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Chemoenzymatic Synthesis and Enzymatic Analysis of 8-Modified Cytidine Monophosphate-Sialic Acid and Sialyl Lactose Derivatives

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Abstract: The sialic acids found on eukaryotic glycans have remarkably diverse core structures, with a range of modifications at C5, C7, C8 and C9. These carbohydrates have been found to play key roles in cell−cell interactions within eukaryotes and often serve as the initial site of attachment for viruses and bacteria. Consequently simple changes to the structures of the sialic acids can result in profoundly different and often opposing biological effects. Of particular importance are modifications at the 8-position. These include *O*-acetylation, carried out by an acetyl transferase, and particularly polysialylation, catalyzed by a polysialyltransferase. As part of a structural and mechanistic study of sialyltransferases and polysialyltransferases, access was needed to sialic acid-containing oligosaccharides that are modified at the 8-position of the sialic acid to render this center non-nucleophilic. The free 8-modified sialic acid analogues were synthesized using a concise, divergent chemical synthetic approach, and each was converted to its cytidine monophosphate (CMP) sugar donor form using a bacterial CMP-sialic acid synthetase. The transfer of each of the modified donors to lactose by each of two sialyltransferases was investigated, and kinetic parameters were determined. These yielded insights into the roles of interactions occurring at that position during enzymatic sialyl transfer. A transferase from *Campylobacter jejuni* was identified as the most suitable for the enzymatic coupling and utilized to synthesize the 8′′-modifed sialyl lactose trisaccharides in multimilligram amounts.

Introduction

Sialic acid, or *N*-acetyl neuraminic acid, is a functionally dense nine-carbon sugar that is normally found as the terminal sugar of the glycan part of glycoproteins and glycolipids in the extracellular matrix of mammalian cells.[1] Currently over 50 variations of the core structure of sialic acid are known,[2,3] and it has been shown that even subtle changes can have drastic biological effects. The 8-position is particularly important because sialylation at this position[4] gives rise to the α-2,8-linked sialic acid polymers (PSA) that play key roles in brain development[5,6] and are found to be overexpressed in some cancerous tumors,[7] as well as being essential to the pathogenicity of some bacteria.[8,9] The 8-position has also been found to be acetylated,[10] methylated, and sulfated,[11] with each such modification possessing its own biological activity (see Figure 1). Convenient access to sialic acids bearing modifications at the 8-position would therefore be very useful in investigating the role played by sialic acid in each of these systems.

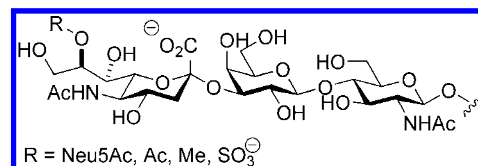

Figure 1. Biologically relevant modifications to the 8-position of sialic acid containing glycans.

Of particular interest to us is ready access to sialic acid containing oligosaccharide derivatives in which the 8-position of the terminal sialic acid has been rendered non-nucleophilic by deoxygenation, deoxyfluorination, or *O*-methylation or by complete removal through truncation of the glycerol side chain. Such minimally modified analogues might act as useful "incompetent" acceptor analogues for the study of enzymes that transfer sialic acids or other substituents to the 8-position of sialic acid. They should act as inhibitors of these enzymes, allowing measurement of binding affinities through kinetic analyses and permitting X-ray crystallographic structural analy-

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ses of ternary complexes in which both donor and acceptor substrate (analogues) are bound.[12] Sialic acids in extracellular glycoconjugates are commonly found conjugated to lactose or *N*-acetyl-lactosamine as shown in Figure 1.[13] 8″-Modified sialyl lactose derivatives were chosen as our synthetic targets because the enzymes under study work well on lactose and aryl lactosides are more readily prepared than the analogous *N*-acetyl glucosamine-containing derivatives. The synthesis of such 8″-modified sialyl lactose derivatives could, in principle, be carried out by chemical modification of sialyl lactose. However, the complexity of the protecting-group chemistry required and the presence of multiple functional groups would render such a route challenging. A more promising alternative is a chemoenzymatic approach in which a chemical modification of the 8-position on the sialic acid monosaccharide is carried out first and then enzymes are employed to transfer the modified sugar onto the lactose moiety.

Despite the relevance of the 8-hydroxyl group of sialic acids in biology, there have been few reports of chemical manipulation of this position. Of these studies most have concentrated on deoxygenation,[14,15] sulfonation[16] and simple alkylation,[17,18] and reports of halogenation at this position, with the exception of iodination,[19,20] are entirely absent. These syntheses utilize lengthy reaction sequences to obtain a protected sialic acid derivative with only OH8 free, which is further manipulated as required. Lengthy reaction sequences have been necessary because direct regioselective functionalization of OH8 is difficult and thus multiple deprotection and reprotection steps have been required to gain access to a sugar that has only OH8 free.[21] We present a relatively short reaction sequence, exploiting the different chemical reactivities of each of the five hydroxyl groups, to gain access to OH8 starting from a simple sialic acid glycoside. This route allows the ready manipulation and installation of multiple functionalities at this position.

Once the modified sialic acids are chemically synthesized, the second step of this chemoenzymatic approach requires access to two classes of enzymes that will, respectively, synthesize the CMP-sialic acid derivatives (CMP-sialic acid synthetase) and transfer the sialic acid (sialyltransferase). Because enzymes are often rather specific for their substrates, this approach carries the risk that the enzymes might well not function with such modified species. However, previous studies have shown that CMP-sialic acid synthetases can be remarkably tolerant to substrate modification,[22−24] and because the 8-position is relatively remote from the anomeric center, we were optimistic that this approach could work. Similarly our experience with a range of sialyltransferases gave us confidence that the CMP derivatives so formed could act as substrates for at least one of these enzymes. If successful, such an approach would not only provide us access to our desired sialyl lactose analogues but also provide further insights into the specificities of representatives of the two classes of enzymes employed in their synthesis.

This manuscript describes the chemical synthesis of 8-deoxy, 8-deoxy-8-fluoro and 8-*O*-methyl analogues of sialic acid, as well as a truncated heptose analogue of sialic acid in which C8 and C9 have been removed. It further describes the conversion of each of these analogues to their activated CMP donor sugar using a bacterial CMP-sialic acid synthetase and then use of these derivatives as substrates for two different bacterial sialyltransferases to prepare multimilligram quantities of each modified sialyl lactose derivative. Insights into the specificities of these two sialyltransferases, Cst-I from *Campylobacter jejuni*[25] and Pm0188h from *Pasteurella multocida*,[26] were provided by determination of kinetic parameters for transfer by each enzyme.

Materials and Methods

All enzymes were obtained from the Sigma-Aldrich company, with the exception of CMP-sialic acid synthetase,[22] Cst-I[27] and Pm0118h,[28] which were expressed and purified as previously described.

The general method for synthesis of 8-modifed CMP-donor sugars is as follows. Sialic acid analogue (1 equiv) and CTP disodium salt (1.05 equiv) were dissolved in Tris buffer (100 mM, pH 8.5) containing magnesium chloride (20 mM) and DTT (0.1 mM) to give a final sialic acid concentration of 15 mM. CMP-sialic acid synthetase (1 U/μmol) and inorganic pyrophosphatase (1 U/mmol) were added, and the mixture was tumbled at ambient temperature. The pH of the solution was checked regularly, and aqueous sodium hydroxide solution (1 M) was added as appropriate to keep the pH constant. On completion of reaction, as observed by TLC analysis (ethyl acetate/methanol/water/concd ammonia solution in a 4:3:2:1 ratio mobile phase), the mixture was cooled to −80 °C. Once it had thawed, it was filtered (0.44 μm) and incubated with alkaline phosphatase (20 U/mmol) for 10 min. The mixture was filtered (0.44 μm) again and loaded directly onto an ion-exchange column, pre-equilibrated with ammonium formate (50 mM). After an initial wash, using the same buffer, a stepped gradient (50 mM to 1 M) was performed, and products containing fractions were identified by TLC, pooled, and lyophilized. This crude product was dissolved in the minimum volume of buffer (20 mM ammonium formate buffer, pH 8.1) and loaded onto a size exclusion column. The column was run at 10 mL/h, and fractions were collected every 15 min. Product-containing fractions were identified by TLC analysis, pooled, and lyophilized.

All Michaelis−Menten kinetic parameters were determined using a CMP release assay.[29] Cst-I kinetics were performed at 37 °C in HEPES pH 7.5 buffer (20 mM) containing sodium chloride (50 mM), manganese chloride (10 mM), and magnesium chloride (10 mM) at substrate concentrations from 0.1 to 2 mM. Pm0118h

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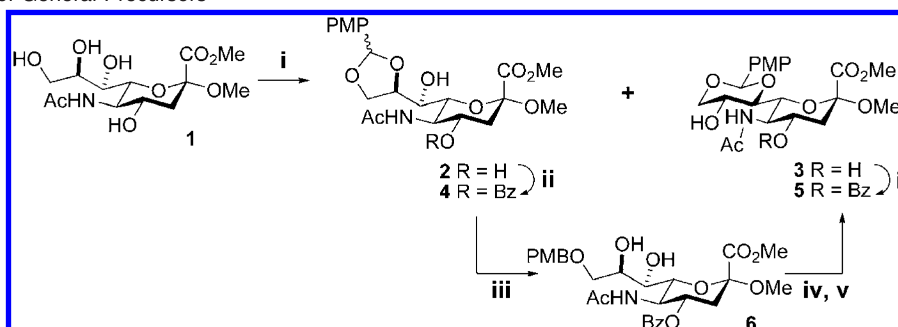
Scheme 1. Synthesis of General Precursors[a]



[a] Reagents and conditions: (i) anisaldehyde dimethyl acetal, TsOH (cat.) in MeCN, 54% of **2**, 37% of **3**. (ii) BzCl in pyr/CH_2Cl_2, −40 to 0 °C, 91% for **4**, 44% for **5**. (iii) $NaBH_3CN$ and HCl·Et_2O in THF at 0 °C, 72% of **6**. (iv) Et_3SiCl and NEt_3 in CH_2Cl_2 at 0 °C, then (v) DDQ in CH_2Cl_2 with Na_2SO_4 then TBAF, 82% over 2 steps.

kinetics and data were performed at 37 °C in HEPES pH 8.5 buffer (20 mM) containing sodium chloride (50 mM) and magnesium chloride (20 mM) at substrate concentrations from 0.02 to 1 mM. Data were analyzed using GraFit from Erithacus software.

The general method for synthesis of 8″-modified sialyl lactoses derivatives is as follows. β-Phenylsulfanyl lactoside[30] (1.1 equiv) was incubated with 8′-modified CMP-sialic acid (1.0 equiv) donor in HEPES (50 mM, pH 7.5) buffered solution containing manganese chloride (10 mM) in the presence of Cst-I (65 μM) and alkaline phosphatase (300 U/mL) at room temperature for 2 h. The mixture was filtered (0.44 μm) and loaded onto a pre-equilibrated Waters SepPak C18 (2 g) column and washed with water (10 column volumes), and products were eluted with 5% acetonitrile in water. Product-containing fractions were identified by TLC (ethyl acetate/methanol/water, in a 7:2:1 ratio mobile phase), pooled and lyophilized. Further purification was performed using reverse-phase HPLC as described in the Supporting Information.

Results and Discussion

Selective chemical modification at the 8-position of sialic acid requires that suitably partially protected derivatives of sialic acid be prepared in which the 8-hydroxyl is uniquely exposed, or at least preferentially reactive. To achieve this it is necessary to first protect the anomeric center, and in our case the simple methyl glycoside derivative **1** was chosen.[31] Since the relative reactivities of the remaining hydroxyls in **1** generally fall in the order OH9 > OH4 > OH8 > OH7,[21] it is essential only to protect OH9 and OH4 in order to carry out selective reactions at the 8-position, but in some cases having only OH8 exposed would be advantageous.

A convenient strategy is to protect both OH9 and OH7 simultaneously using an acetal, and such a method was described by Magnusson[32] on an analogous system that involved the use of a benzylidene acetal to protect part of the glycerol side chain, followed by benzoylation at C4. Thus, treatment of **1** with anisaldehyde dimethyl acetal under acidic conditions gave a (3:2) mixture of the 9,8- and 9,7-acetals (**2** and **3**, respectively), with the five-membered ring product predominating (Scheme 1). This five-membered acetal **2** was isolated as a (4:3) mixture of diastereoisomers, epimeric at the benzylidene center, whereas the six-membered acetal **3** was isolated as a single isomer in which all the substituents occupied equatorial positions. The large ($J = 10$ Hz) coupling constants between H8 and each of the H7 and H9 protons indicates that H8 is in an axial position, and the conformation of **3** shown in Figure 1 is the most likely. Benzoylation of the 4-hydroxyl of **2** proceeded smoothly to give compound **4**, but benzoylation of **3** was less selective, giving a mixture of 4- and 8-*O*-benzoyl compounds, favoring the desired compound **5**. This derivative (**5**) now contains OH8 as the only free hydroxyl and was used for some of the modification steps described below. Compound **5** could also be obtained from the five-membered acetal **4** via the common intermediate, 9-*O*-PMB derivative **6**, which was formed by reductive ring opening of the benzylidene acetal. Protection of the 8-hydroxyl group of diol **6**, using a triethylsilyl group, followed by oxidative ring closure under anhydrous conditions,[33] gave the six-membered acetal **5** after TBAF treatment to remove the silyl protecting-group. The 8-*O*-TES group is required to force the formation of the six-membered acetal, since oxidative ring closure in its absence results in preferential reformation of the five-membered acetal **4**. Importantly diol **6** is also a very useful intermediate for modification of the 8-position, since the reactivity of OH7 is so low.

Replacement of the 8-hydroxyl by fluorine to give a product of the same stereochemistry requires a double inversion of stereochemistry to be performed at C8. This was achieved relatively simply by taking advantage of the greater reactivity of the 8-hydroxyl over the 7-hydroxyl in **6** in order to form the mono-*O*-triflate at C8 by reaction with trifluoromethane sulfonic anhydride (2 equiv in pyridine at −20 °C). Warming of this intermediate to ambient temperature resulted in formation of the L-*glycero*-D-*galacto* configured 7,8-epoxide **7**, by intramolecular nucleophilic displacement of the 8-*O*-triflate by the 7-hydroxyl.[34] This one-pot reaction proceeds smoothly to yield a 25:1 ratio of the desired compound **7** and the D-*glycero*-L-*altro* configured 7,8-epoxide, which arises from displacement of the 7-*O*-triflate by the 8-hydroxyl. All attempts to open epoxide **7** with fluoride to install fluorine at the 8-position failed. However, removal of the 9-*O*-PMB group, using ceric ammonium nitrate, to give alcohol **8** greatly increased the reactivity of the epoxide toward nucleophilic addition. The successful ring opening was achieved using hydrogen fluoride-pyridine as the fluoride source in the presence of a catalytic amount of boron trifluoride to give the desired 8-fluoro derivative **9**, along with equal quantities of a diol byproduct **10**, with undetermined stereochemistry at C8 and C7. Monitoring of this transformation

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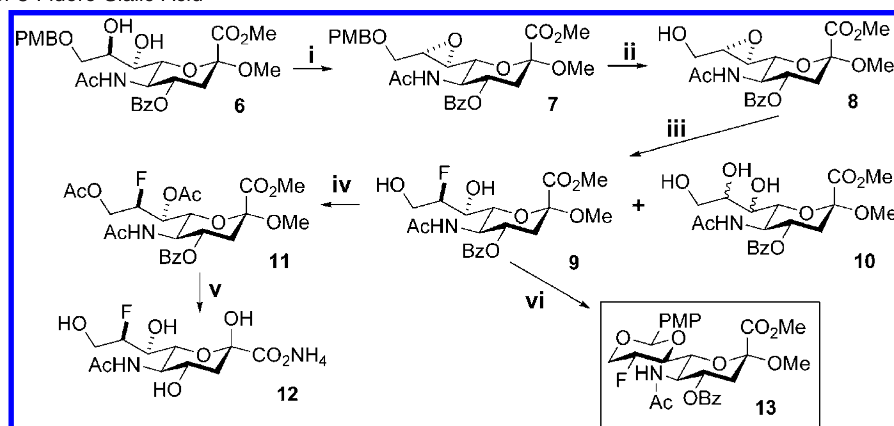
Scheme 2. Synthesis of 8-Fluoro Sialic Acid[a]



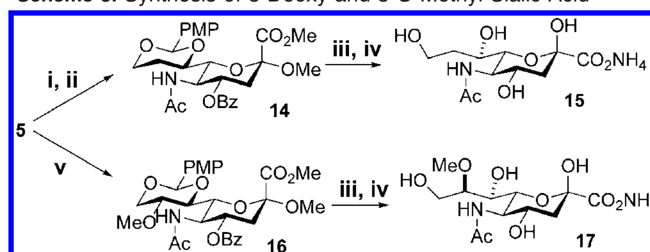
[a] Reagents and conditions: (i) Tf_2O in pyridine from −20 °C to rt, 82%. (ii) CAN in $MeCN/H_2O$, 96%. (iii) HF•pyr in CH_2Cl_2 at 0 °C, then immediately $BF_3 \cdot Et_2O$, 44% **9** and 42% **10**. (iv) Ac_2O in pyr, 80%. (v) $Mg(OMe)_2$ in MeOH, then LiOH (1 M) in THF and water, followed by HCl (aq) (25 mM) at 60 °C, 66% over 3 steps. (vi) Anisaldehyde dimethyl acetal with TsOH (cat.) in MeCN, 40%.

by TLC proved to be difficult because of coelution of starting material and product in all solvent systems attempted. Similar problems were encountered in purification, so after an initial silica gel chromatography step the crude product **9** was converted to the 9,7-diacetate **11**, which could be purified to homogeneity. Removal of the acetyl and benzoyl groups presented some problems because basic conditions tended to lead to degradation. These difficulties were overcome by using magnesium methoxide in methanol, a much milder method of acetate removal.[35] Subsequent saponification of the methyl ester was also carried out at low temperature to minimize any degradation, and the 8-fluoro sialic acid product (**12**) was purified by ion-exchange chromatography (Scheme 2).

The stereochemistry at C8 of compound **9** was confirmed by conversion to the 9,7-PMB acetal **13**, allowing a direct comparison with the spectra of the 8-hydroxy parent compound **5**. Again a large ($J = 10$ Hz) coupling constant is observed for H8 to H7 and H9a, indicating that H8 is in the desired axial position. The ^{19}F NMR spectrum of the 8-fluoro compound **13** shows a single resonance as a double triplet with one large ($J = 49$ Hz) coupling to H8 and two small ($J = 5.5$ Hz) couplings to each of H9-axial and H7, indicating that the fluorine is in an equatorial position. The stereochemistry at C7 and C8 of diol byproduct **10** was not determined, though comparison with spectra of a later compound, **18**, confirmed that **10** did not have the natural C7/C8 stereochemistry. This byproduct could be either the L-*glycero*-L-*altro* derivative, from ring opening by water at the C7 position, or the L-*glycero*-D-*galacto* derivative from a Payne-rearrangement,[36] followed by ring opening at the 8-position by water.

9,7-*O*-PMB acetal **5** provided the ideal departure point to deoxygenate C8. This was achieved using a Barton−McCombie protocol to give the 8-deoxy acetal **14** in a reasonable yield. Most of the loss in yield was due to hydrolysis of the thiocarbonate intermediate during workup, resulting in isolation of the 8-*O*-phenyl-carbonate (not shown). Treatment of **14** with sodium methoxide, followed by saponification, removed the 4-*O*-benzoyl group and the C1-ester. Subsequent acidic hydrolysis to remove both the benzylidene acetal and methyl glycoside gave the desired 8-deoxy product (**15**), which was isolated by ion-exchange chromatography and possessed spectroscopic data identical to those previously published,[37] but in fewer steps and higher overall yield (Scheme 3).

Scheme 3. Synthesis of 8-Deoxy and 8-*O*-Methyl Sialic Acid[a]



[a] Reagents and conditions: (i) PhOC(S)Cl with DMAP (cat.) in pyr at 0 °C. (ii) Bu_3SnH with AIBN (cat.) in toluene at reflux, 44% over 2 steps. (iii) NaOMe in MeOH, then NaOH (0.1 M). (iv) HCl (aq.) (25 mM) at 60 °C, 62% for **15** and 60% for **17**, over 2 steps. (v) MeI with NaH in DMF at 0 °C, 72%.

Synthesis of 8-*O*-methyl sialic acid (**17**) was also achieved from the common intermediate **5** by simple methylation of the 8-hydroxyl to give ether **16**, plus a small amount of the overmethylated *N*-methyl-acetamide, which was isolated as a minor byproduct. Deprotection of **16** was achieved in a manner similar to that used for the 8-deoxy compound to give **17** after ion-exchange chromatography, with identical characteristics to that previously published, and again in a higher yield and fewer steps.[17,38]

Oxidative cleavage of the vicinal-diols in **18** using sodium periodate initially gave an aldehyde at C7, which was reduced in situ to give alcohol **19** (Scheme 4). Removal of the esters under basic conditions proceeded smoothly; however, under the acidic conditions required to hydrolyze the methyl glycoside, a deacetylated byproduct **21** was surprisingly also obtained. Because *N*-deacylation of this kind is not seen in other deprotections of this type, it seems probable that this byproduct arises from acetate migration from the C5-nitrogen to the primary alcohol at C7. The subsequent deacetylation of the 7-*O*-acetate can then occur under the acidic conditions of the reaction. The desired C7-truncated compound **20** was obtained as a minor

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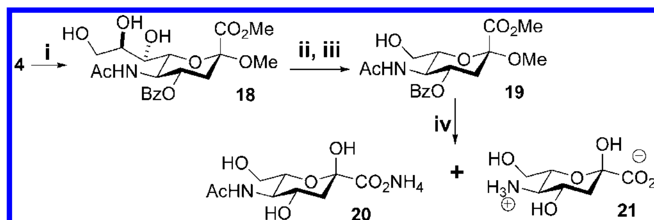
Scheme 4. Synthesis of a C7-Truncated Heptose Analogue of Sialic Acid



[a] Reagents and conditions: (i) TFA/H_2O (3:1 ratio), 96%. (ii) $NaIO_4$ in MeOH, then (iii) $NaBH_4$ in MeOH at 0 °C, 59% over 2 steps. (iv) NaOMe in MeOH at 0 °C, then NaOH (1M) at rt, followed by HCl (aq.) (25 mM) at 60 °C, 11% for **20** and 49% for **21**.

product but could be readily generated from **21**. Spectroscopic data for **20** were consistent with those previously reported.[39]

Although the CMP-derivative of each sialic acid analogue could be accessed by chemical synthesis,[40] enzymatic syntheses are far more attractive because they are more direct and less laborious.[23,24,41] The CMP-sialic acid synthetase from *Neisseria* has proved to be useful in this regard, and the equilibrium can be driven to completion by inclusion of inorganic pyrophosphatase to degrade the pyrophosphate generated during the reaction.[22] Indeed it is possible to combine the sialic acid aldolase, CMP-sialic acid synthetase and sialyltransferase enzymes in a single reaction mixture to generate sialic acid-containing oligosaccharides from the corresponding *N*-acetyl mannosamine sugars, although reports of this type have mainly centered on sialylglycosides that are modified at C5 or C9.[28,42−47] Our approach was to synthesize and isolate each of the 8-modified CMP-sialic acids, which could be subsequently evaluated kinetically as substrates for the available sialyltransferases.

This approach required the isolation and purification of the nucleotide phosphosugar. The enzymatic synthesis of the CMP-derivatives was achieved by incubating each of the 8-modified sialic acid analogues (up to a 50 mg scale) with CTP (1.05 equiv) in the presence of the CMP-sialic acid synthetase (1 U/μmol substrate) and inorganic pyrophosphatase (1 U/mmol substrate) in a Tris buffer (100 mM, pH 8.5) containing magnesium chloride (20 mM) and DTT (0.1 mM) (Scheme 5). Reactions were monitored by TLC and once complete were terminated by cooling the reaction mixture to −80 °C, after which they were thawed, filtered to remove protein precipitates, and then treated with alkaline phosphatase to degrade any CMP to neutral cytosine. Initial isolation of each of the crude 8′-modified CMP-sialic acid conjugate products was achieved by ion-exchange chromatography on Dowex 1x2-200 resin. Products were further purified and desalted by size-exclusion (Bio-Gel P2) chromatography to yield the desired, labile products in excellent yields as shown in Table 1.

To provide insight into the importance of the 8-hydroxyl in the enzyme-catalyzed sialyl transfer, as well as to identify the optimal catalyst for the synthesis of sialyl lactose derivatives, two different sialyltransferases from different GT families and of different three-dimensional fold were studied. In each case an initial assessment was performed by monitoring the enzymatic transfer reaction by TLC, using a fluorescent BODIPY-lactose acceptor. This was followed, in each case, by measurement of Michaelis−Menten kinetic parameters for the transfer reaction, as well as for the enzyme-catalyzed hydrolysis in the absence of the lactose acceptor, using a previously established CMP-release assay.[29]

Cst-I is a member of CAZy[48] family GT-42 and catalyzes the formation of α-2,3 linkages to galactose residues using CMP-sialic acid as the donor substrate. The three-dimensional structures of this enzyme, both free and as its binary and ternary complexes, have been published and show that the enzyme adopts a GT-A fold.[27] The TLC experiments (Table 2) show that Cst-I transfers each of the modified sialic acid donors to BODIPY-lactose, albeit with a reduced efficiency compared to the natural donor substrate. The transfer of the natural donor (lane **b**) was complete in less than 2 min, whereas transfer of each of the modified sugars (lanes **c** to **f**) required 60 min for completion under the same conditions.

Kinetic parameters determined using a CMP-release assay[29] reveal that the modified donors bind to the enzyme with similar affinities to the parent substrate, with the exception of the 8-*O*-methyl derivative **23**, which binds some 4-fold more weakly. This is a surprisingly small effect given the potential for the bulk of the methyl group to cause detrimental interactions within the binding pocket of the enzyme and suggests that ground state interactions with the substrate at this position are minimal. A more significant effect is seen on k_{cat}, with an approximate 10-fold drop, implying that more important interactions at this position are formed at the transition state. It is also interesting to note that while the parent substrate undergoes enzyme-catalyzed hydrolysis at around 10% of the rate of transfer in the absence of lactose acceptor, no significant transfer to water was observed for any of the modified sugar donors. This could imply that interactions formed at this position are particularly important to the hydrolysis reaction but that binding of acceptor can partially overcome any deficiency caused by their absence. The crystal structure of Cst-I, solved in the presence of CMP-3-fluoro sialic acid (see Figure 2)[27] shows that the donor sugar binding site is not solvent-accessible and that there is only one significant contact (Gln47) made between the 8- and 9-hydroxyls of the donor sugar and the enzyme. This may explain the tolerance of the enzyme toward the modifications at the 8-position. Even more surprising is the relatively high k_{cat}/K_m value obtained for the truncated compound **25**, whose kinetic parameters are essentially identical to those of the 8-deoxy analogue **22** despite the complete absence of the C8 and C9 hydroxymethyl groups. Presumably the ground state interactions of OH8 and OH9 with Cst-I are weak enough that their absence is not deleterious, but interactions at the transition state are satisfied by the presence of only one hydroxyl of the glycerol side chain. Indeed the crystal structure shows that OH7 interacts

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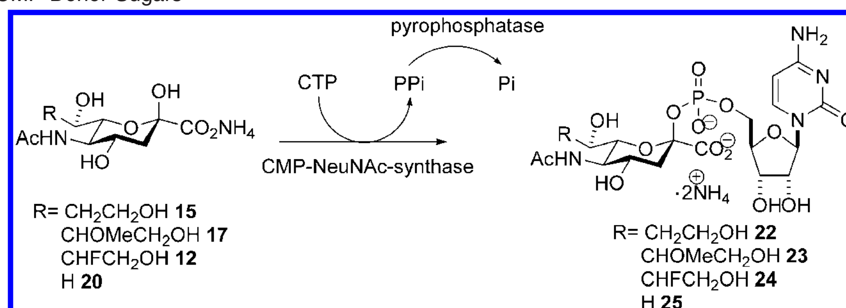
Scheme 5. Synthesis of CMP Donor Sugars[a]



[a] Reagents and conditions: (i) TFA/H_2O (3:1 ratio), 96%. (ii) $NaIO_4$ in MeOH, then (iii) $NaBH_4$ in MeOH at 0 °C, 59% over 2 steps. (iv) NaOMe in MeOH at 0 °C, then NaOH (1 M) at rt, followed by HCl (aq.) (25 mM) at 60 °C, 11% for **20** and 49% for **21**.

Table 1. Summary of Enzymatic Syntheses of 8-Modified-CMP-Sialic Acid Derivatives Using CMP-Sialic Acid Synthetase and 8-Modified Sialic Acids

product	no.	scale (mmol)	time (h)	yield (%)
CMP-8-*H*-NeuNAc	**22**	0.11	0.5	83
CMP-8-*O*-Me-NeuNAc	**23**	0.20	1	78
CMP-8-*F*-NeuNAc	**24**	0.03	6	82
CMP-C7-NeuNAc	**25**	0.08	4	72

with Asn66, and these kinetic results could suggest a significant contribution of this residue to stabilization of the transition state.

Quite different results were obtained with the *Pasteurella* enzyme, which is a member of the GT-80 family and has a GT-B fold. This is a much more efficient enzyme than Cst-I, with k_{cat}/K_m values approximately 250-fold higher. Earlier studies[28] have revealed a much broader specificity, with the ability to form both α-2,3 and α-2,6-linkages to galactose along with having α-2,3-sialidase and α-2,3-*trans*-sialidase activities. The precise mechanisms of these latter activities, along with their different pH-dependences, remain unclear.

The experiments described in this paper were performed at pH 8.5, conditions described as being optimal for the α-2,3-sialyltransferase activity. Both the TLC and kinetic data in Table 3 reveal that the *Pasteurella* enzyme will transfer all of the modified sugars to lactose, although the TLC studies revealed that, even in the presence of a 2-fold excess of donor sugar over acceptor, reactions with **22**, **23** and **24** did not proceed to completion. This may be due to the higher ratio of hydrolysis to transfer measured for **22** and **23** (about 1:1.8) versus that of **25** (about 1:3.2), meaning that a larger proportion of the donor is being hydrolyzed by the enzyme. In fact prolonged incubation times resulted in significant hydrolysis of the BODIPY-sialyl-lactose transfer products back to BODIPY-lactose such that after 18 h all sialyl-lactoside conjugates, with the exception of the truncated transfer product (lane **f**), had been degraded back to their lactoside derivatives. Interestingly two product spots are seen in lane **c** corresponding to the transfer of 8-deoxysialic acid, suggesting the formation of two different products. The faster running product has an R_f consistent with that of the transfer product of Cst-I, the α-2,3-linked product, and the lower running product is most likely the α-2,6-linked sialoside, since the *Pasteurella* enzyme is known to have this alternative transferase activity. Furthermore, incubation of the 8-deoxy-sialyl lactose products at a lower pH (5.3) in the presence of the *Pasteurella* enzyme resulted in exclusive degradation of the faster running product. This is further evidence that this product is the α-2,3-linked sialoside, since the enzyme is known to cleave α-2,3-linked sialosides at low pH values but leaves α-2,6-linked sialosides untouched.[28]

The kinetic parameters reveal essentially no effect at all of 8-position modification on k_{cat} and only very minimal effects upon K_m. This would imply that no significant interactions are formed between the enzyme and the 8-position at either the ground state (Michaelis complex) or at the reaction transition state. Further, and also in marked contrast to what was seen with Cst-I, there was no deleterious effect upon the hydrolytic

Table 2. Kinetic Parameters for the Transfer of Modified CMP-Sialic Acid Derivatives to Lactose and to Water by Cst-I[a]

Compound	#	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$) (transfer)	k_{cat}/K_m ($mM^{-1}min^{-1}$) (hydrolysis)	
CMP-SA		0.75 (±0.04)	188 (±5)	250 (±13)	22 (±1)	abcdef
CMP-8-H	**22**	0.75 (±0.09)	29 (±2)	38 (±5)	<0.1	
CMP-8-*O*-Me	**23**	2.93 (±0.25)	32 (±2)	11 (±1)	<0.1	
CMP-8F	**24**	1.04 (±0.10)	17 (±2)	10 (±1)	<0.1	
CMP-C7	**25**	0.72 (±0.05)	28 (±1)	39 (±3)	<0.1	

[a] Also shown are the transfers of each of the modified donors to bodipy-lactose by Cst-I (15 μM), performed at ambient temperature, as monitored by TLC (ethyl acetate/methanol/water/acetic acid mobile phase in a 4:2:1:0.1 ratio): lane **a**, bodipy lactose acceptor; **b**, CMP-sialic acid donor; **c**, donor **22**; **d**, donor **23**; **e**, donor **24**; **f**, donor **25**.

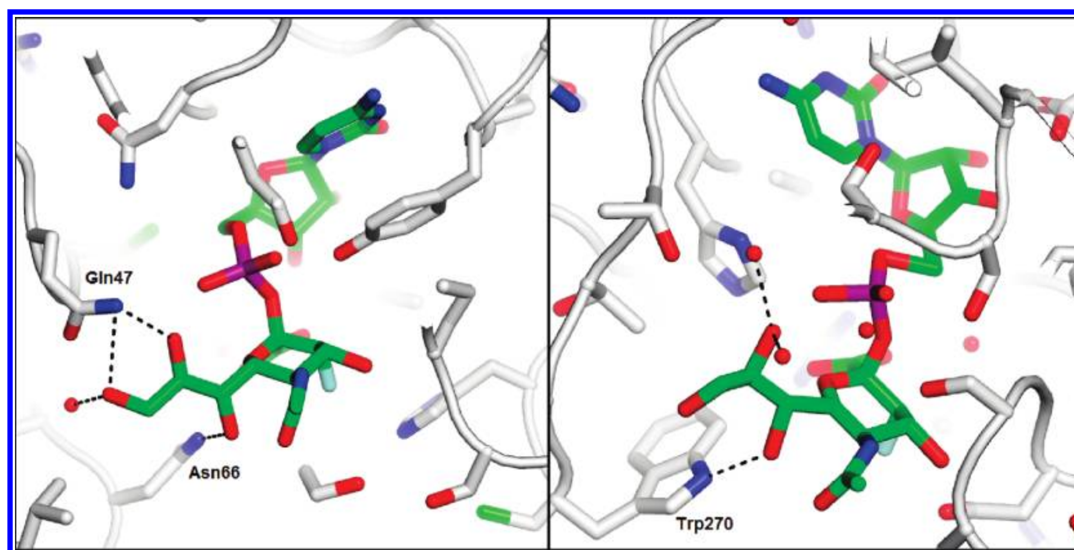


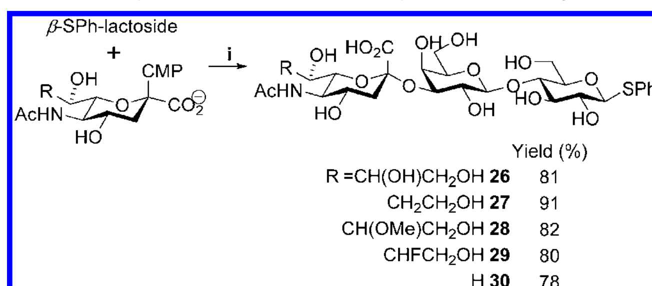
Figure 2. Interaction of glycerol side chain with Cst-I (left) and Pm0188h (right) sialyltransferases.

Table 3. Kinetic Parameters for the Transfer of Modified CMP-Sialic Acid Derivatives to Lactose and to Water by PM0188h[a]

Compound	#	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$) (transfer)	k_{cat}/K_m ($mM^{-1}s^{-1}$) (hydrolysis)
CMP-SA		0.14 (±0.01)	143 (±5)	1008 (±93)	206 (±19)
CMP-8-H	**22**	0.17 (±0.02)	141 (±7)	837 (±88)	469 (±49)
CMP-8-*O*-Me	**23**	0.14 (±0.01)	117 (±3)	810 (±69)	478 (±41)
CMP-8F	**24**	0.22 (±0.03)	121 (±7)	537 (±55)	ND
CMP-C7	**25**	0.24 (±0.01)	142 (±3)	593 (±27)	187 (±8)

[a] Also shown are the transfers of each of the modified donors to bodipy-lactose by PM0118h (5 μM), performed at ambient temperature, as monitored by TLC (ethyl acetate/methanol/water/acetic acid mobile phase in a 4:2:1:0.1 ratio): lane **a**, bodipy lactose acceptor; **b**, CMP-sialic acid donor; **c**, donor **22**; **d**, donor **23**; **e**, donor **24**; **f**, donor **25**.

Scheme 6. Synthesis of 8″-Modified Sialyl Lactose Analogues[a]



[a] Reagents and conditions: (i) Cst-I, 50 mM HEPES pH 7.5, 10 mM $MnCl_2$ and $MgCl_2$, alkaline phosphatase, 4 mM β-thiophenyl lactoside and 5 mM CMP-8-modified donor sugar.

reaction; indeed for two of the substrates the rate of sialyl transfer to water was stimulated 2-fold. These findings are entirely consistent with the published three-dimensional structures of the *Pasteurella* sialyltransferase in complex with substrates,[26] which showed that both the 8- and 9-hydroxyls are essentially fully solvent-exposed, with the OH7 being the only hydroxyl within the glycerol side chain to make significant contacts with the enzyme through a hydrogen bond to Trp270 (see Figure 2).

Table 4. ^{1}H NMR Chemical Shifts of H3 Protons of Sialyl Lactose Conjugates **26**−**30** in Comparison with Previously Published α-Sialosides

compound	$H3_{ax}$	$H3_{eq}$
26	1.66	2.62
27	1.64	2.54
28	1.62	2.54
29	1.63	2.56
30	1.62	2.57
α-OH-Neu5Ac[50]	1.59	2.70
α-*O*-Me-Neu5Ac[51]	1.69	2.69
α-*O*-Gal-Neu5Ac[52]	1.68	2.63

An important consequence of this behavior is that, although the *Pasteurella* enzyme is an excellent catalyst for syntheses with the natural as well as C5- and C9-modified CMP-sialic acid donors, especially given its high efficiency, it is not as useful for substrates modified at the 8-position. The shift from a 5:1 ratio of transfer rate to lactose:hydrolysis rate constant for the natural sugar to a ratio of under 2:1 with **22** and **23** renders the enzyme less useful for such syntheses, especially given the need to very carefully monitor reaction progress in order to minimize hydrolysis of the sialylated product. Thus the transferase from *Campylobacter* (Cst-I) was chosen

Table 5. ^{1}H and ^{13}C NMR Chemical Shifts of the Galactose Residue in Sialyl Lactose Conjugates **26**−**30** in Comparison with *β*-PhS-Lactoside

compound	C2′	C3′	C4′	C6′	H2′	H3′	H4′	H6′a	H6′b
β-PhS-lactoside	70.4	72.0	68.0	60.5	3.40	3.52	3.78	3.61	3.65
26	68.8	74.9	66.9	59.5	3.43	3.97	3.81	3.69	3.83
27	68.9	74.9	67.2	59.6	3.43	3.96	3.85	3.69	3.84
28	68.8	75.2	67.2	59.5	3.43	3.94	3.84	3.69	3.85
29	68.9	75.0	67.0	59.6	3.43	3.97	3.84	3.69	3.83
30	69.0	74.9	66.9	59.6	3.42	3.97	3.85	3.68	3.82

as the optimal catalyst to synthesize 8″-modifed sialyl lactose analogues. It is also worth noting that Cst-I can also transfer to GM1-glycan, T-antigen and lactosamine acceptors,[49] indicating that further sialic acid containing oligosaccharides could be synthesized using this methodology, which may not be limited to lactose derivatives.

Completion of the synthesis of the 8″-modified sialyl lactose derivatives was carried out using a thiophenyl lactoside as the acceptor sugar, since the aryl glycoside can be used to aid purification via reversed-phase chromatography, and also to monitor the progress of the reaction. Simple alkyl and aryl galactosides can also be used as acceptors. A slight excess of acceptor was used to ensure that all of the valuable donor was converted to product, and the concentrations of donor and enzyme were optimized to minimize the reaction time. On completion of the reaction, as monitored by TLC, each of the sialyl lactose conjugates was isolated using a C18-Sep-pak cartridge and further purified by reversed-phase HPLC. The isolated yields obtained (Scheme 6) in each case are good to excellent, even for the C7-truncated analogue **30**.

To confirm the stereochemistry and position of the sialyl lactose glycosidic bond of compounds **26** through **30**, the ^{1}H and ^{13}C NMR spectra of each were unambiguously assigned. To address the stereochemistry of the glycosidic bond, the chemical shifts of both of the H3″ protons were compared to those of previously reported α-sialosides. The chemical shifts of the H3 protons of sialic acid are extremely sensitive to, and thus diagnostic of, the anomeric configuration at C2. Most α-configured sialosides display a significantly downfield-shifted H3-equatorial proton (2.9−2.5 ppm) compared with the same proton of a *β*-sialoside (2.3−1.9 ppm). Furthermore α-sialosides possess a larger difference in chemical shift between H3-equatorial and H3-axial protons (1.1−0.9 ppm difference), compared with *β*-sialosides (0.3−0.6 ppm difference).[51] The chemical shifts of the H3-equatorial protons of all of the synthesized sialyl lactose conjugates (**26**−**30**) are entirely consistent with those of an α-sialoside (see Table 4), as is the difference in chemical shift between the two H3 protons, confirming that Cst-I transfers 8-modified sialic acids to form α-sialosides.

To address the position of attachment of the sialic acid, the ^{1}H and ^{13}C chemical shifts of the galactose residues in each of the sialyl lactose conjugates (**26**−**30**) were compared with those of the *β*-thiophenyl lactoside acceptor (Table 5). For clarity only data for those positions on the galactose that bear a free hydroxyl are shown for comparison. It is clear from the ^{13}C NMR data that C3′ shows the largest deviation in chemical shift (approximately 3 ppm downfield) from the parent lactoside (72.0 ppm), with C2′ and C4′ showing smaller (less than 1 ppm) but significant differences in chemical shift, consistent with sialylation at the 3-position of galactose. Further evidence is provided by comparison of the ^{1}H NMR spectra whereby the H3′ proton in each case has moved downfield, by approximately 0.4 ppm to around 3.95 ppm, compared with the parent acceptor compound (3.52 ppm). Yet further evidence is the strong correlation observed in the HMBC spectra between H3′ and C2″ for each trisaccharide. These data confirm that the modified sialic acid-containing donors do not affect or change the specificity of Cst-I and that the 8″-modified-sialyl lactose products possess the natural α-2,3-glycosidic linkage.

Conclusion

A series of 8-modified sialic acids has been synthesized and each converted to their CMP-derivative using a bacterial CMP-sialic acid synthetase. These modified donors were evaluated as substrates for two sialyltransferases. Cst-I from *C. jejuni* was found to transfer each of the modified sugars to lactose with a 10-fold lower catalytic efficiency compared with the natural donor substrate. By contrast Pm0188h from *P. multocida* was found to transfer the modified sugars at the same rates as the natural donor but catalyzed their hydrolysis more efficiently than is the case with the natural substrates. Of these two enzymes Cst-I, with its minimal hydrolysis of the modified donors and good transfer rates, has the most desirable characteristics for efficient synthesis of a number of 8″-modified sialyl lactoses and was used as such, with yields of around 80% being obtained. The conditions used are compatible with other biological systems and may be used to incorporate such modified sialic acids onto glycoproteins and other sensitive biomolecules, a clear advantage over chemical glycosylation techniques. The 8-modified sialyl lactose derivatives prepared will be employed in kinetic and structural studies on polysialyltransferases to probe substrate binding modes and energetics.

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Supporting Information Available: Experimental details and ^{1}H and ^{13}C NMR data for the chemical synthesis of all modified sialic acid derivatives, the chemoenzymatic synthesis of all sialyl-lactosides, and the kinetic analysis of all modified sialic acid donors. This material is available free of charge via the Internet at http://pubs.acs.org.

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