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# Synthesis of Sialidase-Resistant Oligosaccharide and Antibody Glycoform Containing $\alpha$2,6-Linked 3F$^{ax}$-Neu5Ac

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*Supporting Information*

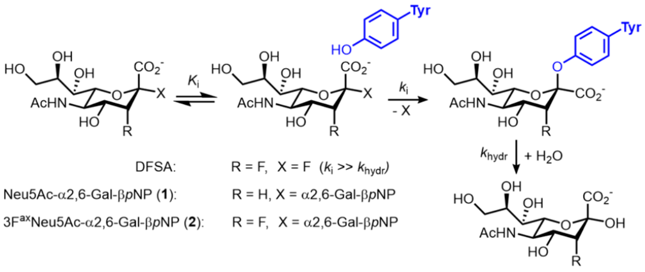
**ABSTRACT:** Fluorinated glycosides are known to resist the glycosidase-catalyzed glycosidic bond cleavage; however, the synthesis of such glycans, especially 3-fluoro-sialic acid (3F-Neu5Ac) containing sialosides, has been a major challenge. Though the enzymatic synthesis of $\alpha$-2,3-linked 3F-sialosides was reported, until recently there has not been any effective method available for the synthesis of 3F-sialosides in the $\alpha$-2,6-linkage. In order to understand the biological effect of such modification, we report here a chemical synthesis of 3F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal as a building block for the assembly of 3F$^{ax}$-Neu5Ac-containing sialosides and a representative homogeneous antibody glycoform. Our results showed that the sialosides are stable under sialidase catalysis and the rituximab glycoform with a sialylated complex-type biantennary glycan terminated with 3F$^{ax}$-Neu5Ac in the $\alpha$-2,6-linkage ($\alpha$2,6-F-SCT) has a similar binding avidity as its parent glycoform. These findings open up new opportunities for the development of therapeutic glycoproteins with improved pharmacokinetic parameters.

Sialic acid is a negatively charged monosaccharide often displayed at the outermost end of glycans on glycolipids and glycoproteins, which are involved in many physiological intra- and intercellular processes, including interactions with other biomolecules and receptors on cells, viruses, and bacteria.[1] In addition, sialylation plays an important role in regulating the function and fate of secreted glycoproteins and membrane-bound receptors. For example, sialylation of the epidermal growth factor receptor (EGFR) was shown to inhibit EGFR dimerization, thus interfering with EGF binding and phosphorylation, which is associated with tumorgenesis.[2] Also, sialylation modulates the half-life of glycoproteins in blood circulation as desialylation of *N*-glycans exposes the underlining galactose, which is recognized by the hepatic asialoglycoprotein receptors, leading to a rapid removal of the glycoprotein from circulation.[3] Hence, increasing the degree of sialylation could improve the half-life and undesirable effects of glycoprotein therapeutics.

In recent years, the role of glycosylation on protein structure and function has been intensively studied inspiring the development of new methods for glycan synthesis[4] and glycoengineering of proteins, particularly therapeutic monoclonal antibodies (mAbs).[5] For example, our group has demonstrated that the afucosylated sialylated complex-type biantennary *N*-glycan with terminal $\alpha$-2,6-linked sialic acids ($\alpha$2,6-SCT) is the optimal glycan structure for the enhancement of antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and anti-inflammatory activities.[6]

Sialic acid derivatives with fluorine at the C-3 position have been known to inhibit sialyltransferases and sialidases (or neuraminidases).[7] By introducing the fluorine atoms to the anomeric and C-3 positions, a 2,3-difluorosialic acid (DFSA) was developed (Scheme 1)[8] and used as a biochemical probe,[9] and the activity-based protein profiling probe to study sialidases.[10] With the help of DFSA, it was shown that the fluorine atom at the axial position at C-3 (3F$^{ax}$) has a greater effect on slowing down both the deactivation ($k_i$) and reactivation ($k_{hydr}$) of the enzyme than the 3F$^{eq}$ substituent.[9b] Inspired by this observation, we wanted to investigate the stability of sialosides with 3-fluorosialic acids for their potential use in glycoprotein therapeutics. Specifically, we intended to study if incorporation of a 3F$^{ax}$-Neu5Ac motif at the terminal end of *N*-glycan on a mAb could increase its stability toward sialidases and sustain its effector functions. However, glycosylation with fluorinated sugars as donors is a major challenge due to the strong electronic effect of the fluorine group that inactivates glycosylation reaction. Although there is a sialyltransferase capable of transferring the 3F$^{ax}$-sialic acid residue from the corresponding CMP-sialic acid to form the $\alpha$-2,3-linked sialoside, there is no corresponding $\alpha$-2,6-sialyltransferase available.[11]

**Scheme 1. Steps of Hydrolysis with Retaining *exo*-$\alpha$-Sialidase**



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Toward our goal, we developed a preparative method for the synthesis of $\alpha$-2,6-linked 3F$^{ax}$-Neu5Ac oligosaccharides, including the biantennary *N*-glycan. We also showed that the synthetic 3F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal linkage is stable in the presence of sialidases and the antibody bearing the biantennary glycan with 3F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal has the same binding avidity as a nonfluorinated counterpart.

Several methods were reported for the synthesis of 3F-Neu5Ac, including (a) fluorination of protected glycals with $XeF_2-BF_3 \times OEt_2$,[12] molecular fluorine,[13] and Selectfluor;[7c] (b) inversion of equatorial hydroxyl group at C3 in a sialic acid derivative;[7f] and (c) aldolase-catalyzed enzymatic transformation of ManNAc and 3-fluoro-pyruvate into 3F$^{eq}$-Neu5Ac and 3F$^{ax}$-Neu5Ac.[7a,11a,14] However, to the best of our knowledge, there is no method describing the synthesis of *N*-glycans terminated with 3F$^{ax}$-Neu5Ac so far. The only account disclosing the preparation of oligosaccharides with 3F$^{ax}$-Neu5Ac was limited to the enzymatic synthesis of 3F$^{ax}$-Neu5Ac-$\alpha$2,3-Lac-$\beta$OMe, and not the $\alpha$-2,6-linkage.[11a] We, therefore, focused our effort on the chemical synthesis of this linkage.[15]

After screening a variety of glycosylation conditions using 3F$^{ax}$-Neu5Ac-based donors without any success (Figure 1a and Table S1, Supporting Information), we investigated alternative strategies, which encompassed the $S_N2$ reaction of the OH$^{eq}$ to F$^{ax}$ in 3OH$^{eq}$-Neu5Ac-$\alpha$2,6-Gal-STol (Figure 1b). Starting with the sialylation conditions reported by Goto et al. (Table S2, Supporting Information),[16] we were able to optimize the sialylation reaction to give **6** in 35% (99% brsm) yield with excellent $\alpha$-selectivity ($\alpha$:$\beta$ = 13:1) (Scheme 2).

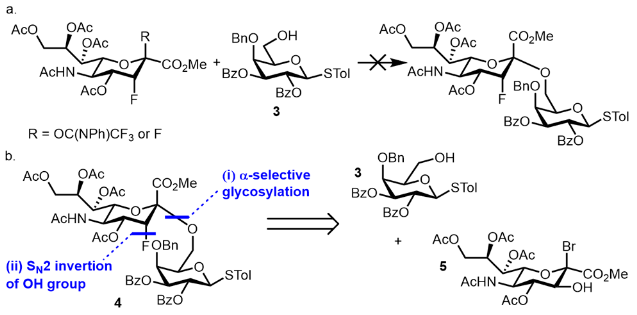
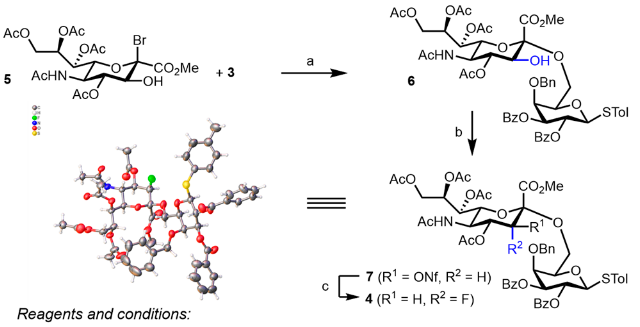


**Figure 1.** Synthetic routes toward 3F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal.

The inversion of OH$^{eq}$ to F$^{ax}$ turned out to be a challenging task. Substitution of OTf and OMs by fluorine using tris(dimethylamino)sulfonium difluorotrimethylsilicate (TASF) led to decomposition of the starting material. After screening a variety of fluorinating reagents,[17] we observed that treatment of **6** with perfluoro-1-butanesulfonyl fluoride (NfF) in the presence of 1,8-diazabicyclo[5,4,0]-undec-7-ene (DBU) in anhydrous toluene for 2 days at 90 °C gave **4** in 6% yield (Table S4, Supporting Information).[18] Further optimization of the reaction conditions, such as decreasing reaction temperature and increasing reaction times, improved the overall yield of **4**. However, the transformation of **7** to **4** seemed to be a rate-limiting step, probably due to steric hindrance. Thus, we were able to isolate **7** in the presence of NfF and DBU at room temperature within 1 day; however, conversion of **7** to **4** (49%, or 77% brsm yield) required long reaction times (15 days). The attempts to improve the conversion by elevating reaction temperature resulted in decomposition of **7**. Finally, we found that addition of TASF has helped improve the reaction efficiency reducing the reaction time to only 2 days. The stereochemistry of fully protected 3F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal-STol disaccharide (**4**) was confirmed by the X-ray diffraction analysis.

**Scheme 2. Synthesis of 3-F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal-STol**



*Reagents and conditions:*
(a) **5** / **3** / AgOTf / $Na_2HPO_4$ (1 / 1 / 1.5 / 4.2 equiv.), toluene, - 50 °C, 16 h (**6**: 35% (99% brsm), $\alpha$:$\beta$ = 13:1). (b) NfF / DBU / TASF (4 / 4 / 2 equiv. per day), toluene, 40 °C, 48 h (**4**: 60%, and **7**: 8%). (c) NfF / DBU (4 / 4 per day), toluene, 40 °C, 15 d. (**4**: 49% (77% brsm)).

The 3F$^{ax}$-Neu5Ac-disaccharide donor **4** was coupled with the acceptor (**8**) using NIS/TMSOTf (Scheme 3) to give **9**. Next, the *O*-allyl group at the anomeric position was removed by isomerization with $PdCl_2$ in AcOH/NaOAc, and the anomeric hydroxyl group (**10**) was further transformed into fluoride (**11**) and imidate (**12**). The glycosylation of the core disaccharide (**13**) at *O*-3 with 3F$^{ax}$-Neu5Ac-terminated fluoride donor (**11**) using $Cp_2HfCl_2$/AgOTf conditions gave hexasaccharide **14** in 85% yield. After removal of the benzylidene group, **15** was glycosylated at the *O*-6 position to give the desired decasaccharide (**16**) in 70% yield with excellent regio- and $\alpha$-stereoselectivity. We also tested the TfOH-promoted glycosylation with *N*-phenyl trifluoroacetimidate donor (**12**), which, however, gave the product in a poor yield. Next, the fully deprotected glycan (**17**) was obtained in 40% overall yield following a sequence of steps: (a) saponification with LiOH to remove the esters and the NHTroc group; (b) acetylation of free amines and alcohols; (c) removal of the OAc groups with sodium methoxide; and (d) hydrogenolysis of the *O*-benzyl groups with Pd/C in a mixture of MeOH/water/$HCO_2H$.[4b]

Having established a protocol for the stepwise synthesis, we streamlined the glycan assembly by developing a programmable [2 + 2 + 2] one-pot synthesis of hexasaccharide (**14**), which is a precursor of **17** (Scheme 4). The one-pot protocol was initiated by coupling of the 3F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal-STol donor (**4**) (RRV = 2053) with a less reactive acceptor (**18**) (RRV = 537) at −40 °C, followed by injection of the reducing-end acceptor **13** at −20 °C. After 1 h at −10 °C and a standard purification protocol, the hexasaccharide **14** was isolated in 26% yield.

In order to gather preliminary data about the stability of the 3F$^{ax}$-Neu5Ac-$\alpha$2,6-Gal motif in the presence of sialidases, we prepared Neu5Ac-$\alpha$2,6-Gal-*p*NP (**1**) and the 3F$^{ax}$-Neu5Ac analog (**2**) as substrates (Scheme 1) for the *in vitro* assay[19] with the commercially available sialidases from *C. perfingens* and *V. cholera*. Both enzymes showed the expected hydrolytic activity for the native substrate **1** but were inactive toward the 3F$^{ax}$-analog **2** (Figure S1, Supporting Information). We also observed that **2** did not significantly inhibit the hydrolysis of **1** as DANA did.

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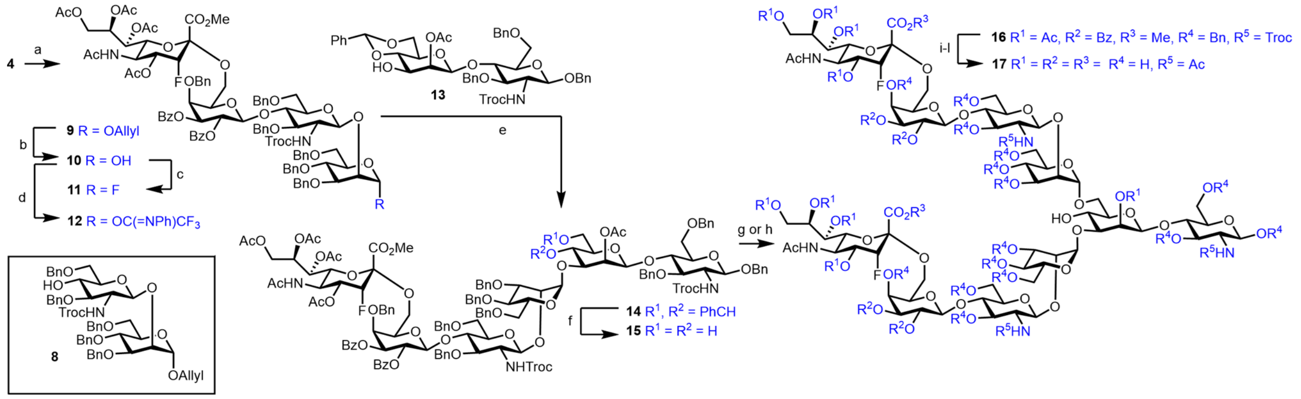
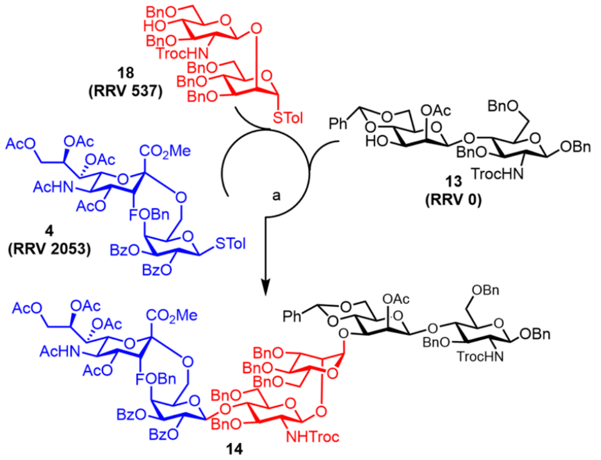
**Scheme 3. Synthesis of 3F$^{ax}$-Neu5Ac-Terminated Biantennary *N*-Glycan (17)**



*Reagents and conditions:*
(a) **8**, TfOH, NIS, 4Å MS, $CH_2Cl_2$, -40 °C, 2 h, 64%. (b) $PdCl_2$, $CH_3COONa$, $AcOH/H_2O$, 20 h, 82%. (c) DAST, $CH_2Cl_2$, -20 °C, 73%. (d) $ClC(=NPh)CF_3$, $Cs_2CO_3$, $CH_2Cl_2$, 0 °C to r.t., 3 h, 56%; (e) **11**, AgOTf, $Cp_2HfCl_2$, toluene, 4Å MS, 0 °C, 3 h, 85 %; (f) pTSA•$H_2O$, $CH_3CN$, 6 h, 75%; (g) **11**, AgOTf, $Cp_2HfCl_2$, toluene, 4Å MS, -15 °C, 3 h, 70 % (80% brsm); (h) 12, TfOH, $CH_2Cl_2$, 4Å MS, -60 to -20 °C, 3 h, 33 % (55% brsm); (i) LiOH, dioxane/$H_2O$ (4:1), 90 °C, 16 hrs; (j) $Ac_2O$, Py, 16 h; (k) NaOMe, MeOH, 16 h; (l) $Pd(OH)_2$, $MeOH/H_2O/HCOOH$ (6:3:1), $H_2$, 16 h, 40% (4 steps).

**Scheme 4. Programmable One-Pot Synthesis of Hexasaccharide (14)**



*Reagents and conditions:*
(a) TfOH, NIS, 4Å MS, $CH_2Cl_2$, -40 to -10 °C, 3 h, 26%

To prepare a homogeneous glycoform of mAb, compound **17** was converted into the oxazoline donor and ligated to the GlcNAc-primed IgG (without core fucose) in the presence of Endo S2 (D184Q) following a standard protocol (Scheme 5).[6]

**Scheme 5. Synthesis of α2,6-F-SCT Glycoform of Rituximab**

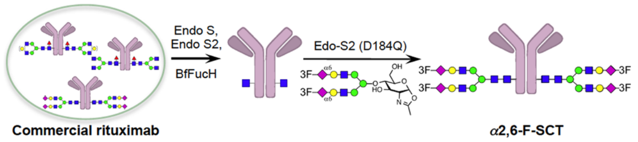


**Table 1. Binding Avidity of Glycoengineered Rituximab IgG1 to FcγRIIIa[a]**

| Sample IgG1 | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) | $R_{max}$ (RU) | Fold |
|---|---|---|---|---|---|
| Rituximab[b] | $2.31 \times 10^5$ | 0.07054 | $3.06 \times 10^{-7}$ | 32.33 | 1 |
| α2,6-F-SCT-Rit | $2.44 \times 10^5$ | 0.001996 | $8.18 \times 10^{-9}$ | 71.28 | 37.4 |
| α2,6-SCT-Rit | $2.68 \times 10^5$ | 0.002059 | $7.67 \times 10^{-9}$ | 60.64 | 39.9 |

[a] Analyzed antibodies were captured by the human Fab capture kit and detected with the single cycle kinetic method. [b] Commercial sample of rituximab contains several glycoforms (Figure S3, Supporting Information).

The binding avidity of the mAb 3F$^{ax}$-Neu5Ac-glycoform to FcγRIIIa was measured by the surface plasmon resonance analysis[6] together with the parent nonfluorinated glycoform (G2S2) and a commercial sample of rituximab (major glycoforms: G1F1, G0F1, G2F1). When compared to the commercial sample of rituximab, the homogeneous glycoforms of IgG1 bearing α2,6-SCT without core fucose demonstrated 39.9-fold (α2,6-SCT-Rit) and 37.4-fold (α2,6-F-SCT-Rit) improvement in binding avidity (Table 1). The fact that the avidity of the 3F$^{ax}$-Neu5Ac-modified glycoform was similar to that of the parent glycan provides a premise for the *in vivo* studies of 3F$^{ax}$-Neu5Ac-glycosylated mAb. These results will be reported in due course.

In conclusion, we have developed a chemical synthesis of 3F$^{ax}$-Neu5Ac-α2,6-Gal-STol building block for the synthesis of sialidase-resistant oligosaccharides and α2,6-F-SCT, which was used for modification of a representative mAb. When compared with the commercial rituximab sample, the homogeneous glycoform modified with α2,6-F-SCT showed a 37.4-fold improvement in binding to the FcγRIIIa. The parent nonfluorinated and 3F$^{ax}$-Neu5Ac-modified antibody glycoforms demonstrated similar binding avidity to the FcγRIIIa receptor. Overall, our results have revealed a new general strategy for the improvement of half-lives of therapeutic glycoproteins.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b01991.

Synthetic procedures, characterization of compounds and crystallographic data for **4**, as well as protocols for biological assays (PDF)
Crystallographic data for **4** (CIF)

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**Notes**

The authors declare no competing financial interest.

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