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Sialyltransferase inhibitors: consideration of molecular shape and charge/hydrophobic interactions

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ABSTRACT

In order to evaluate the importance of molecular shape of inhibitor molecules and the charge/H-bond and hydrophobic interactions, we synthesized three types of molecules and tested them against a sialyltransferase. The first type of compounds were designed as substrate mimics in which the phosphate in CMP-Neu5NAc was replaced by a non-hydrolysable, uncharged 1,2,3-triazole moiety. The second type of compound contained a 2-deoxy-2,3-dehydro-acetylneuraminic moiety which was linked to cytidine through its carboxylic acid and amide linkers. In the third type of compound the sialyl phosphate was substituted by an aryl sulfonamide which was then linked to cytidine. Inhibition study of these cytidine conjugates against *Campylobacter jejuni* sialyltransferase Cst 06 showed that the first type of molecules are competitive inhibitors, whereas the other two could only inhibit the enzyme non-competitively. The results indicate that although the binding specificity may be guided by molecular shape and H-bond interaction, the charge and hydrophobic interactions contributed most to the binding affinity.

1. Introduction

Sialylated glycoproteins and glycolipids are ubiquitous in mammalian cells and play important roles in various fundamental physiological and pathological processes, such as cell–cell adhesion, immune defense, tumor cell metastasis, and inflammation.[1–5] Biosynthesis of sialylated glycoconjugates can be achieved by using sialyltransferases (ST), a family of glycosyltransferases, that catalyze the transfer of sialic acid residues to non-reducing terminal galactose or another terminal sialic acid attached to oligosaccharide chains of glycoproteins and glycolipids, using cytidine monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) as the donor. Studies have shown elevated sialyltransferase activity leads to over expression of sialylated glycoconjugates in human colorectal cancer, breast carcinoma, leukemia cell lines, and metastatic tissues.[6–10] Characteristic differences in the occurrence of sialic acids in healthy and in diseased human tissues have also been reported.[11,12] Thus, sialyltransferase inhibitors may provide an alternative chemotherapeutic treatment of cancer and also an invaluable tool to study ST-dependent biological processes.[13]

A common approach toward the development of effective sialyltransferase inhibitors is to create mimic analogs based on the donor[14–22] (CMP-Neu5Ac), acceptor[23–27] or the transition state[28–31] involved in the glycosyl transfer within the active site of the enzyme. Schmidt[15,32–34] has designed and synthesized inhibitors based on transition state analogs. They reported compounds **A** and **B** (Fig. 1), transition-state mimics, based on the proposed mechanism of sialyl transfer involving partial dissociation of the CMP moiety and the formation of a planar oxocarbenium structure in the transition state.[28–31] In compound **A**,[15] they used a 2-deoxy-2,3-dehydroneuraminic acid residue and introduced another negatively charged phosphate group in order to achieve trigonal planar geometry similar to the transition-state and to increase charge interactions. Similarly, in compound **B**[32] they replaced the neuraminyl residue with an aryl ring keeping intact the cytidine residue. Both molecules exhibited good inhibition, but from a therapeutic

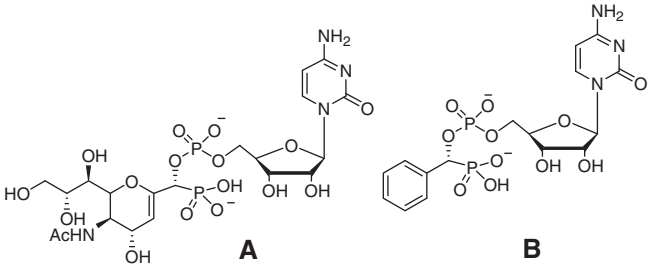

Figure 1. Transition-state sialyltransferase inhibitors: molecules with charge.

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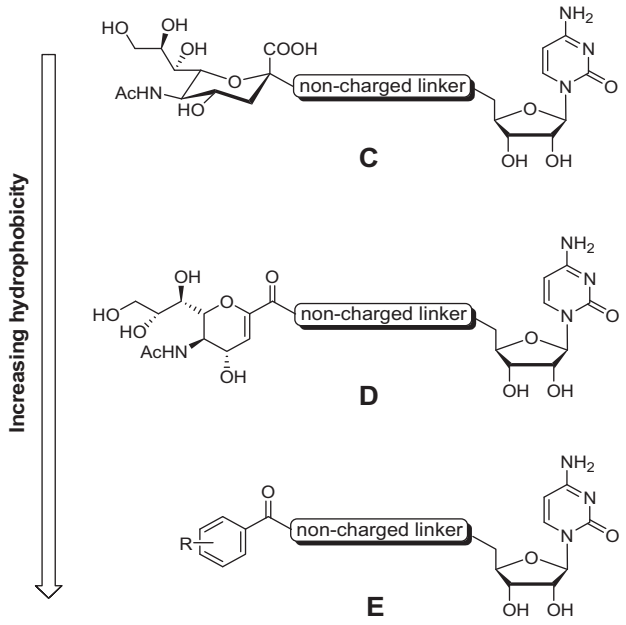


Figure 2. Three types of molecules as potential sialyltransferase inhibitors.

perspective they have serious drawbacks because of their poor bioavailability. The charged phosphate linkage imparts poor cellular permeability and is prone to phosphatase activity resulting in a significant or total loss of activity.

Inhibition studies have shown that the cytidine portion of the donor is important for binding with the active or regulatory site of the enzyme, though sialic acid may not be essential.[35,36] The phosphodiester of CMP-Neu5Ac may also contribute to the binding, but it is unclear to what extent, as derivatives void of the phosphodiester linker also exhibited significant ST inhibiting activity.[37,38] Since non-substrate mimic inhibitors of sialyltransferases (e.g., flavonoids,[39] Soyasaponin,[40] and, Lithocholic acid analogs[41]) do not share the structural characteristics of CMP-Neu5Ac, interaction through their hydrophobic moieties with sialyltransferases becomes critical in the inhibition either competitively or non-competitively.

To address whether non-charged molecules devised from compounds **A** and **B** can be effective competitive inhibitors to sialyltransferase, we designed a library of three distinct classes of molecules containing two distinct features, a non-charged linker and an increase, in type D–E, in hydrophobicity and planarity of the replaced sialic acid unit (Fig. 2). In C-type molecules the phosphodiester linkage of the substrate CMP-Neu5Ac was replaced with a non-ionic triazole moiety. Such mimics still contain both the sialic acid and cytidine residues responsible for binding to the sialyltransferase active site. In the D-type molecules, instead of sialic acid, a 2-deoxy-2,3-dehydroacetylneuraminyl moiety was connected to cytidine by a triazole or a sulfamide linkage. This might help to achieve trigonal planarity at the anomeric center of the transition state involved during glycosylation, while also by slightly increasing hydrophobicity. Type E compounds lack both sialic acid and a phosphodiester linkage that were replaced, respectively, by an aryl group and an isosteric sulfamide linker. The present work describes the synthesis and preliminary inhibition studies of above three types of molecules.

2. Results and discussion

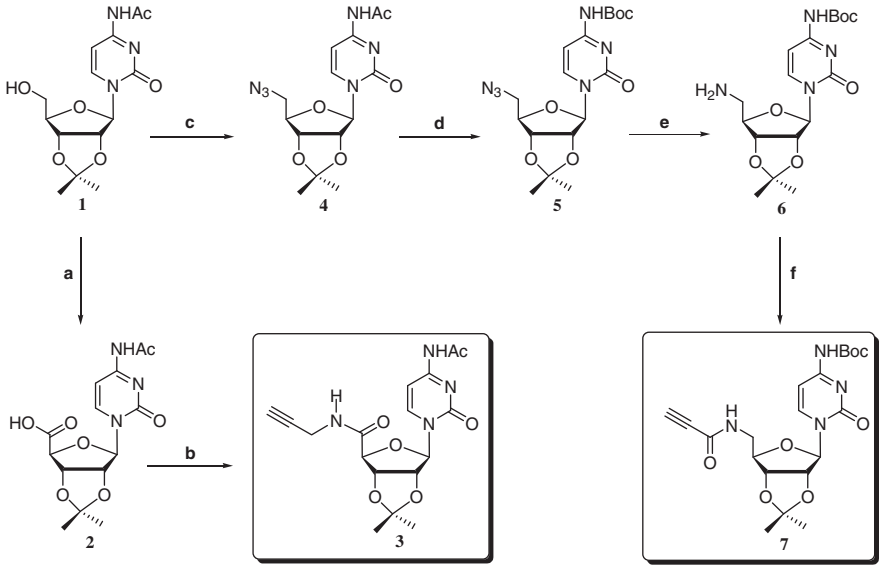
2.1. Synthesis

Using click chemistry, two C-type compounds were obtained from 2-azidosialic acid (**9**) and propargyl derivatives of cytidine (**3** and **7**) (Schemes 1 and 2). *N*-Acetyl-2′,3′-di-*O*-isopropylidene cytidine (**1**) was treated with a catalytic amount of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and stoichiometric amount of the oxidant, [bis(acetoxy)iodo]benzene (BAIB) in acetonitrile–water (1:1) that led to an acid (**2**),[42] which was then treated with propargylamine along with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in DMF to give the desired propargyl derivative (**3**). The other propargyl derivative (**7**) was also synthesized starting from **1** in four steps, including intermediate, *N*-acetyl-5′-azido-2′,3′-di-*O*-isopropylidene cytidine (**4**), obtained by treatment of **1** with methanesulfonyl chloride and pyridine followed by substitution by sodium azide in DMF. N-Deacetylation with base followed by N-protection with di-*tert*-butyloxycarbonate gave *N*-(*t*-butyloxycarbonyl)-5′-azido-2′,3′-di-*O*-isopropylidene cytidine (**5**), which was then reduced to *N*-(*t*-butyloxycarbonyl)-5′-amino-2′,3′-di-*O*-isopropylidene cytidine (**6**). Interchange of *N*-acetyl protection to *N*-Boc protection was necessary as catalytic hydrogenation of **4** failed to produce the desired amine (**6**). Condensation of amine (**6**) with propiolic acid along with coupling reagents EDC–HOBt in DMF yielded the desired propargyl derivative (**7**).

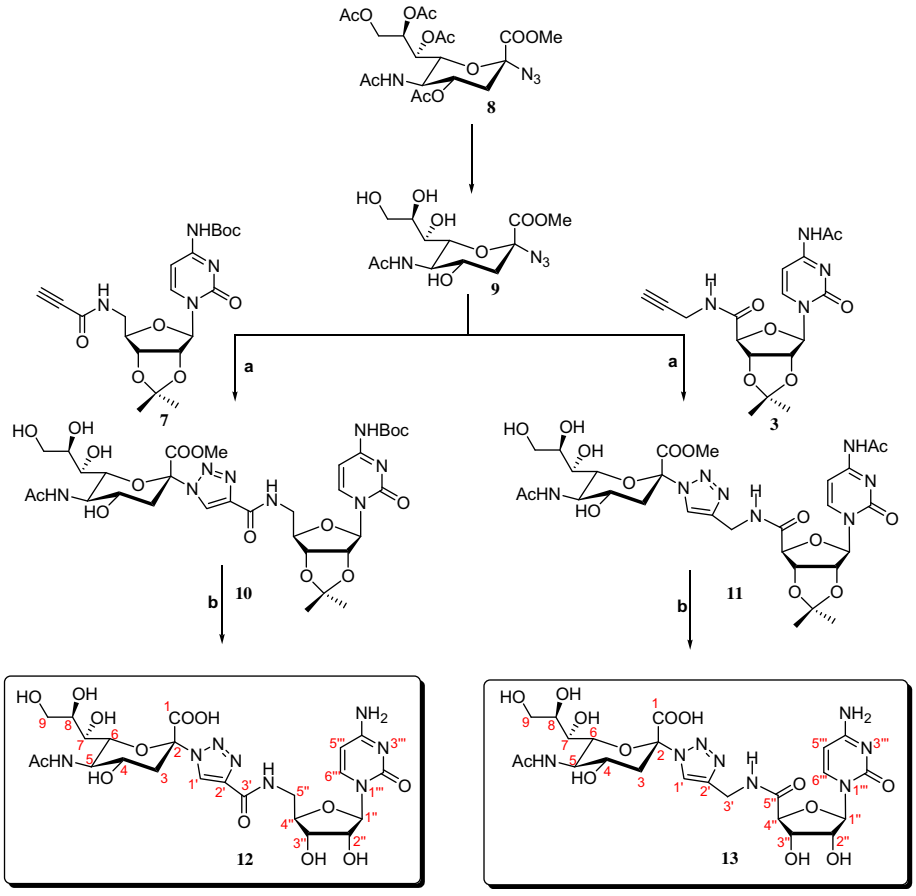
Synthesis of 2-azido-2-deoxy-*N*-acetyl-α-neuraminic acid derivative **9** started from fully acetylated 2-chloro-2-deoxy-β-neuraminic acid methyl ester, which under a phase transfer catalysis process reacted with sodium azide to yield acetylated neuraminyl azide (**8**).[43] Since the [3+2] cycloaddition between alkynes (**3** and **7**) and **8** failed under click-chemistry conditions, as reported by Linhardt,[44] the *O*-acetyl groups of **8** were removed under basic conditions to yield neuraminyl azide **9**. [3+2] Cycloaddition of propargyl derivatives **3** and **9** in the presence of $CuSO_4$ and sodium ascorbate was then performed at 60 °C in a mixture of the *t*-BuOH and water (1:1 v/v), which successfully gave the desired triazole **11** in 78% yield. Similarly, triazole **10** was obtained from the propargyl derivatives **7** and **9** in 81% yield. The addition products, **10** and **11**, were treated with aqueous TFA to yield the deprotected desired C-type compounds, **12** and **13**, respectively, which were fully characterized by spectroscopic analyses. The proton and carbon signals in D_2O were assigned unambiguously with 1D- and 2D-NMR techniques.

Using similar [3+2] cycloaddition a triazole linker was introduced in type-D compound **19** (Scheme 3) which involves the coupling of alkyne derivative **17** and azide **4**. O-Deacetylation of 2,3-dehydroneuraminic methyl ester **14** under basic conditions followed by treatment with 2,2-dimethoxypropane and *p*-toluenesulfonic acid (PTSA) in DMF afforded the 8,9-di-*O*-isopropylidene-2,3-dehydroneuraminic methyl ester **15** in 58% overall yield. When treated with 0.1 N NaOH/MeOH (1:1) the methyl ester was hydrolyzed to afford the free carboxylic acid derivative **16**. Condensation of **16** and propargyl amine using coupling reagents EDC–HOBt in DMF yielded the desired propargyl derivative **17**. Click chemistry was performed on **17** and the azide **4** using CuI in MeCN which successfully gave triazole derivative **18**. Sequential removal of *N*-acetyl and isopropylidene protection in **18** by using NaOMe–MeOH solution and aqueous TFA–water solution afforded the desired triazole linker D-type molecule **19**.

In addition, other linkers such as sulfonamide (Scheme 3) and peptides (Scheme 4) were also introduced in the construction of other D-type molecules (**21** and **24**). Intermediate **20** for sulfonamide linker D-type molecule **21** was synthesized by reacting sulfamoyl chloride with **1** in DMA. This sulfonamide (**20**) was coupled with carboxylic acid **16** and the subsequent deprotection afforded **21** in good yield (Scheme 3). In order to prepare peptide linked type-D molecules (**24a–e**), Fmoc chemistry was employed as outlined in Scheme 4. The synthetic sequence involved condensation of amine derivative **6** with protected amino acids, Fmoc-Gly-OH, Fmoc-Gln(Tr)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBt)-OH, and Fmoc-Trp(Boc)-OH. The coupling reaction was carried out using EDC–HOBt coupling reagents to furnish Fmoc-protected coupled products in 65–70% yields. Removal of the



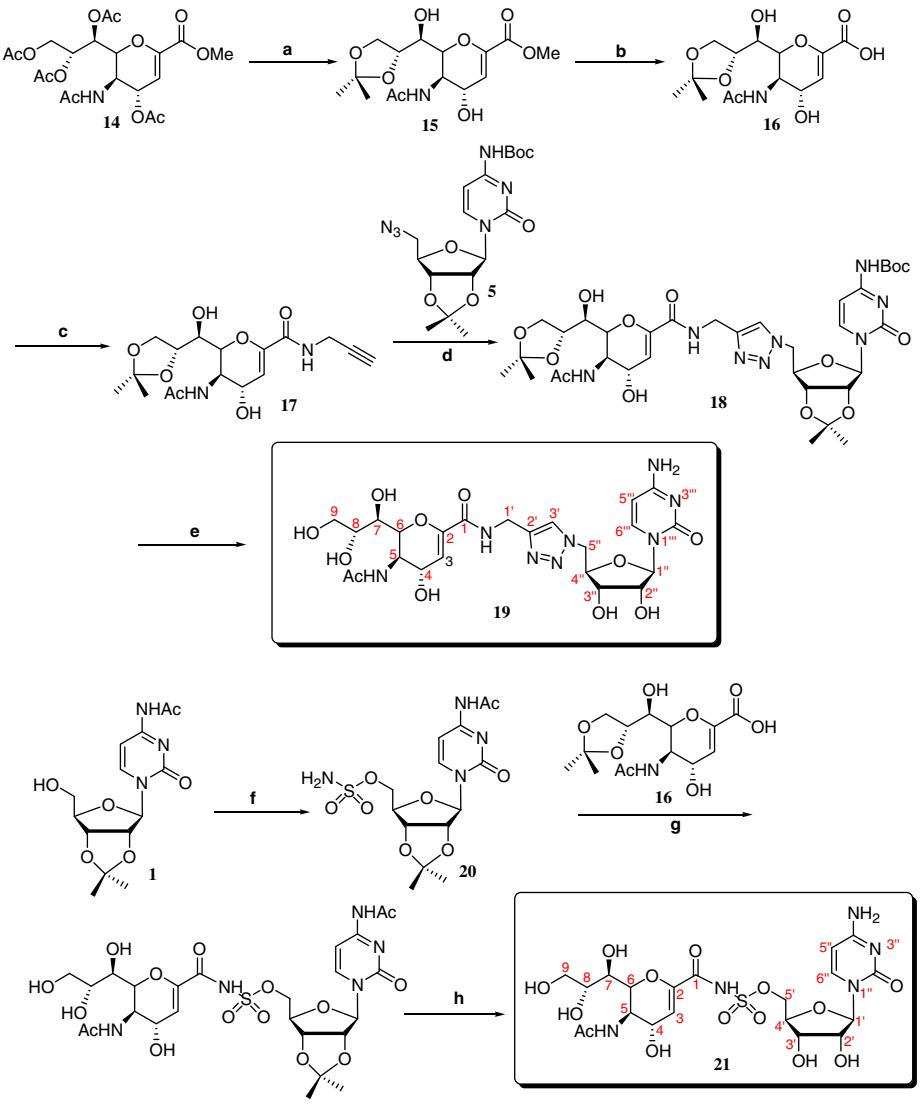
Scheme 1. Reagents and conditions: (a) TEMPO, BAIB, MeCN–H_2O (1:1 v/v), 3 h, 0 °C–rt, 84%; (b) propargylamine, EDC–HOBt, DMF, 24 h, rt, 76%; (c) (i) MsCl/Py, DCM; (ii) NaN_3, DMF, 80 °C, 72%; (d) (i) NaOMe/MeOH, 1 h, 0 °C; (ii) $(Boc)_2O$, Et_3N, THF, 24 h, rt, 69%; (e) Pd/C, MeOH, 92%; (f) propiolic acid, EDC–HOBt, DMF, 24 h, rt, 72%.



Scheme 2. Reagents and conditions: (a) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, ^{t}BuOH/H_2O/DCM (2:1:1 v/v), 12 h, 60 °C, 82% for **10**, 78% for **11**; (b) TFA–H_2O, DCM, 2 h, 0 °C, 1 M, NaOH–H_2O.

Fmoc group using piperidine produced intermediates (**22a–e**), followed by coupling with acid **16** yielded compounds **23a–e**. Global deprotection using aqueous TFA gave another five type-D compounds **24a–e**, which were fully characterized by spectroscopic analyses.

Next, type-E molecules were constructed by coupling various substituted benzoic acids with the sulfamide **20**. The intermediates were subsequently deprotected to afford (**25a–r**). The couplings were achieved indirectly using N-succinimide esters of benzoic acids. Direct coupling between benzoic acids and sulfamide, using



Scheme 3. Reagents and conditions: (a) (i) NaOMe, MeOH; (ii) DMP, PTSA, DMF, 3 h, rt, 58% for two steps; (b) NaOH, MeOH (quantitative); (c) propargylamine, EDC–HOBt, DMF, 24 h, rt, 56%; (d) CuI, DIPEA, MeCN, 1 h, rt, 68%; (e) TFA, H_2O, 1 h, 0 °C, 67%; (f) sulfamoyl chloride, DMA, 0 °C, 4 h, rt, 68%; (g) EDC, HOBt, DMF, 24 h, 0 °C; (h) TFA, H_2O, 1 h, 0 °C, 59% for two steps.

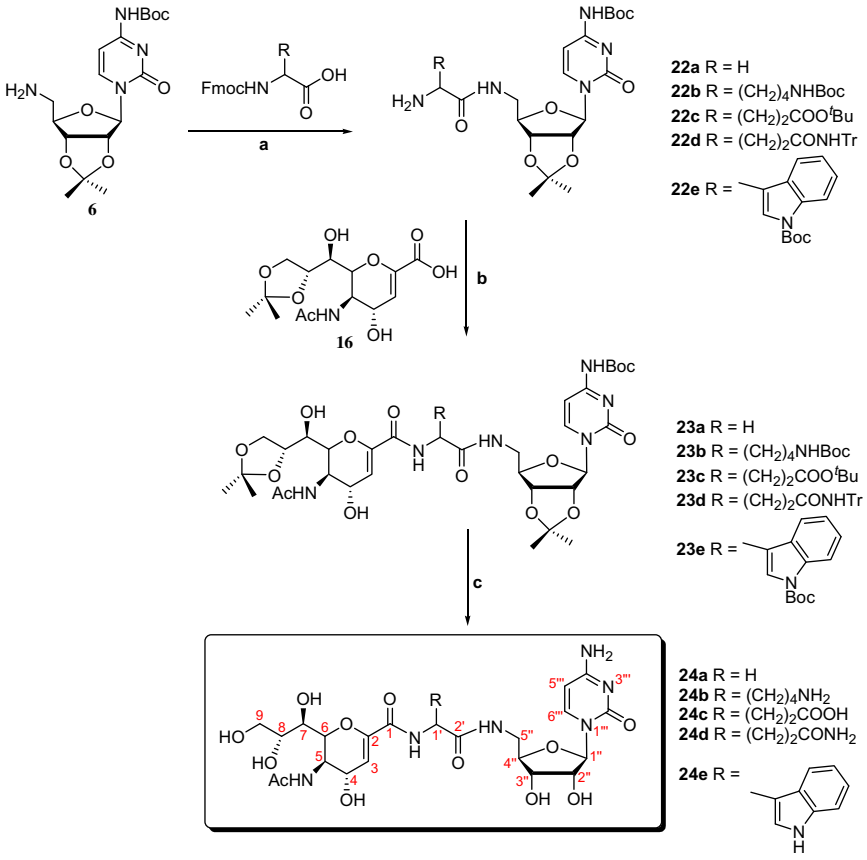
dicyclohexylcarbodiimide (DCC) and DMAP as described by Widlanski[45] also gave **25a**. The synthesis of **25d** was also achieved by activating the salicylic acid with 1,1′-carboxydiimidazole and then using DCC to couple with the sulfamide.

2.2. Inhibition studies

Three different types (C, D, and E) of molecules synthesized above were tested against α-(2,3)-sialyltransferase Cst-06. Cst-06 is a fusion protein with Cst-I of *Campylobacter jejuni* bound with maltose and belongs to GT-42 superfamily.[46] The results of inhibition are presented in Table 1.

C-Type molecules **12** and **13** with a triazole linkage as mimic of the phophodiester linkage[47–49] and a α-anomeric configuration of sialic acid showed more than 50% inhibition at 500 μM. The K_i for **13** is 160 μM which is twice lower than the K_m of CMP-Neu5Ac (400 μM).[46] The Lineweaver–Burk double reciprocal plot of molecule **13** as shown in Figure 3 suggested a mixed inhibition toward Cst-06. The two inhibition constants calculated were K_{ic} = 222.7 μM and K_{iu} = 984.2 μM. Since the K_{ic} is 4–5 times less than the K_{iu}, the inhibition can be considered 'predominantly competitive'.[50] The docking study with the enzyme Cst-I suggests **13** could have two binding sites with two different binding energies, which is corroborated by the two observed inhibition constants. Modeling shows that **13** is able to bind to the enzyme's active site as well as to another site just off the actual active site. The calculated free energy of binding at the active site is −6.75 kcal/mol and −5.19 kcal/mol at the other site. (More detailed discussion can be found in Supplementary data.)

D-Type molecules, in general, were found to be poorer inhibitors than C-type molecules. The highest inhibition observed among the D-type molecules was around 30% for **21** and **19**. These two molecules differ in the linkage structure with the triazole in **19** being replaced with sulfamide in **21**. The poor inhibition was likely due to the lack of charge interaction, as indicated by the better inhibition of **24c** which carries a negative charged amino acid (aspartic acid). However, the kinetics showed that the inhibition was not competitive. We did not further investigate the unknown binding site of the sialyltransferase. Although the compounds are analogs of a known transition state inhibitors (see Fig. 1A) the lack of phosphate group and charge interaction seems to be detrimental to the binding affinity at the active site. The results also suggest



Scheme 4. Reagents and conditions: (a) (i) EDC, HOBt, DMF, 24 h, rt, 52–63%; (ii) piperdine, CH_2Cl_2, 30 min, rt; (b) EDC, HOBt, DMF, 24 h, 0 °C; (c) TFA, H_2O, 2 h, rt, 60–70%.

Table 1
Inhibition of α-(2,3)-sialyltransferase Cst-06[a]

Compound	% Inhibition at 500 μM	Compound	% Inhibition at 500 μM
C-Type		E-Type	
12	51.7 (58.5)[b]	**25a**	27.2 (22.5)
13	58.5 (55.0) (K_i = 160 μM)[b]	**25b**	31.7 (33.5)
		25c	28.0 (29.0)
		25d	38.1
D-Type		**25e**	39.5 (44.1)
19	29.2	**25f**	43.1 (44.6)
21	33.5	**25g**	22.3
24a	10.5	**25h**	28.7
24b	10.7	**25i**	12.7
24c	30.5	**25j**	15.9
24d	19.1	**25k**	40.5 (43.1)
24e	25.1	**25l**	29.5
		25m	78.4 (75.6)[b]
		25n	79.4 (73.0) (K_i = 87 μM)[b]
		25o	9.0
		25p	50.1 (48.6)
		25q	27.4
		25r	33.8

[a] Data were average from a duplicated enzyme inhibition assay performed at 37 °C. Data in bracket were repeated results.

[b] Inhibition was confirmed using FCHASE lactoside as substrate by monitoring the formation of sialyllactoside (data not shown). The assays were performed at 37 °C for 20 min using 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 250 μM CMPNeuAc, 500 μM Lac-FCHASE 6-(5-fluoresceincarboxamido)-hexanoic acid succinimidyl ester. All reactions were stopped with 0.35 M Na_2CO_3. The samples were analyzed by CE with a P/ACE MDQ CE system equipped with a laser module 488 (Beckmann coulter). The percentage conversion of the FCHASE-Lac to product was calculated by the integration of the trace peaks using the MDQ 32 Karat software.

that a combination of cytidine and a neuraminyl group without charge interaction is not enough to have an effective binding at the active site, which may consequently lead to another binding site likely stabilized by hydrophobic and hydrogen bonding interactions. This is supported by the fact that although cytidine is not an inhibitor of sialyltransferases,[51] yet cytidine monophosphate (CMP) does competitively inhibit sialyltransferase[35] and polysialyltransferase activities.[52] The cytidine may play a key role in binding specificity, but the affinity could be largely contributed through charge interaction from additional phosphate. Another example illustrating the importance of charge interaction is Tamiflu which as a prodrug becomes a powerful neuraminidase inhibitor only after the ester is hydrolyzed to allow charge interaction and effective binding.[53]

Non-sugar analogs, such as an unsaturated bicyclic system with a conjugated carboxylate group are used as a replacement of neuraminyl group,[54] and sugar moiety attached to a bulky thiazole like compounds has also been tested as potent inhibitors.[55] These experiments encouraged us to explore E-type compounds as potential sialyltransferase inhibitors. E-type molecules contain a sulfonamide group as a surrogate of phosphate group (see Scheme 5). Meanwhile, the neuraminyl group was replaced by aryl/heteroaryl groups to further explore if the potency of the inhibition could be improved as observed by others,[32] where 5′-triazole nucleoside analogs were found to be competitive inhibitors of α-2,3-sialyltransferase. Compounds **25m** and **25n** gave the best inhibition, 70–80% at 500 μM. But, the kinetics suggest the inhibition is not competitive, which is inconsistent with the results of similar compounds reported.[32] Distinctively, the substitution of *m*-NO_2 of the aryl group is critical, but not the carboxyl/carboxylate group as indicated by comparing the inhibition of **25i**, **25j**–**25m**, and **25n**,

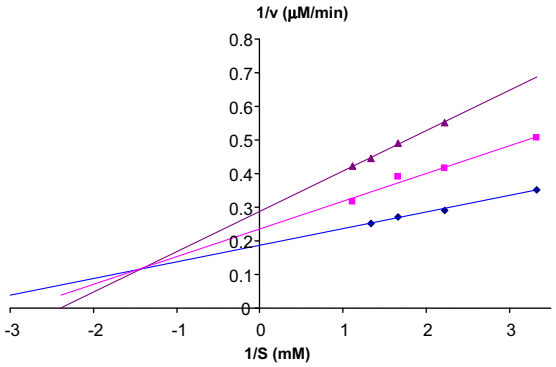


Figure 3. Lineweaver–Burk plot for inhibition of compound **13** against α-2,3-sialyltransferase (*C. jejuni*).

respectively, suggesting a strong interaction occurred only between $-NO_2$ group and the enzyme. Since all other analogs are poor inhibitors, it is plausible that a specific binding between *m*-NO_2 aryl group and the enzyme contributes to the inhibition.

Time based inhibition studies were also carried out to explore whether increasing the pre-incubation of enzyme with inhibitor would affect the inhibition. Although pre-incubation of compounds with the enzyme for 10 min led to slightly better inhibition, prolonged pre-incubation did not further improve inhibition.

3. Conclusions

Through molecular structure variations and its correlation to inhibition against a sialyltransferase it was observed that molecules based on substrate mimics are more likely to be a competitive inhibitor. The results also show that the charge interaction plays a pivotal role in enhancing binding affinity. Increasing hydrophobicity of a molecule may improve its binding but risk sacrificing its specificity. Perhaps, the better way to improve the bioavailability of a sialyltransferase inhibitor is not to eliminate the charge as we tried, but rather to temporary mask the charge and make a pro-inhibitor. It should be noted that inhibitors against a bacterial sialyltransferase often do not inhibit human enzymes due to their limited structural homology.

4. Experimental section

4.1. Inhibition assay

Assays were performed based on procedures described by Gosselin et al. (15) on microtiter plate. The hydrolysis of CMP-NeuAc in the absence of enzyme was excluded from enzyme-catalyzed activities. Pre-incubation for 5 min at 37 °C in a temperature controlled microplate reader (Bio-Rad Benchmark Microplate Reader) was followed prior to the addition of CMP-NeuAc. Each of the microwell contained 10 mM of magnesium chloride, 10 mM of manganese chloride, 55 mM HEPES (Fluka) at pH 7.0, 50 mM of potassium chloride, 0.22 mg/ml Fraction V Bovine Serum Albumin (Sigma–Aldrich), 2.8 mM phospho(enol)pyruvic acid tri(cyclohexyl ammonium) salt (PEP) 4 mM ATP (Sigma), 0.45 mM NADH (Sigma). 3.6 U of pyruvate kinase (Sigma), 5.4 U of Lactic dehydrogenase (Sigma), 5 mU of nucleotide monophosphate kinase, 25 mM of lactose, 250 μM of CMP-NeuAc, and 0.25 mU of sialyltransferase (CST06) in a final volume of 200 μL. All assays were designed to limit the CMP-Neu5Ac consumption to 10–15%, in order to get reliable initial rates. All results were duplicated. Kinetic data were analyzed using LEONARA program.[56]

4.2. General methods

NMR spectra were recorded on a spectrometer with tetramethylsilane or the residual signal of the solvent as the internal standard. Chemical shifts are quoted in ppm and *J* values in Hz. Mass spectra were recorded on a Quadrupole time-of-flight mass spectrometer using electrospray ionization (ESI) or time-of-flight mass spectrometer using matrix-assisted laser desorption/ionization (MALDI). Analytical thin-layer chromatography was performed on precoated plates of silica gel and visualized with H_2SO_4–H_2O (1:20 v/v) followed by heating. Flash column chromatography was performed using silica gel (230–400 mesh). All solvents and reagents were purified and dried according to standard procedures.

4.3. General procedure for synthesis of NHS ester

To a solution of carboxylic acid (1.0 equiv) in THF (10 mL) at 0 °C were added *N*-hydroxysuccinimide (1.0 equiv) and DCC (1.0 equiv). The resulting mixture was stirred for 30 min at 0 °C and 2 h at room temperature. The reaction mixture was filtered to remove the precipitates, and the filtrate was concentrated under reduced pressure. Purification by flash chromatography (hexane/EtOAc) afforded the desired *N*-hydroxysuccinimidyl esters.

4.4. General procedure for acylation from NHS esters

To a solution of *N*-hydroxysuccinimidyl ester (1.0 equiv) in DMF (10 mL) at 0 °C were added protected sulfamoyl cytidine (**20**) (1.5 equiv) and Cs_2CO_3 (2.0 equiv). The reaction mixture was warmed to room temperature and stirred for 16–20 h. The reaction solution was concentrated under reduced pressure to a residue which was extracted with EtOAc (100 mL), and the filtrate was concentrated. Purification by flash chromatography (EtOAc/MeOH 10:1 v/v) afforded the desired compounds.

4.5. General procedure for direct acylation

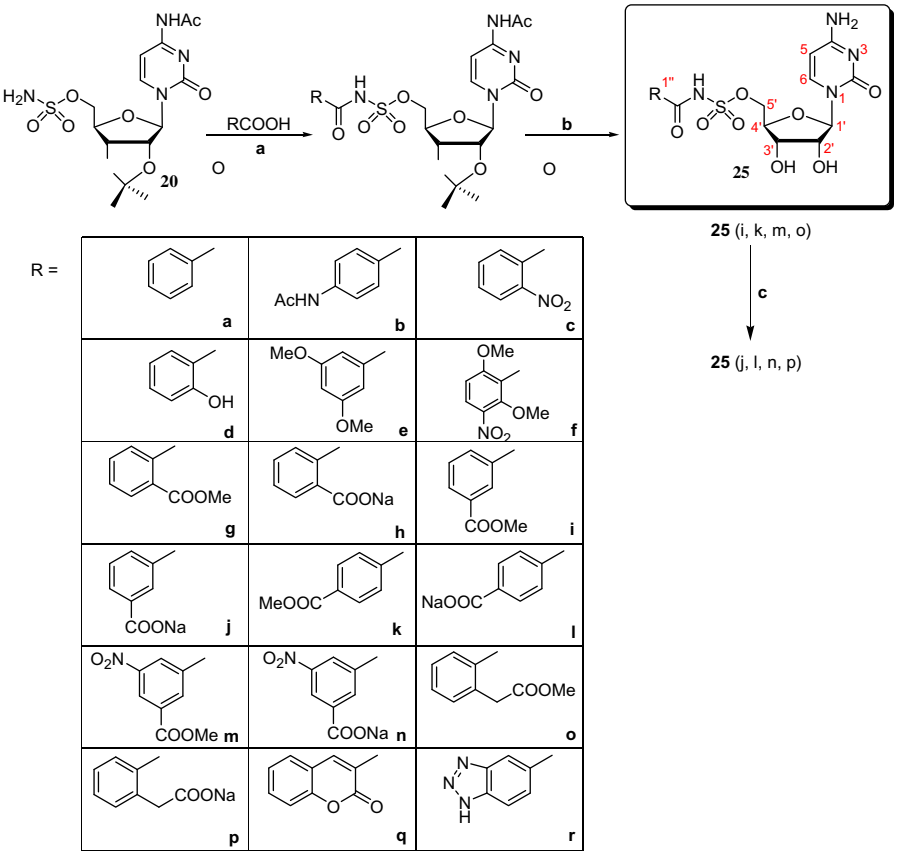
To a solution of carboxylic acid (1.0 equiv) and protected sulfamoyl cytidine (**20**) (1.0 equiv) in DMF (10 mL) at ambient temperature were added DCC (1.0 equiv) and DMAP (1.0 equiv). The resulting mixture was stirred for 3 days at ambient temperature. The reaction mixture was concentrated under reduced pressure. Purification by flash chromatography (EtOAc/MeOH 10:1 v/v) afforded the desired compounds.

4.6. General procedure for TFA deprotection

A solution of *N*-acetyl-5′-*O*-[*N*-acyl(sulfamoyl)]-2′,3′-*O*-isopropylidenecytidine in 30% TFA (MeOH–H_2O 1:1 v/v) was stirred for 2 h at 0 °C to give the desired product. The reaction mixture was concentrated under reduced pressure. Purification by size-exclusion gel chromatography (Biogel-P2, water) afforded the pure compound (**25**).

4.7. 5′-*O*-[*N*-(Benzoyl)sulfamoyl]cytidine (25a)

White solid, $[\alpha]_D^{20}$ +36.9 (*c* 0.27, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.75 (d, *J* = 7.8 Hz, 2H, H-2″, H-6″), 7.61 (d, *J* = 7.6 Hz, 1H, H-6), 7.43 (dd, *J* = 7.8 and 6.7 Hz, 1H, H-4″), 7.32 (dd, *J* = 7.6 and 7.6 Hz, 2H, H-3″, H-5″), 5.76 (d, *J* = 3.4 Hz, 1H, H-1′), 5.69 (d, *J* = 7.8 Hz, 1H, H-5), 4.36 (d, *J* = 11.4 Hz, 1H, H-5a′), 4.26 (dd, *J* = 11.6 and 3.6 Hz, 1H, H-5b′), 4.17–4.13 (m, 3H, H-2′, H-3′, H-4′); ^{13}C NMR (100 MHz, D_2O) δ 166.2 (2 × CO), 144.2 (C-4), 141.3 (C-6), 136.3 (C-1″), 132.3 (C-4″), 128.5 (C-2″, C-3″, C-5″, C-6″), 96.4 (C-5), 89.9 (C-1′), 81.5 (C-4′), 74.1 (C-2′), 69.5 (C-3′), 68.1 (C-5′), MALDI-HRMS: Calcd for $C_{16}H_{19}N_4O_8S$ $[M+H]^+$ 427.0918, Found: 427.0912.



Scheme 5. Reagents and conditions: (a) DCC, DMAP, DMF, 48 h, rt; or (i) NHS, DCC, THF, 4 h, 0 °C; (ii) $CsCO_3$, DMF, 12–15 h, 0 °C–rt; (b) TFA, H_2O, MeOH, 2–4 h, rt; (c) NaOH (pH 11.5), 10 h, rt.

4.8. 5′-*O*-{*N*-[(4″-Acetylamino-phenyl)acetyl]sulfamoyl}cytidine (25b)

White solid, $[\alpha]_D^{20}$ +36.0 (*c* 0.3, H_2O); ^1H NMR (400 MHz, D_2O) δ 7.64 (d, *J* = 7.6 Hz, 1H, H-6), 7.39 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.28 (d, *J* = 8.1 Hz, 2H, Ar-H), 5.95 (d, *J* = 7.6 Hz, 1H, H-5), 5.83 (d, *J* = 2.1 Hz, 1H, H-1′), 4.51 (m, 2H, H-5ab′), 4.31 (m, 1H, H-4′), 4.19 (m, 2H, H-2′, H-3′), 3.65 (s, 2H, CH_2), 2.16 (s, 3H, Ac); ^{13}C NMR (100 MHz, D_2O) δ 172.8, 159.1, 148.4, 143.5, 136.4, 130.2, 130.0, 122.3, 121.5, 95.1, 89.7, 81.3, 73.8, 69.5, 68.8, 43.0, 23.0.

4.9. 5′-*O*-[*N*-(2-Nitrobenzoyl)sulfamoyl]cytidine (25c)

White solid, $[\alpha]_D^{20}$ +15.0 (*c* 0.04, H_2O); ^1H NMR (400 MHz, D_2O) δ 7.91 (dd, *J* = 8.2 and 1.0 Hz, 1H, H-6″), 7.74 (d, *J* = 7.6 Hz, 1H, H-6), 7.62 (ddd, *J* = 7.6, 7.4 and 1.2 Hz, H-4″), 7.49 (ddd, *J* = 8.2, 7.4 and 1.6 Hz, 1H, H-5″), 7.46 (dd, *J* = 7.6 and 1.2 Hz, 1H, H-3″), 5.86 (d, *J* = 7.6 Hz, 1H, H-5), 5.84 (d, *J* = 4.3 Hz, 1H, H-1′), 4.39 (dd, *J* = 11.5 and 2.1 Hz, 1H, H-5a′), 4.31 (dd, *J* = 11.5 and 2.7 Hz, 1H, H-5b′), 4.25–4.23 (m, 1H, H-4′), 4.21 (dd, *J* = 4.9 and 4.7 Hz, 1H, H-3′), 4.19 (dd, *J* = 4.9 and 4.5 Hz, 1H, H-2′); ^{13}C NMR (100 MHz, D_2O) δ 175.1 (CO), 165.4 (CO), 156.7 (C-4), 146.0 (C-6), 141.8 (C-2″), 134.5 (C-1″), 134.4 (C-3″), 130.6 (C-6″), 128.6 (C-5″), 24.4 (C-4″), 96.4 (C-5), 89.4 (C-1′), 81.8 (C-4′), 74.1 (C-2′), 69.6 (C-3′), 68.3 (C-5′); MALDI-HRMS: Calcd for $C_{16}H_{18}N_5O_{10}S$ $[M+H]^+$ 472.0768, Found: 472.0746.

4.10. 5′-*O*-[*N*-(2-Hydroxybenzoyl)sulfamoyl]cytidine (25d)

White solid, $[\alpha]_D^{20}$ +25.8 (*c* 0.15, H_2O). ^1H NMR (400 MHz, D_2O) δ 7.75 (d, *J* = 8.0 Hz, 1H, H-6), 7.69 (dd, *J* = 7.9 and 1.8 Hz, 1H, H-6″), 7.32 (ddd, *J* = 8.2, 7.2 and 1.8 Hz, 1H, H-4″), 6.81 (ddd, *J* = 7.9, 7.3 and 1.1 Hz, 1H, H-5″), 6.77 (dd, *J* = 8.2 and 1.1 Hz, 1H, H-3″), 5.85 (d, *J* = 8.0 Hz, 1H, H-5), 5.64 (d, *J* = 3.5 Hz, 1H, H-1′), 4.41 (dd, *J* = 11.9 and 1.9 Hz, 1H, H-5a′), 4.32 (dd, *J* = 11.7 and 3.9 Hz, 1H, H-5b′), 4.22 (dd, *J* = 5.1 and 3.7 Hz, 1H, H-2′), 4.18–4.14 (m, 2H, H-3′, H-4′); ^{13}C NMR (100 MHz, D_2O) δ 174.2, 159.1 (CO), 148.3 (C-4), 144.4 (C-6), 134.9 (C-1″), 130.1 (C-2″), 119.9 (C-3″), 118.2 (2C, C-6″, C-5″), 117.1 (C-3″), 94.9 (C-5), 91.1 (C-1′), 81.9 (C-4′), 73.7 (C-2′), 69.3 (C-3′), 68.8 (C-5′); MALDI-HRMS: Calcd for $C_{16}H_{18}N_4NaO_9S$ $[M+Na]^+$ 465.0686, Found: 465.0637.

4.11. 5′-*O*-[*N*-(3,5-Dimethoxybenzoyl)sulfamoyl]cytidine (25e)

Amorphous solid, $[\alpha]_D^{20}$ +6.0 (*c* 0.4, H_2O); ^1H NMR (400 MHz, D_2O) δ 7.90 (d, *J* = 7.6 Hz, 1H, H-6′), 7.08 (br s, 2H, Ar-H), 7.73 (br s, 1H, Ar-H), 6.00 (d, *J* = 7.6 Hz, 1H, H-5), 5.89 (d, *J* = 3.9 Hz, 1H, H-1′), 4.56 (dd, *J* = 2.0 and 12.1 Hz, 1H, H-5a′), 4.49 (dd, *J* = 4.2 and 12.1 Hz, 1H, H-5b′), 4.30 (m, 3H, H-2′, H-3′, H-4′), 3.87 (s, 6H, OMe); ^{13}C NMR (100 MHz, D_2O) δ 173.6, 160.1, 159.1, 148.6, 144.2, 138.0, 109.9, 109.3, 106.6, 104.4, 94.9, 90.7, 81.8, 73.7, 69.1, 68.7, 55.7, 55.6; MALDI-HRMS: Calcd for $C_{18}H_{23}N_4O_{10}S$ $[M+H]^+$ 487.1129, Found: 487.1152.

4.12. 5′-*O*-[*N*-(2,6-Dimethoxy-3-nitrobenzoyl)sulfamoyl] cytidine (25f)

Amorphous solid, $[\alpha]_D^{20}$ +20.0 (*c* 0.4, H_2O); ^1H NMR (400 MHz, D_2O) δ 8.32 (d, *J* = 9.4 Hz, 1H, Ar-H), 7.89 (d, *J* = 8.2 Hz, 1H, H-6), 7.11 (d, *J* = 9.4 Hz, 1H, Ar-H), 5.81 (br s, 1H, H-1′), 5.75 (d, *J* = 8.2 Hz, 1H, H-5), 4.88 (dd, *J* = 12.1 and 2.0 Hz, 1H, H-5a′), 4.67 (dd, *J* = 12.1 and 4.2 Hz, 1H, H-5b′), 4.46 (m, 1H, H-4′), 4.40 (m, 1H,

H-3′), 4.29 (m, 1H, H-2′), 3.94 (s, 3H, OMe), 3.89 (s, 3H, OMe); ^{13}C NMR (100 MHz, D_2O) δ 161.2, 157.0, 155.4, 148.1, 144.8, 139.4, 131.0, 126.2, 113.8, 103.7, 89.6, 87.0, 77.0, 69.4, 64.5, 59.6, 59.3, 52.5.

4.13. 5′-*O*-[*N*-(2-Carboxylatobenzoyl)sulfamoyl]cytidine sodium salt (25h)

Amorphous solid, $[\alpha]_D^{20}$ +14.2 (*c* 0.18, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.58 (d, *J* = 7.5 Hz, 1H, H-6), 7.48 (m, 2H, Ar-H), 7.35 (m, 2H, Ar-H), 5.92 (d, *J* = 7.6 Hz, 1H, H-5), 5.76 (d, *J* = 3.7 Hz, 1H, H-1′), 4.40 (dd, *J* = 11.5 and 2.2 Hz, 1H, H-5a′), 4.30 (dd, *J* = 11.5 and 3.7 Hz, 1H, H-5b′), 4.19–4.13 (m, 3H, H-2′, 3′, 4′); ^{13}C NMR (100 MHz, D_2O) δ 175.7 (2 × CO), 165.6 (CO), 156.7 (C-4), 141.7 (C-6), 135.6, 130.0, 128.2, 96.4 (C-5), 90.7 (C-1′), 80.9 (C-4′), 73.7 (C-2′), 69.2 (C-3′), 68.8 (C-5′); MALDI-HRMS: Calcd for $C_{17}H_{18}N_4NaO_{10}S$ $[M+H]^+$ 493.0636, Found: 493.0635.

4.14. 5′-*O*-[*N*-(3-Methoxycarbonylbenzoyl)sulfamoyl]cytidine (25i)

Amorphous solid, $[\alpha]_D^{20}$ +53.8 (*c* 0.07, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 8.29 (dd, *J* = 1.8 and 1.6 Hz, 1H, H-2″), 8.02 (ddd, *J* = 7.8, 1.6 and 1.4 Hz, 1H, H-4″), 7.97 (ddd, *J* = 7.8, 1.8 and 1.2 Hz, 1H, H-6″), 7.76 (d, *J* = 8.0 Hz, 1H, H-6), 7.45 (dd, *J* = 7.8 and 7.8 Hz, 1H, H-5″), 5.83 (d, *J* = 8.0 Hz, 1H, H-5), 5.65 (d, *J* = 3.6 Hz, 1H, H-1′), 4.45 (dd, *J* = 11.7 and 1.8 Hz, 1H, H-5a′), 4.33 (dd, *J* = 11.9 and 3.9 Hz, 1H, H-5b′), 4.21–4.13 (m, 3H, H-2′, 3′, 4′), 3.80 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, D_2O) δ 173.0, 168.6, 159.1, 148.3 (C-4), 144.3 (C-6), 135.9 (C-1″), 133.5 (C-3″), 133.2 (C-6′), 133.1 (C-4″), 129.4 (C-2″), 129.2 (C-5″), 94.9 (C-5), 90.7 (C-1′), 81.8 (C-4′), 73.8 (C-3′), 69.2 (C-2′), 68.8 (C-5′), 53.0 (OCH_3); MALDI-HRMS: Calcd for $C_{18}H_{21}N_4O_{10}S$ $[M+H]^+$ 485.0973, Found: 485.0935.

4.15. 5′-*O*-[*N*-(3-Carboxylatobenzoyl)sulfamoyl]cytidine sodium salt (25j)

Amorphous solid, ^{1}H NMR (400 MHz, D_2O) δ 8.20 (br s, 1H, H-2″), 7.88–7.85 (dd, *J* = 7.6 and 1.0 Hz, 2H, H-4″, H-6″), 7.60 (d, *J* = 7.6 Hz, 1H, H-6), 7.36 (dd, *J* = 7.8 and 7.8 Hz, 1H, H-5″), 5.75 (d, *J* = 3.7 Hz, 1H, H-1′), 5.68 (d, *J* = 6.1 Hz, 1H, H-5), 4.37 (dd, *J* = 11.7 and 2.0 Hz, 1H, H-5a′), 4.27 (dd, *J* = 11.7 and 3.7 Hz, 1H, H-5b′), 4.20–4.12 (m, 3H, H-2′, 3′, 4′); ^{13}C NMR (100 MHz, D_2O) δ 175.4 (2 × CO), 174.8 (CO), 165.7 (C-4), 157.1 (C-6), 141.4 (C-1″), 136.4 (C-3″), 132.4 (C-4″), 131.1 (C-6″), 128.8 (C-2″), 128.5 (C-5″), 96.4 (C-5), 90.0 (C-1′), 81.6 (C-4′), 74.1 (C-2′), 69.4 (C-3′), 68.1 (C-5′); MALDI-HRMS: Calcd for $C_{17}H_{18}N_4NaO_{10}S$ $[M+H]^+$ 493.0636, Found: 493.0691.

4.16. 5′-*O*-[*N*-(4-Methoxycarbonylbenzoyl)sulfamoyl]cytidine (25k)

White amorphous solid, $[\alpha]_D^{20}$ +60.0 (*c* 0.4, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.90 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.81 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.72 (d, *J* = 7.7 Hz, 1H, H-6), 5.80 (d, *J* = 7.7 Hz, 1H, H-5), 5.69 (d, *J* = 3.7 Hz, 1H, H-1′), 4.38 (dd, *J* = 11.8 and 1.8 Hz, 1H, H-5a′), 4.29 (dd, *J* = 11.8 and 3.7 Hz, 1H, H-5b′), 4.20–4.12 (m, 3H, H-2′, 3′, 4′), 3.80 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, D_2O) δ 174.6, 168.8, 161.0, 150.9 (C-4), 143.5 (C-6), 140.8 (C-1″), 132.4 (C-4″), 129.5 (C-6″, C-2″), 128.6 (C-3″, C-5″), 95.3 (C-5), 90.5 (C-1′), 81.9 (C-4′), 74.0 (C-2′), 69.3 (C-3′), 68.3 (C-5′), 52.9 (OCH_3); MALDI-HRMS: Calcd for $C_{18}H_{21}N_4O_{10}S$ $[M+H]^+$ 485.0973, Found: 485.0939.

4.17. 5′-*O*-[*N*-(4-Carboxylatobenzoyl)sulfamoyl]cytidine sodium salt (25l)

Amorphous solid, $[\alpha]_D^{20}$ +20.0 (*c* 0.08, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.76 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.71 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.56 (d, *J* = 7.5 Hz, 1H, H-6), 5.74 (d, *J* = 3.4 Hz, 1H, H-1′), 5.64 (d, *J* = 7.4 Hz, H-5), 4.36 (dd, *J* = 11.6 and 1.9 Hz, 1H, H-5a′), 4.28 (dd, *J* = 11.6 and 3.8 Hz, 1H, H-5b′), 4.19–4.11 (m, 3H, H-2′, 3′, 4′); ^{13}C NMR (100 MHz, D_2O) δ 175.3 (2 × CO), 165.5 (CO), 156.8 (C-4), 141.5 (C-6, C-4″), 138.6 (C-1″), 128.8, 128.4, 96.3 (C-5), 90.1 (C-1′), 81.5 (C-4′), 74.1 (C-2′), 69.5 (C-3′), 68.3 (C-5′); MALDI-HRMS: Calcd for $C_{17}H_{17}N_4Na_2O_{10}S$ $[M+Na]^+$ 515.0455, Found: 515.0435.

4.18. 5′-*O*-[*N*-(3-Methoxycarbonyl-5-nitrobenzoyl)sulfamoyl] cytidine (25m)

Amorphous solid, $[\alpha]_D^{20}$ +12.0 (*c* 0.01, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 8.75–8.73 (m, 2H), 8.64 (dd, *J* = 1.6 and 1.4 Hz, 1H), 7.87 (d, *J* = 8.0 Hz, 1H, H-6), 5.93 (d, *J* = 7.8 Hz, 1H, H-5), 5.71 (d, *J* = 3.3 Hz, 1H, H-1′), 4.41 (dd, *J* = 11.7 and 1.8 Hz, 1H, H-5a′), 4.31 (dd, *J* = 11.7 and 3.1 Hz, 1H, H-5b′), 4.21–4.18 (m, 3H, H-2′, 3′, 4′), 3.85 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, D_2O) δ 171.8 (2 × CO), 165.3 (CO), 162.4 (C-4), 148.2 (C-6), 143.9 (C-1′), 138.9 (C-5″), 135.0 (C-3″), 131.7 (C-2″), 127.6 (C-6″), 127.1 (C-4″), 95.1 (C-5), 90.4 (C-1′), 81.9 (C-4′), 74.0 (C-2′), 69.2 (C-3′), 68.2 (C-5′), 53.4 (OCH_3); MALDI-HRMS: Calcd for $C_{18}H_{20}N_5O_{12}S$ $[M+H]^+$ 530.0824, Found: 530.0793.

4.19. 5′-*O*-[*N*-(3-Carboxylato-5-nitrobenzoyl)sulfamoyl]cytidine sodium salt (25n)

Amorphous solid; ^{1}H NMR (400 MHz, D_2O) δ 8.66–8.65 (m, 2H), 8.56–8.54 (m, 1H), 7.77 (d, *J* = 8.0 Hz, 1H, H-6), 5.82 (d, *J* = 8.0 Hz, 1H, H-5), 5.69 (d, *J* = 3.4 Hz, 1H, H-1′), 4.41 (dd, *J* = 11.9 and 1.9 Hz, 1H, H-5a′), 4.32 (dd, *J* = 11.9 and 3.6 Hz, 1H, H-5b′), 4.20–4.15 (m, 3H, H-2′, 3′, 4′); ^{13}C NMR (100 MHz, D_2O) δ 172.5, 172.0, 161.8 (CO), 148.0 (C-6), 143.4 (C-4), 138.2, 135.1, 134.7, 126.7, 126.2, 125.7, 95.4 (C-5), 90.5 (C-1′), 81.8 (C-4′), 73.9 (C-2′), 69.3 (C-3′), 68.3 (C-5′); MALDI-HRMS: Calcd for $C_{17}H_{17}N_5NaO_{12}S$ $[M+H]^+$ 538.0487, Found: 538.0458.

4.20. 5′-*O*-{[*N*-(2-Methyl ethanoate)benzoyl]sulfamoyl}cytidine (25o)

Amorphous solid, $[\alpha]_D^{20}$ +37.3 (*c* 0.08, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.74 (d, *J* = 8.0 Hz, 1H, H-6), 7.51 (dd, *J* = 7.6 and 1.2 Hz, 1H, H-6″), 7.42 (ddd, *J* = 7.5, 7.4 and 1.4 Hz, 1H, H-4″), 7.30 (ddd, *J* = 7.6, 7.4 and 1.2 Hz, 1H, H-5″), 7.21 (dd, *J* = 7.1 and 0.4 Hz, 1H, H-3″), 5.94 (d, *J* = 8.0 Hz, 1H, H-5), 5.65 (d, *J* = 3.7 Hz, 1H, H-1′), 4.54 (dd, *J* = 11.8 and 2.0 Hz, 1H, H-5a′), 4.45 (dd, *J* = 11.8 and 3.1 Hz, H-5b′), 4.22–4.15 (m, 3H, H-2′, 3′, 4′), 3.78 (d, *J* = 2.4 Hz, 2H, CH_2), 3.53 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, D_2O) δ 174.7, 171.5, 159.1 (CO), 148.3 (C-4), 144.6 (C-6), 133.4 (C-1″), 133.2 (C-2″), 132.5 (C-6″), 132.4 (C-3″), 128.8 (C-4″), 128.0 (C-5”), 95.0 (C-5), 90.4 (C-1′), 81.2 (C-4′), 73.1 (C-3′), 70.1 (C-2′), 68.8 (C-5′), 52.7 (OCH_3), 39.0 (CH_2); MALDI-HRMS: Calcd for $C_{19}H_{23}N_4O_{10}S$ $[M+H]^+$ 499.1129, Found: 499.1099.

4.21. 5′-*O*-{[*N*-(2-Ethanoxylato)benzoyl]sulfamoyl}cytidine sodium salt (25p)

Amorphous solid, $[\alpha]_D^{20}$ +51.4 (*c* 0.04, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.69 (d, *J* = 7.6 Hz, 1H, H-6), 7.47 (dd, *J* = 7.6 and 1.2 Hz, 1H, H-6″), 7.30 (ddd, *J* = 7.6, 7.4 and 1.4 Hz, 1H, H-4″), 7.19 (ddd, *J* = 7.6, 7.4 and 1.2 Hz, 1H, H-5″), 7.13 (d, *J* = 7.6 Hz, 1H, H-3″), 5.82–5.80 (m, 2H, H-1′, 5), 4.35 (dd, *J* = 11.7 and 2.1 Hz, 1H, H-5a′), 4.28 (dd, *J* = 11.7 and 3.3 Hz, 1H, H-5b′), 4.22–4.14 (m, 3H, H-2′, 3′, 4′), 3.68 (d, *J* = 8.8 Hz, 2H, CH_2); ^{13}C NMR (100 MHz, D_2O) δ 173.8, 170.7, 165.6, 156.9 (C-4), 142.1 (C-6), 141.7 (C-1″), 131.7 (C-2″), 131.2 (C-5″), 130.9 (C-3″), 130.5 (C-4″), 128.9 (C-6″), 96.3 (C-5), 90.6 (C-1′), 80.9 (C-4′), 73.7 (C-2′), 69.2 (C-3′), 68.8 (C-5′),

41.7 (CH_2); MALDI-HRMS: Calcd for $C_{18}H_{20}N_4NaO_{10}S$ $[M+H]^+$ 507.0792, Found: 507.0818.

4.22. 5′-*O*-[*N*-(2″-Oxo-2*H*-chromene-3″-carbonyl)sulfamoyl] cytidine (25q)

Amorphous solid, $[\alpha]_D^{20}$ +17.0 (*c* 0.4, H_2O); ^1H NMR (400 MHz, D_2O) δ 8.45 (s, 1H, Ar), 7.96 (d, *J* = 7.6 Hz, 1H, H-6), 7.76 (m, 2H, Ar), 7.45 (m, 2H, Ar), 6.10 (d, *J* = 7.6 Hz, 1H, H-5), 5.89 (d, *J* = 2.1 Hz, 1H, H-1′), 4.49 (m, 2H, H-5ab′), 4.36 (br s, 1H, H-4′), 4.31 (m, 2H, H-2′, 3′); ^{13}C NMR (100 MHz, D_2O) δ 172.8, 159.1, 151.9, 147.0, 143.8, 134.3, 129.7, 125.6, 123.2, 118.3, 116.5, 95.2, 90.3, 81.8, 73.9, 69.2, 68.6; MALDI-HRMS: Calcd for $C_{19}H_{19}N_4O_{10}S$ $[M+H]^+$ 495.0816, Found: 495.0836.

4.23. 5′-*O*-[*N*-(1*H*-Benzotriazole-5″-carbonyl)sulfamoyl]cytidine (25r)

Amorphous solid, $[\alpha]_D^{20}$ +26.0 (*c* 0.3, H_2O); ^1H NMR (400 MHz, D_2O) δ 8.47 (s, 1H, Ar), 8.03 (d, *J* = 8.2 Hz, 1H, H-6), 7.89 (m, 2H, Ar), 5.89 (d, *J* = 7.6 Hz, 1H, H-5), 5.81 (d, *J* = 3.9 Hz, 1H, H-1′), 4.55 (dd, *J* = 2.0, and 12.1 Hz, 1H, H-5a′), 4.47 (dd, *J* = 4.2 and 12.1 Hz, 1H, H-5b′), 4.32 (m, 3H, H-2′, 3′, 4′); ^{13}C NMR (100 MHz, D_2O) δ 174.3, 159.5, 149.1, 143.9, 134.4, 126.8, 125.7, 116.8, 116.3, 113.9, 94.8, 90.6, 81.9, 73.9, 69.2, 68.4; MALDI-HRMS: Calcd for $C_{16}H_{17}N_7NaO_8S$ $[M+Na]^+$ 490.0757, Found: 490.0779.

4.24. Synthesis of compounds (24a–e)

The coupled products (**23a–e**) were dissolved in CH_2CH_2 (5 ml) and treated with aqueous TFA (90%, 2 mL). The mixture was stirred for 2 h after which solvents were removed and the residue was purified by silica gel column chromatography to give the target compounds **24a–e**.

Compound **24a**: Amorphous solid, $[\alpha]_D^{20}$ −17 (*c* 0.5, H_2O): ^1H NMR (400 MHz, D_2O) δ 7.64 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 6.15 (d, 1H, H-5‴), 5.89 (d, $J_{3,4}$ = 2.4, 1H, H-3), 5.81 (d, $J_{1'',2''}$ = 4.4 Hz, 1H, H-1″), 4.52 (dd, $J_{4,5}$ = 8.8 Hz, 1H, H-4), 4.36 (d, $J_{6,5}$ = 10.8 Hz, 1H, H-6), 4.32 (dd, $J_{2'',3''}$ = 4.4 Hz, 1H, H-2″), 4.13 (m, 3H, H-3″, 4″, 5″), 4.05 (d, *J* = 6.4 Hz, 2H, 1′-CH_2), 3.95 (m, 1H, H-8), 3.88 (dd, $J_{9,8}$ = 2.8, $J_{9a,9b}$ = 16.0 Hz, 1H, H-9a), 3.67 (m, 2H, H-9b, 7), 3.61 (m, 2H, H-5′a, 5′b), 2.12 (s, 3H, NAc); ^{13}C NMR (100 MHz, D_2O) δ 178.0, 174.9, 173.4, 166.3, 163.5, 157.7, 145.3, 142.0 (C-6‴), 109.0 (C-3), 96.3 (C-5‴), 91.0 (C-1″), 81.7 (C-3″), 76.5 (C-6), 73.5 (C-2″), 70.6 (C-4″), 69.9 (C-8), 67.9 (C-7), 67.1 (C-4), 63.1 (C-9), 49.9 (C-5), 42.7 (C-1′), 40.7 (C-5″), 22.2 (NAc); MALDI-HRMS: Calcd for $C_{22}H_{32}N_6O_{12}Na$ $[M+Na]^+$ 595.1970, Found: 595.1960.

Compound **24b:** Amorphous solid, $[\alpha]_D$ −31 (*c* 0.5, H_2O); ^1H NMR (400 MHz, D_2O) δ 7.87 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 6.21 (d, 1H, H-5‴), 5.84 (d, $J_{3,4}$ = 2.4, 1H, H-3), 5.78 (d, $J_{1'',2''}$ = 4.4 Hz, 1H, H-1″), 4.54 (dd, $J_{4,5}$ = 8.8 Hz, 1H, H-4), 4.36 (m, 3H, H-2″, H-6, H-1′), 4.16 (m, 3H, H-3″, 4″, 5″), 3.91 (m, 2H, H-8, H-9a), 3.68 (m, 4H, H-7, 9b, 5′a, 5′b), 2.98 (t, 2H, 2 × H-5′), 2.08 (s, 3H, NAc), 1.85 (m, 2H, 2 × H-2′), 1.69 (m, 2H, 2 × H-4′), 1.45 (m, 2H, 2 × H-3′); ^{13}C NMR (100 MHz, D_2O) δ 175.0, 174.0, 166.3, 164.2, 145.4, 144.6 (C-6‴), 109.0 (C-3), 95.1, 91.5, 82.3, 76.5, 73.5, 70.6, 70.1, 67.7, 67.1, 63.1, 61.8, 53.8, 49.9, 40.8, 39.3, 30.7, 26.4, 22.2, 22.1, 13.3.

Compound **24c**: Amorphous solid; ^1H NMR (400 MHz, D_2O) δ 7.82 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 6.20 (d, 1H, H-5‴), 5.88 (d, $J_{3,4}$ = 2.4, 1H, H-3), 5.79 (d, $J_{1'',2''}$ = 4.4 Hz, 1H, H-1″), 4.76 (m, 1H, H-1′), 4.54 (dd, $J_{4,5}$ = 8.8 Hz, 1H, H-4), 4.35 (m, 2H, H-4, 6), 4.13 (m, 3H, H-3″, 4″, 5), 3.91 (m, 2H, H-8, 9a), 3.67 (m, 2H, H-7, 9b), 3.62 (m, 2H, H-5″a, 5″b), 2.88 (d, 2H, H-2′a, 2′b), 2.08 (s, 3H, NAc). ^{13}C NMR (100 MHz, D_2O) δ 175.8, 174.9, 173.1, 166.3, 160.8, 150.4, 145.2, 144.1 (C-6‴), 109.0 (C-3), 95.4 (C-5‴), 91.1 (C-1″), 82.3 (C-3″), 76.5 (C-6), 73.5 (C-2″), 70.3 (C-4″), 70.0 (C-8), 67.8 (C-7), 67.1 (C-4), 63.2 (C-9), 50.6 (C-1′), 49.9 (C-5), 42.5 (C-5″), 36.6 (C-2′), 22.2 (–NAc).

Compound **24d:** Amorphous solid, $[\alpha]_D^{20}$ +9 (*c* 0.3, H_2O); ^1H NMR (400 MHz, D_2O) δ 7.60 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 6.02 (d, 1H, H-5‴), 5.85 (d, $J_{3,4}$ = 2.4, 1H, H-3), 5.81 (d, $J_{1'',2''}$ = 4.4 Hz, 1H, H-1″), 4.52 (dd, $J_{4,5}$ = 8.8 Hz, 1H, H-4), 4.44 (dd, 1H, H-1′), 4.34 (m, 2H, H-2″, 6), 4.13 (m, 3H, H-3″, 4″, 5), 3.97 (m, 1H, H-8), 3.89 (dd, $J_{9,8}$ = 2.8, $J_{9a,9b}$ = 16.0 Hz, 1H, H-9a), 3.69 (m, 2H, H-9b, 7), 3.60 (m, 2H, H-5″a, 5″b), 2.38 (m, 2H, 2 × H-3′), 2.09 (s, 3H, NAc), 2.10 (m, 2H, H-2′), ^{13}C NMR (100 MHz, D_2O) δ 178.0, 174.9, 173.4, 166.3, 163.5, 157.6, 145.3, 142.0 (C-6‴), 109.0 (C-3), 96.3 (C-5‴), 91.0 (C-1″), 81.7 (C-3″), 76.5 (C-6), 73.5 (C-2″), 70.7 (C-4″), 69.9 (C-8), 67.9 (C-7), 67.1 (C-4), 63.1 (C-9), 53.6 (C-1′), 49.9 (C-5), 40.7 (C-5″), 31.1 (C-3‴), 26.8 (C-2‴), 22.2 (–NAc); MALDI-HRMS: Calcd for $C_{25}H_{38}N_7O_{13}$ $[M+H]^+$ 644.2522, Found: 644.2491.

Compound **24e:** Amorphous solid, $[\alpha]_D^{20}$ +9 (*c* 0.4, H_2O); ^1H NMR (400 MHz, D_2O) δ 7.61, 7.48, 7.32, 7.24, 7.17 (tryptophan), 7.30 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 5.86 (d, 1H, H-5‴), 5.86 (d, $J_{3,4}$ = 2.4, 1H, H-3), 5.59 (d, $J_{1'',2''}$ = 4.4 Hz, 1H, H-1″), 4.76 (t, 1H, H-1′), 4.50 (dd, $J_{4,5}$ = 8.8 Hz, 1H, H-4), 4.30 (d, 1H, H-6), 4.10 (m, 1H, H-5), 3.95 (m, 3H, H-2″, 3″, 4″), 3.87 (m, 1H, H-9a), 3.68 (m, 3H, H-7, 8, 9b), 3.52 (dd, 1H, H-5″a), 3.40 (dd, 1H, H-5″b), 3.34 (d, 2H, 2 × H-2′), 2.10 (s, 3H, NAc); ^{13}C NMR (100 MHz, D_2O) δ 175.8, 174.9, 173.1, 166.3, 160.8, 150.4, 145.2, 144.1 (C-6‴), 109.0 (C-3), 95.4 (C-5‴), 91.1 (C-1″), 82.3 (C-3″), 76.5 (C-6), 73.5 (C-2″), 70.3 (C-4″), 70.0 (C-8), 67.8 (C-7), 67.1 (C-4), 63.2 (C-9), 50.6 (C-1′), 49.9 (C-5), 42.5 (C-5″), 36.6 (C-2′), 22.2 (NAc).

4.25. Synthesis of compound (21)

To a solution of **16** (212 mg, 0.64 mmol) and **20** (303 mg, 0.83 mmol) in anhydrous DMF were added WSC (1 mmol) and HOBt (1 mmol) at 0 °C. The reaction mixture stirred under nitrogen for overnight. The solvent was removed and residue was purified using silica gel column chromatography. The resulted product was dissolved in CH_2CH_2 (5 ml) and treated with aqueous TFA (90%, 2 ml). The mixture was stirred for 2 h after which solvents were removed and the residue was purified by silica gel column chromatography to give **21** (227 mg, 59%). $[\alpha]_D^{20}$ +32 (*c* 0.5, H_2O); ^1H NMR (400 MHz, D_2O) δ 7.85 (d, $J_{6'',5''}$ = 7.6 Hz, 1H, H-6″), 6.08 (d, $J_{6'',5''}$ = 7.6 Hz, 1H, H-5″), 5.95 (d, $J_{3,4}$ = 3.6 Hz, 1H, H-3), 5.84 (d, $J_{1',2'}$ = 2.4 Hz, 1H, H-1′), 4.49 (m, 1H, H-4′), 4.46 (m, 1H, H-2′), 4.39 (m, 1H, H-3′), 4.29 (m, 4H, H-4, 5, 6, 5a′), 4.07 (dd, $J_{5b',5a'}$ = 11.2, $J_{5b',4'}$ = 8.8 Hz, 1H, H-5b′), 3.94 (ddd, $J_{8,7}$ = 3.9, $J_{8,9a}$ = 3.0, $J_{8,9b}$ = 13.6 Hz, 1H, H-8), 3.89 (dd, $J_{9a,9b}$ = 12 Hz, 1H, H-9a), 3.49 (m, 2H, H-7, 9b) 2.08 (s, 3H, NAc); ^{13}C NMR (100 MHz, D_2O) δ 174.8 (NAc), 169.8 (C-1), 166.1 (C-4‴), 157.3 (C-2), 141.5 (C-6‴), 108.7 (C-3), 96.4 (C-5‴), 89.8 (C-1′), 81.4 (C-4′), 75.7 (C-6), 74.1, 69.9 (C-8), 69.3 (C-2′), 68.2 (C-4), 67.8 (C-7), 63.1 (C-9), 54.5 (C-5), 22.2 (NAc); MALDI-HRMS: Calcd for $C_{20}H_{30}N_5O_{14}S$ $[M+H]^+$ 596.1505, Found: 596.1514.

4.26. Synthesis of *N*-acetyl-2′,3′-isopropylidine-5′-*O*-sulfamoylcytidine (20)

To a solution of *N*-acetyl-2′,3′-isopropylidinecytidine (5.0 g, 1.0 equiv) in DME (10 ml) at 0 °C was added sulfamoyl chloride (4.4 g, 2.5 equiv) over 15 min under nitrogen atmosphere. The reaction mixture was warmed to room temperature and stirred for 16 h, and was then diluted with ice-cold brine solution and extracted with EtOAc. The organic layer was dried with anhydrous Na_2SO_4 and concentrated. Purification by silica gel chromatography (EtOAc/methanol, 15:1) gave desired product as a white solid (4.7 g, 76% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.04 (d, *J* = 7.4 Hz, 1H, H-6), 7.37 (d, *J* = 7.4 Hz, 1H, H-5), 5.83 (d, *J* = 1.8 Hz, 1H,

H-1′), 5.01 (dd, J = 6.4 and 1.8 Hz, 1H, H-2′), 4.87 (dd, J = 6.3 and 3.5 Hz, 1H, H-3′), 4.79 (br s, 2H, NH_2), 4.46–4.43 (m, 1H, H-4′), 4.36 (dd, J = 10.7 and 3.9 Hz, 1H, H-5′a), 4.29 (dd, J = 10.7 and 5.5 Hz, 1H, H-5′b), 2.15 (s, 3H, $NHCOCH_3$), 1.52 (s, 3H, CH_3), 1.32 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 165.5 (CO), 157.9 (C-4), 150.6 (C-6), 114.8 (CMe_2), 98.3 (C-5), 97.4 (C-1′), 87.3 (C-4′), 86.6 (C-2′), 82.8 (C-3′), 70.2 (C-5′), 27.5 (CH_3), 25.5 (CH_3), 24.7 ($NHCOCH_3$); MALDI-HRMS: Calcd for $C_{14}H_{20}N_4NaO_8S$ $[M+Na]^+$ 427.0900, Found: 427.0912.

4.27. Synthesis of compound (19)

A solution of aqueous trifluoroacetic acid (90%, 2 mL) was added drop wise to a solution of the compound **18** (190 mg, 0.264 mmol) in CH_2Cl_2 at 0 °C, and the mixture was stirred for 1 h at rt. Then, solvents were removed under reduced pressure and co-evaporated with toluene. The residue was purified by column chromatography to give **19** (86 mg, 58%). $[\alpha]_D^{20}$ +11 (c 0.5, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.96 (s, 1H, H-1′), 7.10 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 5.94 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-5‴), 5.84 (d, $J_{3,4}$ = 3.1 Hz, 1H, H-3), 5.79 (d, $J_{1'',2''}$ = 2.5 Hz, 1H, H-1″), 4.88 (d, $J_{5a'',5b''}$=15.2, $J_{5a'',4''}$ = 3.2 Hz, 1H, H-5a″), 4.78 (m, 1H, H-5b″), 4.58 (dd, 1H, H-1′), 4.51 (dd, $J_{2'',3''}$ = 8.9 Hz, 1H, H-2″), 4.38 (m, 1H, H-4′), 4.33 (d, $J_{6,5}$ = 10.5 Hz, 1H, H-6), 4.22 (dd, $J_{4,5}$ = 5.6 Hz, 1H, H-4), 4.12 (m, 2H, H-3″, 5), 3.91 (ddd, $J_{8,7}$ = 4.0, $J_{8,9a}$ = 3.2, $J_{8,9b}$= 13.6 Hz, 1H, H-8), 3.87 (d, 1H, H-9a), 3.67 (m, 2H, H-9b, 7) 2.10 (s, 3H, NAc); ^{13}C NMR (100 MHz, D_2O) δ 174.9 (CO), 166.2 (C-1), 164.9 (CO), 157.4 (C-2), 145.9 (C4‴), 144.6 (C-2′), 141.6 (C-6‴), 125.3 (C-1′), 108.6 (C-3), 96.2 (C-5‴), 91.4 (C-1″), 80.5 (C-4″), 76.4 (C-6), 73.1, 70.0 (C-8), 69.8 (C-2″), 67.8 (C-4), 67.1 (C-7), 63.1 (C-9), 50.7 (C-5″), 49.9 (C-5), 34.5 (C-3′), 22.2 (NAc); MALDI-HRMS: Calcd for $C_{23}H_{32}N_8O_{11}Na$ $[M+Na]^+$ 619.2083, Found: 619.2043.

4.28. Synthesis of compounds 12 and 13

A solution of aqueous trifluoroacetic acid (90%, 2 mL) was added dropwise to a solution of the compound **10** or **11** in CH_2Cl_2 at 0 °C, and the mixture was stirred for 2 h at rt. Then, solvents were removed under reduced pressure and co-evaporated with toluene. The residue was re-dissolved in methanol (2 ml) and treated with 1 M NaOH solution at 0 °C for 12 h. The reaction solution was adjusted to pH 2 with Amberlite 120 (H^+), the solution was filtered to remove the resin, and the filtrate was concentrated in vacuo. The residue was loaded onto a column of Bio-gel P-2 (2 × 100 cm) and eluted with water. The fractions containing the product were pooled and lyophilized to afford **12** and **13**.

Compound 12: Yield 53%,$[\alpha]_D^{20}$ +8 (c 0.2, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 8.68 (s, 1H, H-1′), 7.84 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 6.03 (d, $J_{5''',6'''}$ = 7.6 Hz, 1H, H-5‴), 5.81 (d, $J_{1'',2''}$ = 3.2 Hz, 1H, H-1″), 4.41 (d, $J_{2'',3''}$ = 5.6 Hz, 1H, H-2″), 4.27 (m, 1H, H-4″), 4.24 (dd, $J_{3'',4''}$ = 4.0 Hz, 1H, H-3″), 3.96 (m, 2H, H-4, 5), 3.92 (m, 4H, H-5″a, 5″b, 8, 9a), 3.66 (m, 2H, H-7, 9b), 3.37 (dd, $J_{3a,3b}$ = 11.5, $J_{3a,4}$ = 4.0 Hz, 1H, H-3a), 2.27 (dd, $J_{3b,4}$ = 10.5, 1H, H-3b), 2.14 (s, 3H, NAc); ^{13}C NMR (100 MHz, D_2O) δ 175.2 (C-1), 170.5 (NAc), 162.5 (C-4′), 160.5 (C-2‴), 144.3 (C-4‴), 140.5 (C-2′), 124.8 (C-1′), 95.1 (C-5‴), 91.8 (C-1″), 91.3 (C-2), 82.3 (C-4″), 74.4 (C-6), 73.5 (C-3″), 71.4 (C-8), 70.2 (C-2″), 68.2 (C-4), 68.0 (C-7), 62.9 (C-9), 51.6 (C-5), 39.6 (C-3), 39.7 (C-3′), 22.2 (NAc); MALDI-HRMS: Calcd for $C_{23}H_{32}N_8O_{13}Na$ $[M+Na]^+$ 651.1987, Found: 651.1970.

Compound 13: Yield 48%, $[\alpha]_D^{20}$ +23 (c 0.3, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 8.17 (s, 1H, H-1′), 8.02 (d, $J_{6''',5'''}$ = 7.6 Hz, 1H, H-6‴), 6.13 (d, $J_{5''',6'''}$ = 7.6 Hz, 1H, H-5‴), 5.95 (d, $J_{1'',2''}$ = 4.4 Hz, 1H, H-1″), 4.68 (d, J = 16 Hz, 1H, H-3a′), 4.58 (d, $J_{3'',2''}$ = 4.4 Hz, 1H, H-3″), 4.47 (m, 3H, H-2″, 4″, 3′), 3.98 (m, 3H, H-4, 5, 6), 3.89 (m, 2H, H-8, 9a), 3.66 (m, 2H, H-7, 9b), 3.29 (dd, $J_{3a,3b}$ = 11.5, $J_{3a,4}$ = 4.0 Hz, 1H, H-3a), 2.26 (dd, $J_{3b,4}$ = 10.5, 1H, H-3b), 2.07 (s, 3H, NAc); ^{13}C NMR (100 MHz, D_2O) δ 175.1 (C-1), 171.8 (NAc), 170.4 (C-5″), 162.9 (C-2‴), 152.3 (C-4‴), 144.2 (C-2′), 124.8 (C-1′), 95.1 (C-5‴), 91.7 (C-1″), 91.2 (C-2), 82.2 (C-4″), 74.3 (C-6), 73.5 (C-3″), 71.4 (C-8), 70.1 (C-2″), 68.2 (C-4), 68.0 (C-7), 62.8 (C-9), 51.6 (C-5), 39.6 (C-3), 34.1 (C-3′), 22.2 (NAc); MALDI-HRMS: Calcd for $C_{23}H_{32}N_8O_{13}Na$ $[M+Na]^+$ 651.1987, Found: 651.1969.

4.29. Molecular docking experiment of compound 13 into the sialyltransferase Cst-I using Autodock4

Coordinates of the α-2,3-sialyltransferase Cst-I were extracted from the X-ray crystal structure of the CMP-3FNeuAc (CSF) complex obtained from the Protein Data Bank (2P2V). All 'hetero' atoms including those belonging to CSF ligand, waters, chloride ions, and ethylene glycol were removed. Hydrogen atoms were added automatically by the Biopolymer module of InsightII (Accelrys, San Diego, 1995).

Using the same InsightII package, the inhibitor structure was built with the Biopolymer module. It was then subjected to a 300-step energy minimization with a quasi-Newton BFGS method using the DISCOVER-3 program (CFF91 forcefield) to alleviate bad atomic contacts.

An AUTODOCK Tool (ADT) program—a graphical interface of Autodock, was used to process the ligands and the receptor for the docking. Atomic charges using the Gasteiger PEOE and Kollman methods were added to the ligand and the receptor, respectively, then non-polar hydrogens were merged. Atom types of the ligand molecule and atomic solvation parameters for the protein were assigned automatically using ADT. The ligand was initially put at the binding location of the CSF ligand by spatially overlaying the corresponding cytidine ring. For each atom type, a grid map centered on the ligand of 61 × 61 × 61 points with a spacing of 0.375 Å was pre-calculated using AUTOGRID4 program as well as an electrostatic potential map and a desolvation map with the same spatial settings were also pre-calculated.

For data production, a set of 100 docking experiments was performed with a modified AUTODOCK4 program on a Linux computer using a Lamarckian Genetic Algorithm (LGA) method. The original AUTODOCK 4 program was modified to remove the overall molecular rotation of the ligand during docking of the ligand to the receptor (H. Jarrell, personal communication). A population of 250 random individuals and a maximum of 10,000,000 energy evaluations or 27,000 generations were used in each LGA docking run where a pseudo-Solis and Wets local search method having a maximum of 300 iterations was also used.

The docking experiment allowed the ligand's tortions to be varied as well molecular translation of the ligand. The docked structures were clustered with a tolerance of 2 Å and ranked based on their estimated free energy of binding.

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