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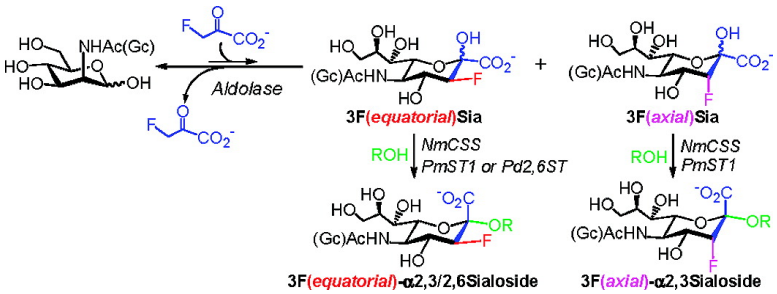

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Enzymatic Synthesis of Fluorinated Mechanistic Probes for Sialidases and Sialyltransferases

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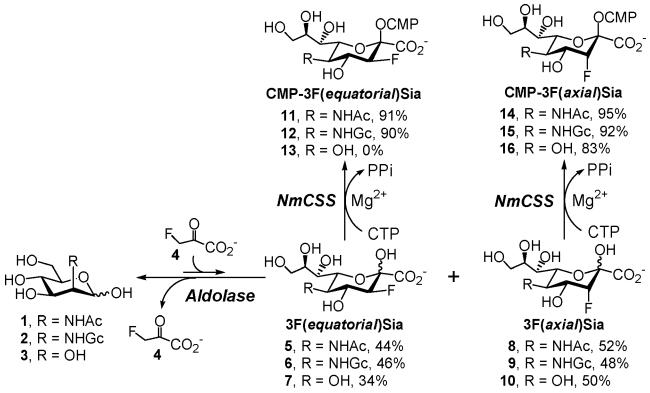
Sialidases and sialyltransferases play important roles in various biological and pathological processes.[1,2] Sialidases (EC 3.2.1.18) are sialic acid-releasing enzymes,[1] and sialyltransferases are enzymes that catalyze the transfer of sialic acid from CMP-sialic acid to an acceptor for the formation of sialic acid-containing structures.[3] Reactions catalyzed by these enzymes are believed to proceed via an oxocarbenium ion-like transition state.[4a,b] Introduction of a strongly electronegative fluorine atom onto the sialyl pyranose ring, especially at C-3 (the carbon adjacent to the anomeric carbon) on sialic acid in the sialidase and sialyltransferase substrates, will strongly destabilize the formation of cationic transition state.[4b,5] These fluorinated compounds serve as important mechanistic probes for kinetic and crystal structural studies of sialidases and sialyltransferases to understand their catalytic mechanisms.[5] For example, 3-fluorinated *N*-acetylneuraminic acid (3FNeu5Ac) has been shown to be a competitive inhibitor for bacterial and viral sialidases.[6] Fluorinated sialosides have been shown to inhibit the activities of hemagglutinins and neuraminidases of influenza virus.[7] CMP-3FNeu5Ac has been confirmed to act as a competitive inhibitor against CMP-Neu5Ac for a commercially available recombinant α-2,6ST (Calbiochem) and CstII, a recombinant bifunctional sialyltransferase from *Campylobacter jejuni*.[4a,8] It has also been used as a mechanistic probe in crystal structural studies of CstII.[4a]

In most fluorinated sialic acid-containing compounds that have been synthesized for sialidase and sialyltransferase mechanistic studies,[4−6,8,9] the fluorine atom at the C-3 of the sialic acid is in the axial configuration. We now report an efficient and general sialic acid aldolase-catalyzed enzymatic synthesis of several 3-deoxy-3-fluoro-sialic acid derivatives with fluorine in either axial or equatorial configuration at C-3. These fluorinated sialic acid derivatives can be activated by a recombinant CMP-sialic acid synthetase to produce a variety of fluorinated CMP-sialic acid derivatives as sialyltransferase mechanistic probes. Some of the fluorinated sialic acids can be further transferred to sialyltransferase acceptors to produce fluorinated sialosides which could be important probes for studying the interaction of sialylated carbohydrates and sialic acid-binding proteins.

Several methods have been reported for chemical synthesis of 3FNeu5Ac by fluorination of the glycal of peracetylated Neu5Ac methyl ester using XeF_2-BF_3-OEt_2,[10] molecular fluorine,[6] or Selectfluor.[9a] These methods, however, are inefficient, lengthy, and tedious. In an attempt to obtain 3FSia and CMP-3FSia (Sia = Neu5Ac; *N*-glycolylneuraminic acid, Neu5Gc; or keto-deoxynonulosonic acid, KDN) as probes for sialidases and sialyltransferases, the substrate specificities of a recombinant *Escherichia coli* sialic acid aldolase (Aldolase)[11] and a CMP-sialic acid synthetase from *Neisseria meningitidis* (NmCSS)[11] were re-evaluated. In contrast to previous reports that *E. coli* sialic acid aldolase is restricted to use only pyruvate as the donor substrate,[12] our results indicated that sodium 3-fluoro-pyruvate can be tolerated by the recombinant aldolase as the donor substrate. To our surprise, further analysis

Scheme 1. Enzymatic Synthesis of Fluorinated Sialic Acids and CMP-Sialic Acid Derivatives: NmCSS, *N. meningitidis* CMP-Sialic Acid Synthetase



indicated that the sialic acid aldolase-catalyzed aldol condensation of *N*-acetylmannosamine (ManNAc), **1** (20 mM), and 3-fluoro-pyruvate, **4** (5 equiv, 100 mM), resulted in a mixture of 3F(*equatorial*)Neu5Ac and 3F(*axial*)Neu5Ac diastereomers with ca. 4:5 ratio in Tris-HCl buffer (100 mM, pH 7.5) at 37 °C for overnight (Scheme 1). This is different from the reports that 3F(*axial*)Neu5Ac was the only or the major product from the aldolase reaction.[4] Pure 3F(*equatorial*)Neu5Ac, **5** (44%), and 3F(*axial*)Neu5Ac, **8** (52%), can be isolated from the reaction mixture by simple flash chromatography. Performing a similar aldolase reaction followed by silica gel purification produced 3F(*equatorial*)Neu5Gc, **6** (46%), and 3F(*axial*)Neu5Gc, **9** (48%), from *N*-glycolylmannosamine (ManNGc), **2**; and 3F(*equatorial*)KDN, **7** (34%), and 3F(*axial*)KDN, **10** (50%), from mannose (Man), **3**. Time-course studies of the aldolase-catalyzed reaction of ManNAc, **1**, and 3-fluoro-pyruvate, **4**, monitored by fluorine NMR (Figure 1) indicated that the 3F(*axial*)-Neu5Ac was formed first. Longer incubation resulted in a mixture of 3F(*equatorial*)Neu5Ac and 3F(*axial*)Neu5Ac of ca. 4:5 ratio.

It has been shown that 3F(*equatorial*)Neu5Ac[10] and 3F(*axial*)-Neu5Ac[8] are not substrates for *E. coli* CMP-sialic acid synthetase. The 3F(*axial*)Neu5Ac, however, has been used as a substrate by a recombinant *Neisseria* CMP-sialic acid synthetase[13] for high-yield production of CMP-3F(*axial*)Neu5Ac.[4b] CMP-sialic acid synthetase-catalyzed production of CMP-3F(*equatorial*)Neu5Ac has not been reported. Using the recombinant NmCSS cloned in our laboratory,[11] we show here that the enzyme can efficiently catalyze the formation of fluorinated CMP-sialic acid derivatives **11**−**12** and **14**−**16** from their corresponding fluorinated sialic acid derivatives **5**−**6** and **8**−**10**, respectively, with high yields (83−95%, Scheme 1). The only exception is 3F(*equatorial*)KDN **7** which could not be used as an acceptor for the synthesis of the corresponding CMP-3F-(*equatorial*)KDN, **13**.

Productive turnover of sugar nucleotide donors where a hydroxyl group is replaced by a fluorine atom has been reported for a recombinant human FucT III (GDP-2F-Fuc),[14a] a bovine β1,4GalT

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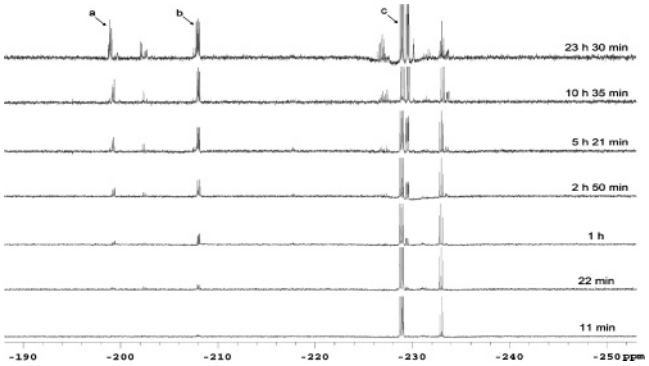
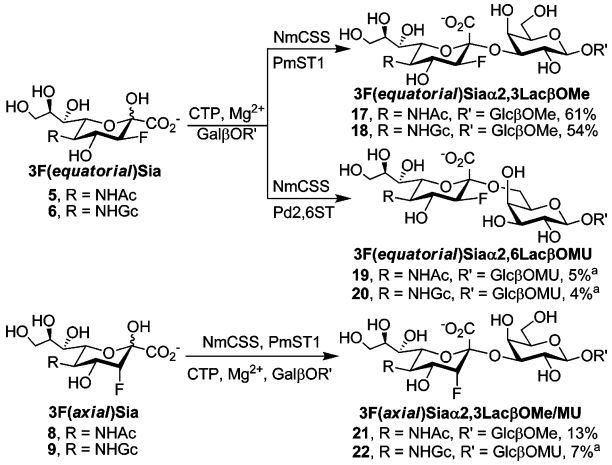


Figure 1. Time course of sialic acid aldolase-catalyzed synthesis of 3FNeu5Ac monitored by ^{19}F-NMR. (a) 3F(*equatorial*)Neu5Ac; (b) 3F(*axial*)Neu5Ac; (c) sodium 3-fluoro-pyruvate.

Scheme 2. One-Pot Two-Enzyme Synthesis of Fluorinated Sialosides: NmCSS, *N. meningitidis* CMP-Sialic Acid Synthetase; PmST1, *Pasteurella multocida* Multifunctional Sialyltransferase; Pd2,6ST, *Photobacterium damsela* α-2,6-Sialyltransferase



[a] Products were characterized by ^{19}F NMR and mass spectroscopy; yields were determined by HPLC analysis. MU = 4-methylumbelliferyl.

(UDP-6FGal),[14b] and a bacterial α-2,6-sialyltransferase (CMP-9FNeu5Ac).[14c] The enzymatic synthesis of sialosides containing a fluorine atom at the C-3 of sialic acid, however, has not been achieved. We show here that CMP-3FNeu5Ac and CMP-3FNeu5Gc could be used as donor substrates by bacterial sialyltransferases for the synthesis of fluorinated sialoside analogues. Fluorinated α-2,3-linked sialosides containing 3F(*equatorial*)Neu5Ac, **17**, and 3F(*equatorial*)Neu5Gc, **18**, were synthesized in good yields in a one-pot two-enzyme system containing NmCSS[11] and a multifunctional sialyltransferase from *Pasteurella multocida* (PmST1)[15a] (Scheme 2). A similar approach was used to obtain fluorinated α-2,6-linked sialosides using NmCSS[11] and an α-2,6-sialyltransferase from *Photobacterium damsela* (Pd2,6ST),[15b] although with low efficiency due to the low tolerance of the CMP-3F(*equatorial*)-Neu5Ac/Neu5Gc by Pd2,6ST. Despite the reported sialyltransferase inhibitory effect, CMP-3F(*axial*)Neu5Ac and CMP-3F(*axial*)-Neu5Gc could be used as donors by PmST1 to produce fluorinated α-2,3-linked sialosides containing 3F(*axial*)Neu5Ac, **21**, and 3F(*axial*)Neu5Gc, **22**, in low yields. This is the very first example of sialyltransferase-catalyzed synthesis of C3-fluorinated sialosides. These compounds would be valuable in investigating, at the molecular level, the involvement of the C-3 in the interaction of sialosides and sialic acid-binding proteins. They could be potential inhibitors and mechanistic probes (due to their resistance to sialidase hydrolysis)[7] for biochemical and crystal structural studies of sialidases. The fluorine label also enables the interaction to be readily monitored by ^{19}F NMR spectroscopy.[16]

In conclusion, we present here a general, convenient, and efficient enzymatic approach for producing fluorinated mechanistic probes for sialidases and sialyltransferases. Other than the reported sialyltransferase inhibitor CMP-3F(*axial*)Neu5Ac, CMP-3F(*equatorial*)Sia derivatives in which Sia is Neu5Ac or Neu5Gc have been synthesized along with CMP-3F(*axial*)Neu5Gc and CMP-3F(*axial*)-KDN as novel inhibitors and important mechanistic probes for sialyltransferases. Both CMP-3F(*axial*)Neu5Ac and CMP-3F(*equatorial*)Neu5Ac have been used in crystal structural studies of the multifunctional sialyltransferase PmST1.[17] Enzymatically synthesized 3-fluoro-sialosides are potential inhibitors and mechanistic probes for sialidases. They can also serve as important probes for sialic acid-binding proteins.

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Supporting Information Available: Experimental details for enzymatic synthesis and NMR and HRMS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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