69.8 (O*C*H_2), 69.2 (C-5″), 63.6 (C-6′), 62.3 (C-6), 58.3 (C-2), 54.4 (*C*H_2N), 42.3 (*C*H_2NH), 38.4 (N*C*H_3), 31.4 (OCH$_2$*C*H$_2$), 24.5 (*C*H_3, *Ac*). MALDI-TOF (M^+) calcd: 821.3; found: 821.4; ESI-HRMS for $C_{34}H_{53}N_4O_{17}S$ (M^+) calcd: 821.3126; found: 821.3132. HPLC retention time = 7.70 min (5–75% acetonitrile/water (0.1% TFA) gradient over a 15 min period).

Enzyme kinetics

Galactosyltransferase assay. To obtain kinetic values for *N*-acetylglucosamine analog **1**, incubations were carried out in 100 μL MOPS buffer (50 mM, pH 8.2) containing $MnCl_2$ (20 mM) and bovine serum albumin (1 mg mL^{-1}), a saturating concentration of UDP-Gal that was held constant at 0.46 mM and enzyme (15 μU) were used. The reaction rates were measured at 6 different concentrations of **1** (0.2 mM to 4 mM). ITagged linker **8** (29 μM) was used as an internal standard to ensure accurate injection volume. The reactions were quenched by boiling at 100 °C and spin down at 6000 rpm in a minicentrifuge to precipitate the protein and the supernatant kept. β-1,4-GalT activity was measured using a LC-MS assay. An aliquot of the supernatant (20 μL) was directly injected onto the LC-MS using a 5–75% acetonitrile/water (0.1% TFA) gradient over a 15 min period. All kinetic assays were performed in duplicate. The time of incubation was set to 20 min at 37 °C. Kinetic parameters were obtained using Origin 7.0 software (Origin lab).

Fucosyltransferase assays. To obtain kinetic values for *N*-acetyllactosamine analog **2**, incubations were carried out in 100 μL MOPS buffer (50 mM, pH 8.2) containing $MnCl_2$ (20 mM) and bovine serum albumin (1 mg mL^{-1}), a saturating concentration of GDP-fucose that was held constant at 0.50 mM and enzyme (60 μU) were used. The reaction rates were measured at 6 different concentrations of **2** (0.05 mM to 2.5 mM). ITagged linker **8** (29 μM) was used as an internal standard to ensure accurate injection volume. The reactions were quenched by boiling at 100 °C and spin down at 6000 rpm in a minicentrifuge to precipitate the protein and the supernatant kept. α-1,3-FucT VI activity was measured using a LC-MS assay. An aliquot of the supernatant (20 μL) was directly injected onto the LC-MS using a 5–75% acetonitrile/water (0.1% TFA) gradient over a 15 min period. All kinetic assays were performed in duplicate. The time of incubation was set to 20 min at 37 °C. Kinetic parameters were obtained using Origin 7.0 software (Origin lab).

Calibration and data acquisition

In all the reactions studied, two ions were monitored in order to quantify the progress of the reaction. The ions monitored (ESI) were disaccharide **2** [M^+] and internal standard **8** [M^+]. The total ion count for each peak was then integrated, and the absolute concentration of each compound was obtained from standard curves after normalising the ionisation efficiencies for each reaction with respect to the internal standard **8**.

A standard curve was constructed for analog **2** at eight different concentrations around those found under the initial reaction conditions needed for initial-rate-method measurements (ESI).

For reaction monitoring, either product formation or acceptor consumption, as judged by their TIC integrated values, were monitored as a function of time. To allow full determination of the kinetic parameters, UDP-Gal and GDP-Fuc were held at a fixed saturating concentration, respectively while the concentration of ITagged-substrates **1** and **2** was varied.

Stability of compound 1 to reductive conditions

To a solution of **1** (0.5 mg) in water (0.5 mL) was added tris-(2-carboxyethyl)phosphine hydrochloride (50 mM in H_2O) and the mixture stirred at room temperature for 24 h, after which the sample was concentrated under reduced pressure and purified on a small plug of C-18 to yield **1** (0.5 mg).

Stability of compound 1 to acidic conditions

To a solution of **1** (0.4 mg) in water (0.5 mL) was added HCl (5% in H_2O) and the mixture stirred at room temperature for 24 h, after which the sample was concentrated under reduced pressure and purified on a small plug of C-18 to yield **1** (0.4 mg).

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