■ **Carbohydrate Chemistry** |*Hot Paper*|

Direct and Regioselective Di-α-fucosylation on the Secondary Rim of β-Cyclodextrin

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Abstract: A straightforward glycosylation method is described to regio- and stereoselectively introduce two α-L-fucose moieties directly to the secondary rim of β-cyclodextrin. Using NMR and MS fragmentation studies, the nonasaccharide structure was determined, which was also visualized using molecular dynamics simulations. The reported glycosylation method proved to be robust on gram-scale, and may be generally applied to directly glycosylate β-cyclodextrins to make well-defined multivalent glycoclusters.

Introduction

Carbohydrate–protein and carbohydrate–carbohydrate interactions are central to many biological processes in all domains of life, including glycoprotein folding, cell signaling, immuno-modulation, and host-pathogen binding.[1] Generally, these interactions are highly multivalent in character, and distinct carbohydrate moieties spaced at specific distances are essential for productive binding.[2] This "multivalency effect" has inspired the design of glycoconjugate clusters based on polymers,[3] nanoparticles,[4] cyclodextrins[5,6] and calixarenes,[7] amongst others, to which a plethora of different glycans are attached.[8] Interestingly, amongst the various attachment methods and linkers, the natural glycosidic linkage is virtually absent. Instead, glycans are often attached through a peptide, thioester, or triazole moiety.

In our on-going program to generate purely carbohydrate-based inhibitors of host–pathogen interactions for clinical and food applications, we set out to glycosylate cyclodextrins (CDs) directly with L-fucose moieties. Cyclodextrins are cyclic oligo-saccharides consisting of six, seven, or eight α-(1→4)-D-gluco-side moieties, to give the so-called α-, β-, and γ-CDs, respectively. Owing to their non-toxic nature, CDs are generally regarded as safe (GRAS) and have received widespread attention in food, agriculture, cosmetics, and pharmacy. As depicted in Scheme 1, CDs generally adopt the shape of a truncated cone,
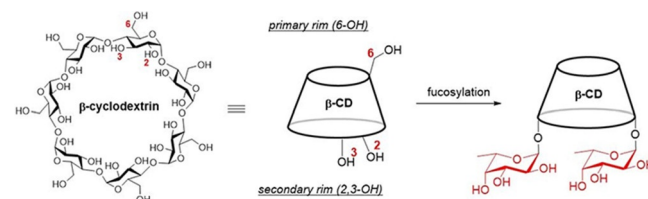
Scheme 1. β-CD cone representation and schematic depiction of secondary rim α-fucosylation.

which has the C-6 hydroxyls on the top (primary rim) and the C-2 and C-3 hydroxyls on the bottom of the cone (secondary rim, Scheme 1).[9] Most examples of chemically synthesized glycoclusters display the glycans on the primary rim,[10–17] and only a few examples are reported of direct glycosylation of the C6 hydroxyls.[18–21] Alternatively, enzymatic transglycosylation strategies resulted in the formation of a mixture of multiply glycosylated CDs, bearing α-galactosyl units on the C-2 or C-6 position of the glucosides.[22,23] When two or more glycoside units were introduced, low regioselectivity was observed. Thus, application of chemical modifications allows for better control over the substitution pattern of CD.

Interestingly, due to the primary alcohols, the primary rim is considered conformationally more flexible, resulting in a reduced effective diameter. In contrast, the alcohols on the secondary rim are sterically more constrained and less flexible, positioning the substituents in a well-defined manner. For the generation of multivalent CD-based glycoclusters, it is highly desirable to have a method to directly glycosylate the secondary rim. The main challenges associated with directly glycosyl-

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ating cyclodextrins arise from the lack of discrimination between the chemically similar hydroxyls on both rims, and the complicated characterization of the resulting products. Illustrative of the first challenge are the seminal reports from Sollogoub and co-workers that deal with the discrimination between the primary hydroxyls to install different functionalities.[24] Also, several methods exist to regioselectively introduce or remove one[25] or two[26–32] protecting groups on the primary rim. To the best of our knowledge, methods to regioselectively liberate hydroxyls on the secondary rim are scarce, with one example reported for permethylated CD.[33] Herein, we report the stereoselective di-α-fucosylation of β-CD on two C-3 positions of the secondary rim. L-Fucose is selected as multivalent CD decoration for its central role in biological glycans,[34–36] where it is attached to blood group antigens, the complex N-glycan of glycoproteins, and human milk oligosaccharides, among others. Fucosylated glycans have shown to block the intestinal adhesion of pathogenic bacteria such as *Salmonella enterica*[37] and *Escherichia coli*,[38] and this makes multivalent fucosylated scaffolds interesting targets for novel antimicrobial therapies.

Results and Discussion

Our method makes use of a β-CD ring (compound **4**) that has the C-3 hydroxyl groups of each glucoside unit available. The synthesis of the acceptors started with perbenzylation of commercially available α- and β-CD, providing the protected CDs **1** and **2** in high yield (Scheme 2).[29] These were then subjected to Et$_3$SiH/I$_2$[39,40] to regioselectively liberate all C-3 hydroxyls to
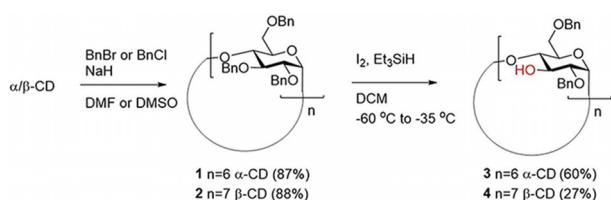
Scheme 2. Synthesis of acceptors **3** and **4**.

give acceptors **3** and **4**, having six and seven free hydroxyls, respectively. For the α-fucosylation of these acceptors, reported thio-fucoside donor **5** was utilized (Scheme 3). While the stereoselective introduction of multiple α-fucosidic bonds simultaneously remains a challenging endeavor, this donor has proven to provide robust α-fucosylation in other glycan syntheses.[41] The first glycosylation experiment was performed using 1.15 equivalents of donor **5** per hydroxyl (total: 6.9 equiv for α-CD, 8.05 equiv for β-CD), which was treated with pre-activation conditions (Ph$_2$SO/Tf$_2$O) at −80 °C (Scheme 3).[42] After complete donor activation, the CD acceptor was added and the reaction was left to proceed at −80 °C overnight, after which time the products were analyzed by UPLC-UV/MS (C4 column). To our surprise, the mixture of products from the α-CD acceptor **3** contained more than 55 % of the twice fucosylated octasaccharide, albeit a mixture of three isomers (peak f =
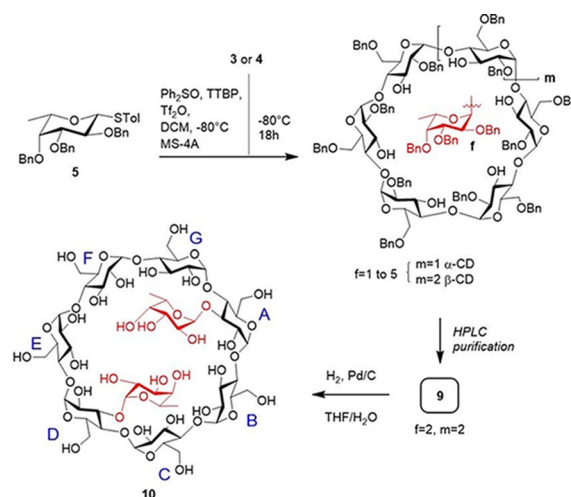
Scheme 3. Fucosylation of CD acceptors **3** and **4**, and synthesis of 2Fuc-β-CD nonasaccharide **10** (numbering of Glc residues is given in blue).

2, Figure 1A, and Figure S1A). The coupling constants of the anomeric fucose H1 signals indicated that the fucosidic linkage was formed stereoselectively, eliminating the possibility that the mixture of octasaccharides resulted from erosion of stereoselectivity (Figure S2). In addition, a significant amount of the (1→1′)-fucosyl disaccharide was isolated, probably resulting from donor hydrolysis and subsequent glycosylation, highlighting the poor reactivity of the CD acceptor. Interestingly, similar experimental conditions with the β-CD acceptor **4** also gave the twice fucosylated nonasaccharide **9** as the major product (50 %), but it appeared as a single peak in the UPLC chromatogram, suggesting the formation of one major nonasaccharide isomer (Figure 1B). The α-stereoselectivity of the newly formed glycosidic bonds was confirmed by ^{1}H NMR (J_{1-2} = 4.0 Hz). To simplify the characterization of the nonasaccharide product, compound **9** was isolated by preparative HPLC (20 % isolated yield), and all benzyl protecting groups were removed by hydrogenolysis using Pd/C in THF/H$_2$O to give product **10** (80 %).

To elucidate the structure of the major nonasaccharide, three different regioisomers were considered (Figure S1B): fu-
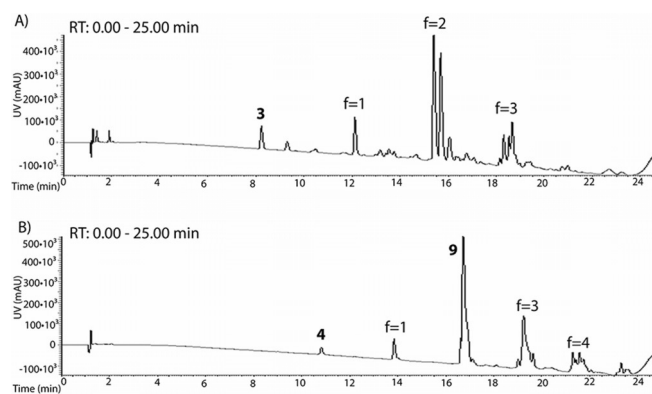
Figure 1. UPLC-UV traces of fucosylated CDs after size-exclusion chromatography: A) Fuc-α-CDs, B) Fuc-β-CDs.

cosides on the 3-OH positions of neighboring glucoside units (termed 3A,3B), two Fuc-Glc motifs separated by one unmodified glucoside (3A,3C), and two Fuc-Glc motifs separated by two unmodified glucose residues (3A,3D). We commenced our characterization efforts with NMR on the purified sample. Signals arising from H-1, H-2 and H-3 within the ring of several Glc and Fuc units were identified with ^{1}H NMR, COSY and TOCSY experiments for each residue (see the Supporting Information). HSQC in combination with HMBC experiments provided the clear identification of the C-3 signals of the two Glc units to which a fucoside was attached. The latter signals shifted significantly downfield in HSQC as compared to the non-modified Glc-C3 signals (from 73.2–72.6 ppm to 77.4 and 77.2 ppm, Figure S11) and had a cross-peak with H-1 of fucoses in HMBC spectrum (Figure 2 B, cross-peak A).

Also using HMBC analysis, we identified the C4 signals of the modified Glc residues (Figure 2, bond and cross-peak B). Interestingly, these C4 signals had a cross-peak to H1 of a non-modified glucose residue (Figure 2 A, bond C) indicating the presence of a non-fucosylated Glc residue linked to each of the Fuc-α(1→3)-Glc motifs, eliminating the possibility of the 3A,3B regioisomer (see the Supporting Information for a detailed explanation). However, further proof to determine the relative positions of the other unmodified Glc residues remained difficult to obtain, since many Glc signals overlapped. Also comparing it to the singly fucosylated β-CD, isolated from HPLC (BnFuc$_1$-βCD, see SI), did not aid in the characterization.

Next, we turned our attention to mass spectrometry fragmentation studies.[43] Ionization of fucosylated glycans in positive mode is notoriously prone to allow migration of the fucosides to neighboring residues upon MS analysis of intact protonated glycan ions, as recently highlighted by Seeberger and co-workers.[44] In contrast, the analysis of the deprotonated species formed with negative-mode ionization reduces the occurrence of side-reactions.[45, 46] Therefore, HILIC chromatography in combination with MS in negative ion-mode was applied to fucosylated cyclodextrin 10, and parent masses of m/z 1425 $[M-H]^-$ and 712 $[M-2H]^{2-}$ were observed (Figure S3). Next, fragmentation of the double charged ion was optimized, resulting in cross-ring fragmentations predominantly. In general, the CD ring is first opened to form linear parent ions, which will then undergo cross-ring fragmentations (Figure 3 A).[47, 48] The observation of the consecutive ^{2,4}A fragments 11, 13, 15, 17, 19 and 21 confirms the presence of two glucoside units between the fucosylated residues, as in the 3A,3D type of modification (Figure 3 B). Additionally, the m/z values corresponding to the ^{0,2}A and ^{0,2}X fragments 23, 25, 26, 27 and 28 support the 3A,3D substitution pattern by demonstrating evidence of three glucosides between the Glc-Fuc moieties (see the Supporting Information for more details). Specific fragments corresponding to the 3A,3C substitution pattern were not observed.
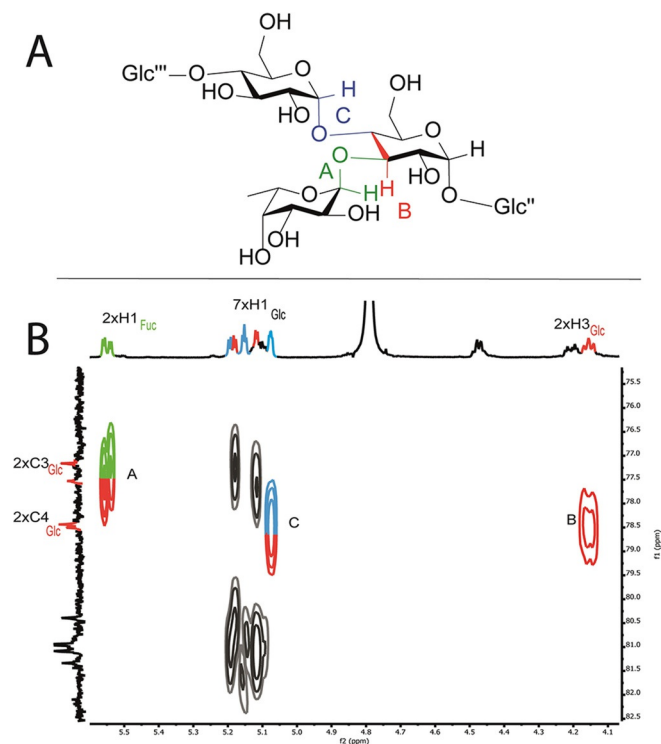

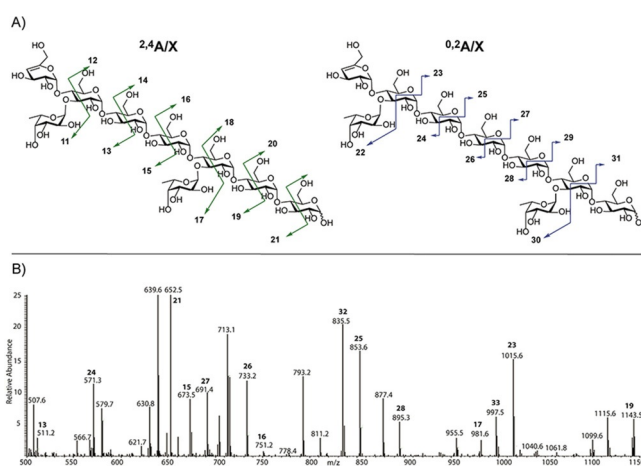Figure 3. A) Fragmentation pattern of two possible parent ions. B) Low-resolution MS/MS spectrum of nonasaccharide 10 (region from m/z 500 to 1150) measured on LCQ Fleet mass spectrometer (Thermo Fisher Scientific) in combination with an ion-trap.

The clear regioselectivity of fucosylation on β-CD encouraged the optimization of the reaction conditions to increase the yield of the twice fucosylated product 9. The reaction times and temperatures were varied, while keeping the equivalents of donor 5, acceptor 4, Tf$_2$O, Ph$_2$SO and TTBP unchanged (Table S1). First, conditions were changed towards a shorter reaction time and higher temperature: $-70\,^\circ$C for 3 hours (compared to $-80\,^\circ$C overnight). These conditions provided a mixture containing 33% of the di-fucosylated product. However, the formation of higher fucosylated compounds, that is, tetra- and pentafucosylation, had significantly increased. Next, the reaction was started at $-80\,^\circ$C followed by warming the mixture to different temperatures (-70, -60 and $-50\,^\circ$C), resulting in

Figure 2. A) Fragment of the structure of compound 10 with the identified cross-peaks H1$_{Fuc}$→C3$_{Glc}$ (A, green bond), H3$_{Glc}$→C4$_{Glc}$ (B, red bond), and C4$_{Glc}$→2×H1$_{Glc''}$ (C, blue bond). B) Fragment of the HMBC NMR of 10.

short reaction times (10–30 minutes). Quenching the reaction at −70 °C resulted in 35 % of the nonasaccharide, while a significant amount of unreacted acceptor was still present (19 %) together with monofucosylated Fuc_1-β-CD product (24 %). Increasing the temperature to −60 °C provided 57 % of the desired di-fucosylated product, while quenching the reaction at −50 °C led to a reduced contribution of difucosylated product (35 %). As a result, the highest yield of nonasaccharide 9 was observed when the reaction was neutralized at −60 °C, and these conditions were used to α-fucosylate the β-cyclodextrin acceptor on a large scale (1 g, 0.42 mmol, Table S1, entry 6).

These optimized conditions provided a mixture of fucosylated CDs in 95 % yield containing 61 % of nonasaccharide, which could be purified by preparative HPLC (purity > 90 %), or by regular flash column chromatography, providing nonasaccharide 9 in 67 % isolated yield and in 69 % purity. After global deprotection, 275 mg of compound 10 was obtained (75 %).

Structural information of the fucoside positioning in space is important to rationalize future carbohydrate–lectin binding, so in an effort to understand the spatial arrangement of di-fucosylated β-CD 10, molecular dynamics (MD) simulations were performed. Since a recent comprehensive study of native CDs in water showed that structural and dynamic properties may differ depending on the choice of force field,[49] two different force fields, that is, GROMOS 53A6[50] and Q4MD,[51] were used in combination with the GROMACS simulation package.[52] In contrast to what is commonly assumed, the cyclodextrin cone is quite flexible, especially in protic solvents.[53]

This is apparent from the tumbling of glucoside units, which bend outwards and distort the conical shape and the hydrogen-bonding network.[49] Indeed, our analysis reveals that specifically the fucosylated glucoside units show enhanced rotation and increased chair flexibility. These tilted states are not frequent, but are stable over several nanoseconds (Figure S4). The tilting of the cyclodextrin ring sugars is more pronounced upon fucosylation (Figure 4), which can be seen from the enhanced probability of finding the dihedral angles connecting Glc units G-A (blue dashed line) and C-D (orange dashed line) at values of 60–90 degrees away from the main peak. Both force fields show this effect, but the Q4MD force field shows it more strongly, which is a reflection of the larger conformational flexibility found using this force field. This flexibility is also apparent from the wider distributions of the dihedral angles (Figure 4 C), and is discussed in more detail in the Supporting Information. The conformations visited during the simulations were grouped into distinguishable conformational states (so-called clusters) by cluster analysis (for details, see SI). Considering the most abundant conformations (Figure S5), the fucosides are attached to a canonical CD ring and prefer to be slightly tilted inward or outward with respect to the CD cavity. The distance between the two centers of the fucosides is peaked at ≈ 7 Å, but the groups can occasionally be considerably closer at ≈ 5 Å, or more distant depending on the tumbling of sugar rings and rotations of the fucoses with respect to the β-CD barrel wall (Figure S6).

In an effort to confirm the structural representation from MD simulations with NMR analysis, NOE intensities were calcu-
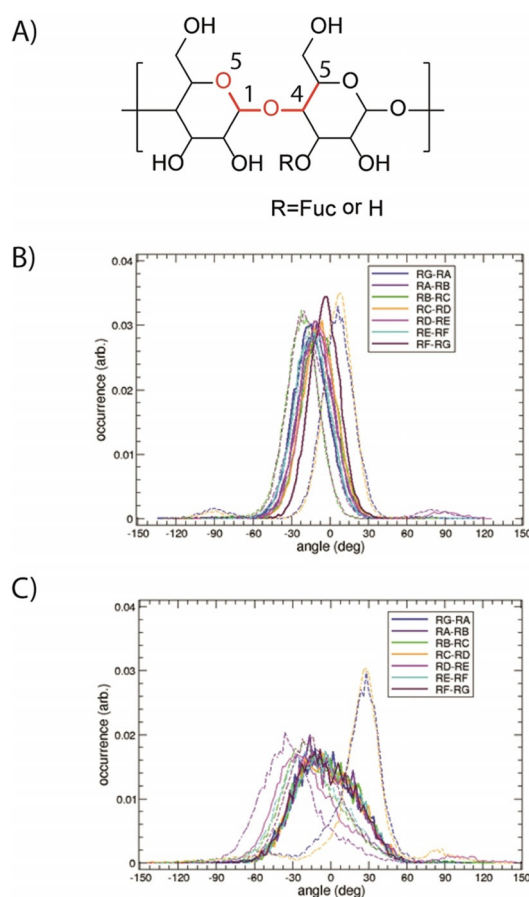

Figure 4. Comparison of the distribution of dihedral angles between two neighboring Glc residues within the β-CD rings between β-CD and nonasaccharide 10. (A) The O5-C1-C4-C5 dihedral angles connecting the ring Glc residues. Dihedral angle distributions for natural β-CD are depicted with solid lines and fucosylated β-CD 10 with dashed lines, using both the GROMOS force field (B) and the Q4MD force field (C).

lated (see the Supporting Information for a detailed description). The MD simulations suggested the presence of weak NOE signals that reflect the conformational flexibility of nonasaccharide 10, in particular regarding the different orientations of the fucosides. The H-atoms on the fucose C5 and C6 positions ($H5Fuc_{A,D}$ and $H6Fuc_{A,D}$) are relatively close to the H-atom on C3 of the upstream glucoside ($H3G_{G,C}$) in many of the most populated clusters (Figures S7 and S8). The closest distance between $H5Fuc_{A,D}$ and $H3G_{G,C}$ is ≈ 1.9 Å in the most populated cluster, and the closest distance between the $H6Fuc_{A,D}$ and $H3G_{G,C}$ is ≈ 2 Å. The H-atoms involved in these expected NOE signals are highlighted in the representative structure of the most populated conformational cluster in Figure 5 A. Excitingly, these NOE cross-peaks were indeed observed experimentally in the NOESY spectrum as shown in Figure 5 B, supporting the dynamical ensemble observed in the MD simulations. The MD analysis also suggests that the bending of the Fuc rings away from the CD barrel is associated with an enhanced chair flexibility of the substituted Glc rings (residues A and D). The relatively low ^3J couplings of 8.3 Hz measured for these Glc H3 hydrogens in compound 10, as compared to 9.5 Hz for the H-3 signals in natural β-CD, are consistent with the MD simulation,
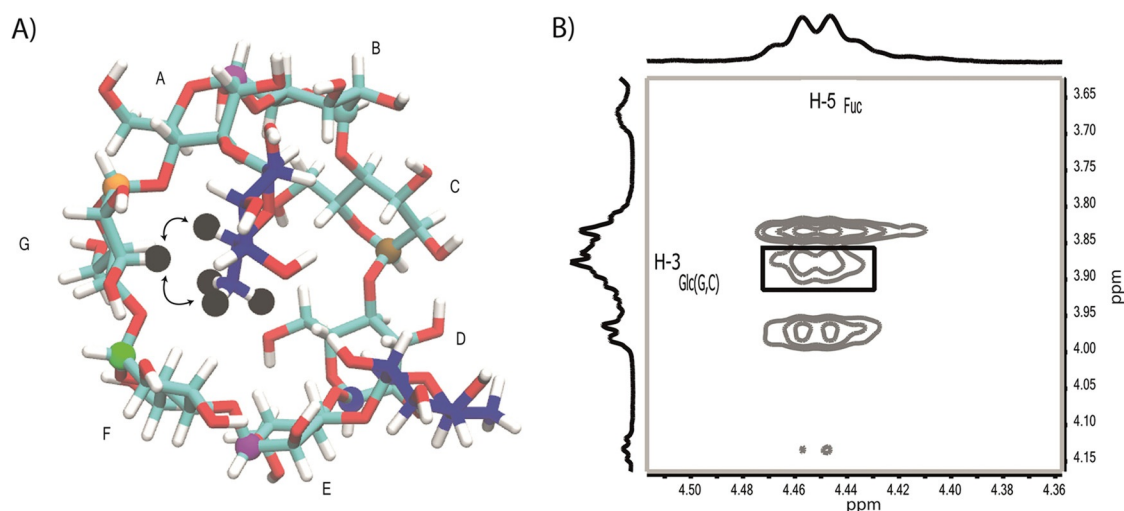
Figure 5. A) Characteristic conformation of **10** with H-5$_{Fuc}$, H-6$_{Fuc}$ and H-3$_{Glc}$ highlighted as black spheres for close contact between fucose on Glc(A) and Glc(G) (fucoside C-atoms are shown in dark blue). B) Observed NOE of H-5$_{Fuc}$(A,D)→H-3$_{Glc}$ (G,C.)

and support the contribution of the unfavored $^1C_4$ conformer (see SI).[54] The MD analysis shown here should be taken as qualitative: the dynamics of the conformational changes is slow on the MD time-scale, and it is not possible to extract converged thermodynamic (equilibrium) data from the extensive simulations of 4 microseconds.

Conclusions

This report presents the regioselective 3A,3D-fucosylation of the secondary rim of semi-protected β-CD, which proved to be robust, reproducible, and scalable. In natural glycans, such as HMOs, the fucosides are often separated by non-fucosylated carbohydrate residues, making this 3A,3D pattern a highly relevant mimic. While the regioselective 6A,6D substitution of CDs is well-developed for decoration of the primary rim,[29,55] only limited examples of di-substitutions on the secondary rim exist,[56] and none of these examples covers a direct glycosylation reaction. We propose that the regioselectivity observed here arises from the kinetic conditions employed in the glycosylation reaction, that is, low temperatures and short reaction times. This may drive the reaction to yield the least sterically hindered 3A,3D nonasaccharide as the kinetic product of the reaction. We hypothesize that the remarkable regioselectivity observed for the β-, but not for the α-CD, is likely to depend on the size of the CD ring. MD simulations to corroborate this hypothesis are currently underway.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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