A kinetic trapping approach for facile access to $3F_{ax}$Neu5Ac and a photo-crosslinkable sialyltransferase probe

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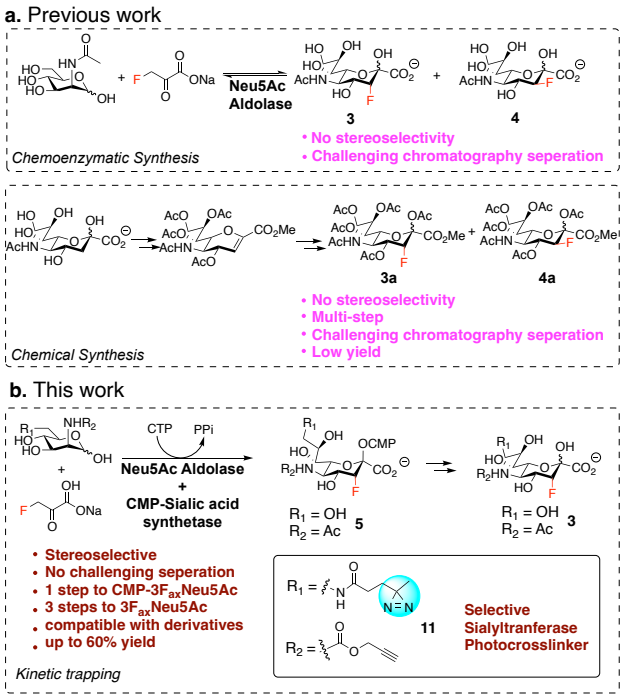
ABSTRACT: Sialic acid (Neu5Ac) is installed onto glycoconjugates by sialyltransferases (STs) using cytidine monophosphate-Neu5Ac (CMP-β-D-Neu5Ac) as their donor. The only class of cell-active ST inhibitors are those based on a $3F_{ax}$Neu5Ac scaffold, which is metabolically converted into CMP-$3F_{ax}$Neu5Ac within cells. It is essential for the fluorine to be axial, yet stereoselective installation of fluorine in this specific orientation is challenging. Sialic acid aldolase can convert 3-fluoropyruvate and 2-acetamido-2-deoxy-D-mannopyranose (ManNAc) to 3FNeu5Ac but stereocontrol of the fluorine in the product has not been possible. We hypothesized that the $3F_{ax}$ kinetic product of a sialic acid aldolase reaction could be trapped by coupling with CMP-sialic acid synthetase, to yield CMP-$3F_{ax}$Neu5Ac. Here, we report that highly active CMP-sialic acid synthetase and short reaction times produces exclusively CMP-$3F_{ax}$Neu5Ac. Removal of CMP from CMP-$3F_{ax}$Neu5Ac, under acidic conditions unexpectedly led to 3-fluoro-β-D-Neu5Ac 2-phosphate ($3F_{ax}$Neu5Ac-2P). Alkaline phosphatase enabled the successful conversion of $3F_{ax}$Neu5Ac-2P to $3F_{ax}$Neu5Ac, enabling the stereochemically-controlled access to $3F_{ax}$Neu5Ac, which is effective in lowering the sialoglycan ligands for Siglecs on cells. Moreover, our kinetic trapping approach could be used to access CMP-$3F_{ax}$Neu5Ac with modifications at the C5, C9, or both positions, which enabled the chemoenzymatic synthesis of a photo-crosslinkable version of CMP-$3F_{ax}$Neu5Ac that selectively photo-crosslinked to ST6Gal1 over two other sialyltransferases.

Sialic acid (Neu5Ac, **1**) is a nine-carbon α-keto acid monosaccharide that caps cellular glycoconjugates on mammalian cells.[1] Addition of Neu5Ac to the underlying glycan is catalyzed by a family of enzymes called sialyltransferases (STs),[2] which use cytidine monophosphate sialic acid (CMP-Neu5Ac, **2)** as their Leloir donor, to transfer **1** to a specific hydroxyl group on a glycan acceptor (**Figure S1a**).[3] The catalytic mechanism of mammalian STs involves a general-base catalyzed attack of the hydroxyl nucleophile at the anomeric center of **2**, displacing CMP as the leaving group, leading to inversion of stereochemistry at the anomeric center (**Figure S1b**).[1, 4, 5] Positive charge accumulation on the proposed oxocarbenium ion-like transition state,[6, 7] is destabilized by a nearby electron withdrawing group.[8, 9] For example, CMP-$3F_{ax}$Neu5Ac **5** is a ST inhibitor[10] and can be co-crystallized with STs.[11] **5** cannot cross the plasma membrane, but an appropriately protected version of $3F_{ax}$Neu5Ac **3** diffuses into cells where it is enzymatically converted to **5**, and decreases cell surface sialoside levels.[10] It is noteworthy that the C3-fluoro epimer of **3**, $3F_{eq}$Neu5Ac **4**, is not active in cells.[10] However, a stereoselective route to access the 3F axial epimer **3** has not yet been reported.

Several strategies have been advanced for the preparation of protected versions of **3**. Chemical synthesis involves fluorination of a glycal using a Selectfluor,[12] XeF_2-BF_3-OEt_2, or molecular fluorine.[13] These chemical methods involve multiple steps, are low yielding and require a challenging chromatography purification for separating the $3F_{ax}$ and $3F_{eq}$ epimers.[14] An alternative to chemical synthesis is a chemo-enzymatic approach using ManNAc and 3-fluoropyruvate catalyzed by sialic acid aldolase.[15–17] It was first reported that **3** is the major product of this reaction,[8] but a subsequent study revealed **3** was the kinetic product, and very quickly equilibrates to a mixture of $3F_{ax}$ and $3F_{eq}$ epimers.[17] Indeed, we found that a one-step reaction with ManNAc, 3-fluoropyruvate, and sialic acid aldolase yielded a mixture of **3** and **4** in a 5:4 ratio (**Scheme 1a**, **Figure S2**). As this aldolase-catalyzed reaction is reversible and thermodynamically controlled, direct access to **3** would still require a challenging chromatographic separation, providing negligible advantage over the chemical method.

Scheme 1. Kinetic trapping of CMP-3F$_{ax}$Neu5Ac (5)



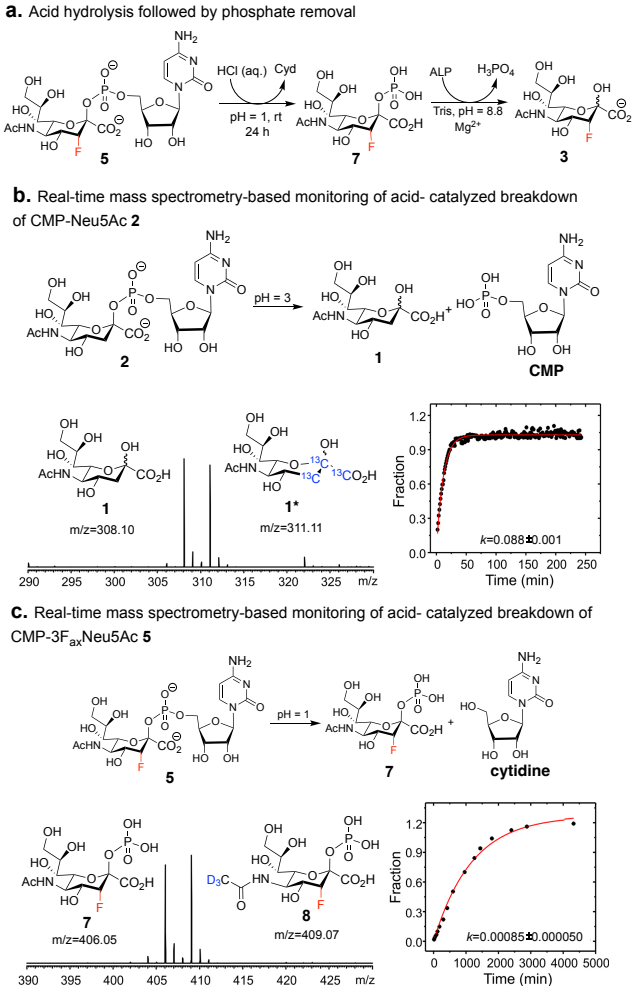
(**a**) Previous chemoenzymatic and chemical strategies to access 3F$_{ax}$Neu5Ac **3** and peracetylated 3F$_{ax}$Neu5Ac **3a**, respectively (**b**) A stereoselective one-pot two-enzyme kinetic trapping approach to access CMP-3F$_{ax}$Neu5Ac **5**.

We hypothesized that trapping the kinetic product **3** by enzymatic installation of CMP onto the anomeric hydroxyl group to generate **5** in a coupled enzymatic reaction could provide a route for the stereoselective installation of fluorine at the axial position (**Scheme 1b**). Initial attempts to couple the sialic acid aldolase reaction with *N. meningitidis* CMP-sialic acid synthetase (CSS) and cytidine triphosphate (CTP),[18] produced an approximately equal mixture of CMP-3F$_{ax}$Neu5Ac **5** and CMP-3F$_{eq}$Neu5Ac **6.** However, we observed only one product formation using the two enzymes in one pot at early time points (<1 h) and two products appeared thereafter (**Figure S3 and S4**). To monitor the products, **5** and **6** were synthesized chemically following a published route with minor modifications to compare their ^{1}H NMR spectra (**Scheme S1**).[10, 19] An aromatic proton in the pyrimidine of cytidine could be used to differentiate **5** and **6** by NMR. Further optimization with highly active CSS enabled the successful isolation of **5** with 60% yield after purification.

We postulated that the above strategy could enable access to **3** through removal of CMP. Native CMP-Neu5Ac **2** is rapidly hydrolyzed under acidic conditions,[20] yet conditions that completely converted **2** into **1** (pH 1, 5 min, RT) were unsuccessful in converting **5** into **3**. With longer reaction times (> 1 h), **5** began disappearing and two new spots appeared on TLC: one that was UV active and co-migrated with cytidine and another that was highly polar and stained with anisaldehyde (**Figure S5**). The spot on the baseline was isolated by gel filtration chromatography and found to have a mass corresponding to 3-fluoro-β-D-Neu5Ac 2-phosphate (3F$_{ax}$Neu5Ac-2P, **7**; **Figure 1a**), which was confirmed by ^{31}P, ^{19}F, ^{1}H, and ^{13}C NMR (**Figures S6-9**). ^{31}P coupled and decoupled ^{19}F NMR confirmed that the phosphate group was still in the β-configuration (**Figures S10**).

Hydrolysis of CMP-Neu5Ac **2** results in Neu5Ac **1** and CMP, while CMP-3F$_{ax}$Neu5Ac **5** appears to hydrolyze through an alternative pathway whereby water attacks the phosphate (**Scheme S2**). We speculate that the fluorine on **5** decreases reactivity at the anomeric, thus favoring this alternative pathway. To test this, we carried out reactions with **2** and **5** in acidified 10% ^{18}O water and find evidence for ^{18}O incorporation into both **1** and **7**, respectively (**Figures S11 and S12**). To quantify the degree to which fluorine slows hydrolysis of **5**, we performed mass spectrometry-based kinetic analysis of the hydrolysis of **2** and **5** into **1** and **7**, respectively, which was enabled by the synthesis of D$_3$-labeled 3F$_{ax}$Neu5Ac-2P **8** (**Scheme S3**). We found that the presence of fluorine substituent at the C3 position decreased the hydrolysis rate by 100-fold (**Figure 1b, c**). However, this difference is likely significantly larger because the reactions had to be carried out at different pH; hydrolysis of **2** was excessively fast at pH 1 and needed to be carried out at pH 3, while hydrolysis of **5** was exceedingly slow at pH 3, and therefore was carried out at pH 1.

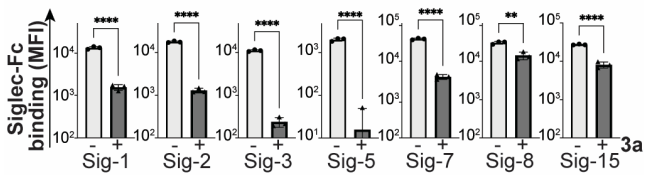
Figure 1. Conversion of CMP-3F$_{ax}$Neu5Ac 5 to 3F$_{ax}$Neu5Ac 3 through an unexpected intermediate 7



(**a**) Acid-catalyzed conversion of CMP-3F$_{ax}$Neu5Ac **5** to 3F$_{ax}$Neu5Ac-2P **7**, followed by enzymatic removal of the phosphate to afford 3F$_{ax}$Neu5Ac **3**. (**b, c**) Real-time mass spectrometry-based monitoring of acid-catalyzed breakdown of (**b**) CMP-Neu5Ac **2** to Neu5Ac **1** or (**c**) CMP-3F$_{ax}$Neu5Ac **5** to 3F$_{ax}$Neu5Ac-2P **7**. Quantification was enabled through spiking of the reaction with a heavy isotope-labelled version of the product **8**.

We assessed the inhibitory activity of **7** against ST6Gal1, ST3Gal1, and ST3Gal4 and found only a small degree of inhibition at a high concentration (**Figure S13**). Moreover, cells treated with **7** for three days showed no effects on the levels of α2-6 and α2-3 linked sialosides, unlike **3** (**Figure S14**). Attempts to remove the phosphate from **7** under acidic or basic conditions and at elevated temperature were unsuccessful, confirming the stabilizing effect that fluorine has on chemistry at the anomeric center. Ultimately, conditions were found that successfully converted **7** to **3** in high yield using alkaline phosphatase (ALP) (**Figure 1a**). Standard conditions were used to install a methyl ester and protect the hydroxyl groups as acetates (**Scheme S1**) to afford **3a** for studies in cells. Testing **3a** in U937 cells, we find that it is highly effective in reducing the level of sialoglycan ligands of Siglec-1, -2, -3, -5, -7, -8, and -15, including in cells overexpressing the carbohydrate sulfotransferase CHST1 (**Figure 2, Figure S15**).[21]

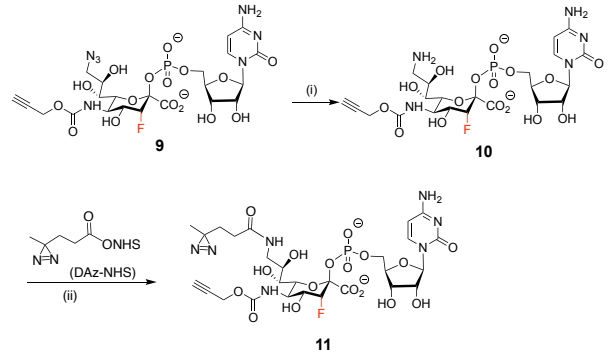
Figure 2. 3F$_{ax}$Neu5Ac decreases Siglec ligands in cells.



CHST1-overexpressing U937 cells were treated with peracetylated 3F$_{ax}$Neu5Ac **3a** or DMSO for three days and assessed for Siglec ligands by flow cytometry. ***P* <0.01; *****P*<0.0001.

We next examined the utility of our kinetic trapping approach to access derivatives of **5**. Accordingly, *N*-propagyloxycarbonyl-D-mannosamine **12**, 6-azido-6 deoxy-*N*-acetyl-D-mannosamine **13**, *N*-azidoacetyl-D-mannosamine **14** and 6-azido-6-deoxy-*N*-propargyloxycarbonyl-D-mannosamine **15** were prepared (**Scheme S3-S5)** and used in the two-step enzymatic reaction with 3-fluoropyruvate. All compounds were successfully converted to their corresponding CMP-3F$_{ax}$Neu5Ac analogues **5a**, **5b**, **5c,** and **9**, respectively (**Scheme S6**). Aiming to create a bifunctionally modified version of CMP-3F$_{ax}$Neu5Ac with a photo-crosslinkable diazirine and click handle, **9** was further elaborated by selectively reducing the 9-azido group by H_2S, followed by amide coupling to introduce the diazirine to produce CMP-9-*N*-DAz-3F$_{ax}$Neu5Al **11** (**Scheme 2**).

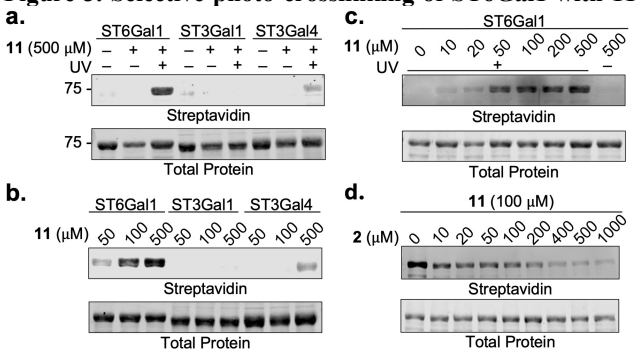
Scheme 2. Synthesis of CMP-9-*N*-DAz-3F$_{ax}$Neu5Al (11)



Reaction conditions: (i) Py. Et_3N, H_2O, H_2S, rt, 12 h, yield 90% (ii) DMF, sat. $NaHCO_3$, rt, 1 h, yield 78%.

Photo-crosslinking probe **11** was tested for UV-mediated crosslinking to three recombinants human STs: ST6Gal1, ST3Gal1, and ST3Gal4 (**Figure 3**). We initially tested **11** against all three STs at a high concentration (500 μM) and strong UV-dependent photo-crosslinking was observed for ST6Gal1, minimal UV-dependent crosslinking for ST3Gal4, and no crosslinking with ST3Gal1 (**Figure 3a**). Titrating **11** down revealed clear selectivity for ST6Gal1 (**Figure 3b**). Indeed, as little as 10 μM of **11** enabled photo-crosslinking to ST6Gal1 (**Figure 3c**). Specificity of **11** for the activity site of ST6Gal1 was demonstrated by competitive inhibition of photo-crosslinking in the presence of increasing concentrations of CMP-Neu5Ac **2** (**Figure 3d**).

Figure 3. Selective photo-crosslinking of ST6Gal1 with 11.

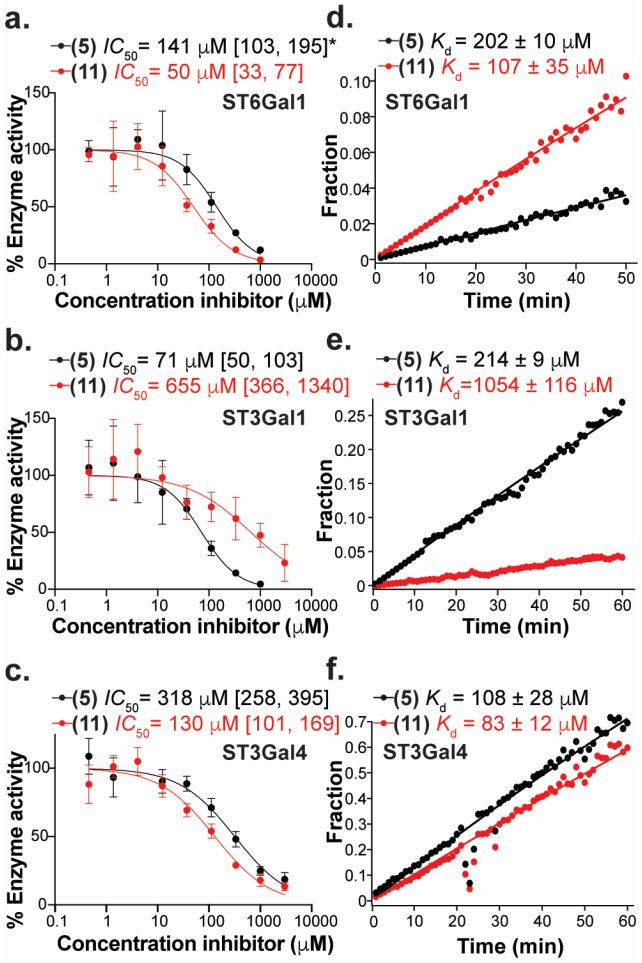


(**a**) Photo-crosslinking of compound **11** (500 μM) with ST6Gal1, ST3Gal1, and ST3Gal4 demonstrates crosslinking. (**b**) Weak photo-crosslinking of **11** with ST3Gal4 is observed at a high concentration (**c**) Dose-dependence for photo-crosslinking of **11** with ST6Gal1 (**d**) Excess CMP-Neu5Ac **2** can reduce photo-crosslinking of **11** (100 μM) with ST6Gal1.

Structures of ST6Gal1[22–24] suggest that there is room for increased extending off the C9 of sialic acid, although the lack of available structures of ST3Gal1 and ST3Gal4 with sialic acid density in the active site make it difficult to fully understand the basis for selectivity of **11** for ST6Gal1. It is noteworthy that a previous study reported that a sialic acid bearing a C9-modified sydnone reporter showed selective usage by ST6Gal1 over ST3Gal1 and ST3Gal4.[25] To understand the basis for the selectivity of **11** for ST6Gal1, we optimized a fluorescence polarization (FP) assay[26] by preparing versions of CMP-Neu5Ac with a fluorophore at C5 **19,** or C9 **24** (**Scheme S7, S8**). In line with the hypothesis that modifications off the C9 position are poorly accommodated by ST3Gal1, we find that **19** is a more active substrate (**Figure S16**). Therefore, we used this assay to assess the inhibitory potency of **11** and **5** against ST6Gal1, ST3Gal1, and ST3Gal4 (**Figure 4a-c**). The results demonstrate that **11,** compared to **5**, shows a modest increase in inhibition potency towards ST6Gal1, similar potency for ST3Gal4, and loss in potency towards ST3Gal1. These trends are roughly in line with selectivities observed in photo-crosslinking results. Lastly, affinity measurements were performed using a newly introduced technique: Concentration-Independent native mass spectrometry (COIN-nMS)[27] and found, like the FP assay, poor binding of **11**, compared to **5**, for ST3Gal1 (**Figure 4d-f, Figure S17**). Similar potencies of **11** for ST3Gal1 and ST3Gal4 do not fully explain the selective photo-crosslinking of **11** for ST6Ga1l, but we speculate that it may result from the diazirine of **11** making closer contacts with active site residues in ST6Gal1 such that the UV-activated carbene is intercepted with solvent at a higher rate in ST3Gal4. Regardless, our results highlight how our kinetic trapping scheme can be used to readily access ST probes

where modifications of the sialic acid scaffold afford selectivity of one ST over another.

Figure 4. Affinity and inhibition measurements of 5 and 11 towards three STs.



(**a-c**) Inhibition and (**d-f**) COIN-mediated affinity measurements of **5** (black) and **11** (red) towards (**a,d**) ST6Gal1, (**b,e**) ST3Gal1, and (**c,f**) ST3Gal4). *95% confidence intervals.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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