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Exploring and Exploiting Acceptor Preferences of the Human Polysialyltransferases as a Basis for an Inhibitor Screen

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Abstract

α2,8-linked polysialic acid (polySia) is an oncofetal antigen with high abundance during embryonic development and reappearance in malignant tumors of neuroendocrine origin. Responsible for polySia biosynthesis are the two polysialyltransferases (polySTs) ST8SiaII & IV. During development, both enzymes are essential to control polySia expression. However, in the tumor situation ST8SiaII is the prevalent enzyme. Consequently, ST8SiaII is an attractive target for the development of novel cancer therapeutics. A major challenge thereby is the high structural and functional conservation of ST8SiaII & IV. To search for specific inhibitors an assay system that enables differential testing of ST8SiaII & IV would be of high value. Here we exploited the different modes of acceptor recognition and elongation for this purpose. With DMB-DP3 and DMB-DP12 (fluorescently labeled sialic acid oligomers with a degree of polymerization of 3 and 12, respectively) we identified stark differences between the two enzymes. The new acceptors enabled the simple comparative testing of the polyST initial transfer rate for a series of CMP-activated and *N*-substituted sialic acid derivatives. Of these derivatives, the non-transferable CMP-Neu5Cyclo was found to be a new, competitive ST8SiaII inhibitor.

Introduction

In mammals, the term polysialic acid (polySia) is used to describe a homopolymer of the negatively charged nine-carbon sugar sialic acid (Sia), each monomer unit conjugated via an α2,8-glycosidic linkage [1]. PolySia is primarily observed as a posttranslational modification of the neural cell adhesion molecule (NCAM) and has, in numerous studies, been demonstrated to regulate cell-cell distances and cellular plasticity by physically increasing the intercellular space (for review see [2]). This function as a mediator of cellular plasticity explains the abundance of polySia-NCAM during mammalian development [3-5]. PolySia is gradually reduced during tissue maturation [6;7] and becomes virtually undetectable in peripheral adult tissues [7]. However, as an oncofetal antigen, polySia reappears in a series of different tumors (for a review see [8]) and the level

of polySia expression in tumors has been demonstrated to negatively correlate with patient prognosis [9;10]. Similar to its function during development, polySia is likely to support neoplasia by destabilizing cell-cell contacts and promoting cellular motility [11-13]. In addition, camouflage with the non-immunogenic polySia helps tumor cells to escape immune surveillance mechanisms [14].

The virtual absence of polySia on adult peripheral tissues and its re-appearance on cancer cells makes the glycan an attractive target for therapeutic approaches and different strategies to realize this goal are currently under investigation [8]. For example, polySia-specific endosialidases have been applied to remove the protecting polySia shell from the tumor [15]. Alternatively, metabolic labelling strategies have been developed, in which the cellular sialylation pathway is hijacked to integrate non-natural Sia-derivatives into polySia chains with the intention to convert polySia into an immunogenic structure (for an extensive review see [16]). In order to specifically target polysialylation pathways, and avoid sialylation pathways which are active in a variety of healthy adult cell types, specific polysialyltransferase inhibitors are required [8]. Of high importance in the search for inhibitors, as well as compounds with which polySia can be metabolically labeled, is a sensitive method that enables the detailed functional evaluation of the mammalian polySTs.

Mammals encode two polySTs (ST8SiaII and ST8SiaIV) [17]. The enzymes share 59% identity at the amino acid sequence level and each has been demonstrated to independently synthesize polySia on NCAM *in vitro* and *in vivo* [18;19]. However, analyses of animal models with different allelic combinations of polySTs demonstrated significant differences in the catalytic functions of these enzymes. Three findings are of particular interest: i) ST8SiaII is the prime enzyme of polySia synthesis during development, it is essential to guarantee exhaustive polysialylation of the NCAM pool [7;20;21]; ii) ST8SiaIV is the major enzyme in the adult brain and the polyST-form expressed in the scattered peripheral tissues that retain dynamic polySia expression in the adult [22-24]; iii) ST8SiaII is the predominant enzyme in tumors [25] and thus the major target for drug design. Consequently, we are searching for test systems that would allow identification of the differential effects of inhibitors on ST8SiaII and ST8SiaIV activity. Recently, we introduced the fluorescent acceptor DMB-DP3 (a 1,2-Diamino-4,5-methylendioxy-benzene-labelled trimer of α2,8-linked sialic acid) as a general acceptor for the sensitive and in depth kinetic testing of polySTs [8;26]. Of note, this test system impressively confirmed data obtained in mouse studies by demonstrating that ST8SiaII has a much higher capacity to initiate polySia chains than ST8SiaIV, the latter preferentially elongating primers of a size of >DP10 [26].

Here we make use of the differential acceptor length preferences of human polySTs to advance the DMB-acceptor based polyST test system. Using DMB-labelled oligo-Sia acceptors of different lengths, we have evaluated the activity of human polySTs towards CMP-activated and *N*-substituted Sia-derivatives. We discovered an inhibitor for ST8SiaII that could serve as a lead structure for the development of further improved inhibitors. These compounds are of interest as potential cancer therapeutics.

Experimental Procedures

Reagents

Chemicals were obtained from Sigma-Aldrich (Poole, UK), Sigma-Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany) unless otherwise stated. The CMP-activated Neu5Ac derivatives *N*-propanoyl neuraminic acid (Neu5Prop), *N*-butanoyl neuraminic acid (Neu5But), *N*-pentanoyl neuraminic acid (Neu5Pent) and *N*-cyclopropylcarbonyl neuraminic acid (Neu5Cyclo) were synthesized as previously described [27]. Human ST8SiaII and ST8SiaIV

were purchased from R&D systems (Minneapolis, USA).

DMB labelling and purification of DMB-labelled oligosaccharides

In this study, DMB-DP3 and DMB-DP12 were used to prime polyST reactions. The compounds were synthesized and isolated according to published protocols [26;28-31]. In brief: 25 mg DP3 (trimer of α2,8-linked Sia) or 100 mg colominic acid (α2,8-linked polySia from bacterial source) were dissolved at 10 mg/ml in 20 mM DMB with 1 M β-mercaptoethanol and 40 mM sodium dithionite. Solutions were then mixed with equal volumes of ice-cold 40 mM trifluoracetic acid (TFA) and incubated for 48 h at 4 °C. Reactions were terminated by the addition of one fifth reaction volume of 200 mM sodium hydroxide. Lactonisation of oligo/polySia was reversed by a second incubation step at pH 10.0 and 4 °C for 48 h. For the purification of DMB-DP3 and DMB-labelled oligo/polySia fragments prepared by this procedure from colominc acid, we used anion exchange chromatography (MonoQ 10/100 column, Amersham Bioscience, Amersham, UK). 50 mM trishydroxymethylaminomethane (Tris)/HCl buffer at pH 8.0 without (M1) or with 1 M sodium chloride (M2) was used as the mobile phase. The flow rate was set to 4 ml / min. After 12 ml of washing with buffer M1, the DMB-labelled compounds were eluted with a linear sodium chloride gradient as follows: From 0 to 8 % M2 over 12 ml, from 8 to 20 % M2 over 56 ml, from 20 to 45 % M2 over 208 ml. 4 ml fractions were collected. Fractions containing DMB-DP3 and DMB-DP12, respectively, were pooled, desalted via a 2,000 MWCO membrane (Satorius, Göttingen, Germany) and concentrated. If single DPs were required, isolated pools were re-chromatographed using high performance liquid chromatography (HPLC; UFLC-RX system, Shimadzu, Kyoto, Japan) with a fluorescence detector (FD; RF10A XL) and a CarboPac PA-100 4x250 mm column (Dionex, Sunnyvale, USA). Elutions were detected with an excitation wavelength of 374 nm and emission at 456 nm. Buffers used were 20 mM sodium nitrate (M3) and 1 M sodium nitrate (M4). Elution was achieved with a curved gradient from 0 % to 26 % M4 for 4.2 ml followed by a linear gradient from 26 % to 50 % for 9 ml. The flow rate was 0.6 ml/min as described [26]. Desalting and concentration of samples was as described above. The chain length of individual fractions was confirmed by the retention time, as described in Keys et al. 2014 [30].

***In vitro* activity testing of polySTs**

Activity testing of human polySTs with different donors and acceptors was carried out in test buffer (10 mM cacodylate HCl pH 6.7, 20 mM $MgCl_2$, 10 mM $MnCl_2$, 50 mM KCl and 5 % glycerol). The acceptors (DMB-DP3 and DMB-DP12) were used at a final concentration of 2.5 µM and CMP-Neu5Ac or derivatives thereof at a final concentration of 500 µM. ST8SiaII was utilized at a concentration of 12.5 µg/ml and ST8SiaIV at 25 µg/ml. Reactions were incubated at 25°C and stopped at distinct time points by the addition of four volumes of stop buffer (80 mM Tris/HCl pH8 containing 16 mM EDTA) and incubation at 50 °C for 10 min. Before HPLC-FD analysis, samples were centrifuged for 10 min at 20,000 *g* and 4 °C. Supernatants were collected and separated via anion exchange chromatography using a 4 x 250 mm DNAPac PA 100 column (Dionex, Sunnyvale, USA) with online UV and fluorescence detection. Results were analyzed with empower 2 software (Waters, Manchester, UK). Reaction products were separated at a flow rate of 1.2 ml/min using different ammonium acetate gradients depending on the priming acceptor used. If DMB-DP3 was used as an acceptor, the gradient consisted of 2 min water, 7 min linear increase to 185 mM NH_4OAc). With DMB-DP12 as the acceptor, the elution gradient was modified to 10 min linear increase of NH_4OAc from 350 mM to 425 mM. The quantification of substrates and products was performed by integrating peaks using empower 2 software.

Measurement of ST8SiaII inhibition

The Neu5Cyclo concentration required to cause half maximum inhibition (K_i) for ST8SiaII was determined in an *in vitro* activity assay with 20 µM DMB-DP3 and three different CMP-Neu5Ac concentrations (50 µM, 100 µM and 150 µM). Each condition was tested in triplicate, as a minimum, with a series of CMP-Neu5Cyclo concentrations from 0 to 10 µM in the described buffer. Aliquots of the reactions were collected after 30, 60, 120, 180 and 240 min and further processed as noted in the previous section. A standard curve obtained from a dilution series of DMB-DP3 (0 to 10 µM) was used to calculate the amount of DMB-DP4 in ng. The first derivatives of the polynomial trend lines for the time dependent increase of DMB-DP4 were used to calculate the initial reaction velocities (v) (ng/min/µg enzyme) for each condition. K_M of CMP-Neu5Ac for ST8SiaII with DMB-DP3 as the acceptor was determined by plotting v^{-1} versus the inverse substrate concentration (s^{-1}) in a Lineweaver-Burk plot. The intersection of the linear regression curve with the x-axis is equivalent to $-K_M^{-1}$. To determine the K_i of CMP-Neu5Cyclo for ST8SiaII, a Dixon plot of the three CMP-Neu5Ac concentrations 50, 100 and 150 µM was calculated by plotting v^{-1} against the inhibitor concentration. The negative K_i is determined from the x-value of the intersection of the linear regression curves.

Results and Discussion

Analysis of the acceptor preferences of ST8SiaII and ST8SiaIV

When we previously tested mammalian polySTs with DMB-DP3, very different patterns of chain elongation were observed. ST8SiaII efficiently and completely consumed the priming DMB–DP3 to produce oligomers with an approximately normal distribution around DP7. In contrast, ST8SiaIV inefficiently extended the priming structure DMB-DP3, leaving >10 % unmodified after overnight reaction, but efficiently extended the intermediate chain lengths to generate long polymers >DP60 [26]. To further investigate the complimentary activity profiles observed for human ST8SiaII and ST8SiaIV, we have synthesized and purified primers of different length, DMB-DP3 and DMB-DP12, to suit each of the enzymes (Scheme 1). To investigate the preference for specific acceptor structures, it was essential to only measure the first transfer onto the supplied acceptor since each transfer generated a new acceptor species with potentially different acceptor quality. To achieve this goal we applied optimized reaction conditions that result in predominantly single transfers, making DMB-DP4 and DMB-DP13 the major products [32;33]. Preliminary tests using equal amounts of the two human polySTs showed that the specific activity of ST8SiaIV was considerably lower than ST8SiaII. To reach conditions that allowed comparative measurements, the ST8SiaIV concentration was increased by a factor of two in subsequent reactions. In a first experiment primed with DMB-DP3, the time-course of DMB-DP4 production was determined. With both polySTs, a continuous synthesis of DMB-DP4 over time was visible, but with remarkable differences in the reaction velocity (Figure 1). Commensurate with our previous results, we observed that ST8SiaII elongates DMB-DP3 more than 10-fold faster than ST8SiaIV. Based on these results, the 4 h time point was chosen for subsequent comparisons of enzymatic activity.

We next compared the activity of each enzyme for the two different acceptors, DMB-DP3 and DMB-DP12 (Figure 2). Changing the priming acceptor from DMB-DP3 to DMB-DP12 reduced ST8SiaII activity by more than 50 % ($p = 0.0071 < 0.05$ in a two-tailed T-test). In sharp contrast, replacement of DMB-DP3 by DMB-DP12 in the case of ST8SiaIV led to a five-fold increase in activity ($p = 0.0096 < 0.5$ in a two tailed T-test) and thus confirmed the increased affinity of ST8SiaIV for longer polySia chains. These experiments are the first clear demonstration that the two human

polySTs have complimentary affinities for polySia chains of different length and are in accordance with earlier *in vivo* and *in vitro* results. Although both polySTs can partially compensate for deletion of the other polyST, the percentage of polysialylated NCAM *in vivo* is higher when only ST8SiaII is expressed than when ST8SiaIV alone is present [6]. This observation is further supported by the three-fold higher NCAM K_M for ST8SiaIV than for ST8SiaII and the observation that the addition of oligoSia to NCAM leads to an increase in ST8SiaIV activity [21;34]. Our *in vitro* results fit perfectly with these studies and provide the biochemical basis for these observations, as well as the polySia chain length profiles observed in mice with genetic deletions of either ST8SiaII or ST8SiaIV [6]. Furthermore, the prominent difference in acceptor affinity will facilitate an improved *in vitro* analysis of the human polyST enzymes and will support the search for specific polyST inhibitors as cancer therapeutics.

Testing of CMP-activated sialic acid derivatives

With optimized acceptors, DMB-DP3 and DMB-DP12 for ST8SiaII and ST8SiaIV, respectively, we set out to explore differences in activity towards CMP-Sia derivatives. We chose a small series of Sia-derivatives with substituents of increasing size in the 5-*N*-position of Sia (*N*-propanoyl-, *N*-butanoyl-, *N*-pentanoyl- and *N*-cyclopropyl-Sia; Scheme 2). Previous metabolic engineering studies using Neu5But and Neu5Pent have demonstrated that these derivatives impair polysialic acid synthesis by ST8SiaII and ST8SiaIV to different extents [35]. Here we applied the new assay conditions to address the ability of each enzyme to catalyze the transfer of 5-*N*-substituted sialic acids from the respective CMP-activated sugars. By examining single transfers onto the acceptor structure, the results are independent of the subsequent effect of these modified sugars on acceptor quality.

We assayed the two polySTs in the presence of each acceptor (DMB-DP3 and DMB-DP12) with the natural substrate CMP-Neu5Ac and the derivatives. ST8SiaII could be assayed effectively with DMB-DP3 or DMB-DP12, the inhibitory effects of each Sia derivative was independent of the specific acceptor used. On the other hand, meaningful data for ST8SiaIV was only obtained using the longer primer, DMB-DP12, thus demonstrating the importance of primer length in the search for inhibitors.

Comparing activities with the natural donor substrate we observed that each of the linear acyl derivatives exerted a comparable reduction of the turnover rate on both enzymes, ranging between 50 % - 70 % reduced turnover at the 4 h reaction time point (Figure 3). In contrast, neither of the human enzymes was able to transfer CMP-Neu5Cyclo and no product was detectable after 4 h reaction.

The data obtained here complement results of earlier metabolic engineering studies. The study by Mahal and colleagues demonstrated that *N*-butanoylmannosamine resulted in a full blockade of cellular polysialylation while *N*-propanoylmannosamine had little effect [36]. Our results indicate that the blockade of polySia synthesis is not due to differences in the transfer of these derivatives, but must result from a decrease in acceptor quality due to the incorporation of derivatives with increasing size of certain *N*-acyl side groups. A study by Horstkorte *et al.* [35] previously indicated that ST8SiaII and ST8SiaIV were differentially sensitive to inhibition by the *N*-acyl sialosides (via *N*-acylmannosamines). Based on our results, we can conclude that enzyme-specific inhibition is not due to differences in nucleotide-sugar binding or sugar transfer *per se*, but results from differences in the acceptor binding properties of the two mammalian polySTs. This conclusion is supported by *in vitro* experiments using an extracellular domain of NCAM with modified glycans as a model acceptor substrate [36].

In contrast to the Sia-derivatives with linear substituents, where transfer rates were not reduced

below 30 %, CMP-Neu5Cyclo was not transferred either by ST8SiaII nor ST8SiaIV. Interestingly, this derivative was also unable to be transferred by the bacterial polyST from *Neisseria meningitidis* serogroup B [27] despite the fact that these enzymes are not homologs, suggesting that the process of convergent evolution has brought these enzymes not only to catalyze the same reaction but also to exhibit similar restrictions towards nucleotide-sugar substrates. CMP-Neu5Cyclo is the first CMP-Sia derivative not to be transferred by the mammalian polySTs. To shed more light on this finding, we have investigated the inhibitory mechanism of CMP-Neu5Cyclo in more detail.

CMP-Neu5Cyclo is a competitive inhibitor of ST8SiaII

To address the question if CMP-Neu5Cyclo is a competitive inhibitor, we measured inhibitor kinetics for CMP-Neu5Cyclo. Due to the absence of ST8SiaII in the adult brain (polySia expression retained in adulthood is synthesized by ST8SiaIV) and the dominant role of ST8SiaII in polySia-associated tumors, ST8SiaII is perhaps the more relevant target for polyST inhibiting anti-tumor drugs [8]. Consequently, the kinetic data was solely measured with human ST8SiaII.

Activity assays were conducted with constant DMB-DP3 and ST8SiaII concentrations and three CMP-Neu5Ac concentrations, as well as a series of inhibitor concentrations. The time-dependent Neu5Ac transfer onto DMB-DP3 was quantified using the HPLC-FD activity assay. The initial reaction velocities were calculated from the time-dependent DMB-DP4 formation. The substrate concentration at which the reaction rate is half maximal, the Michaelis constant (K_M), for CMP-Neu5Ac was determined to be 62 µM using a Lineweaver-Burk plot (v^{-1} vs. s^{-1}) (Figure 4A). The inhibitor concentration necessary to reduce the maximal reaction rate by half, the so-called inhibitory constant (K_i), was calculated using a Dixon plot, v^{-1} vs. inhibitor concentration for varying substrate concentrations (Figure 4B). This analysis revealed that CMP-Neu5Cyclo is a competitive inhibitor with a K_i of 25.9 µM ± 0.4 µM. We conclude that CMP-Neu5Cyclo binds to the donor binding site of ST8SiaII, directly competing with CMP-Sia. The level of inhibition is comparable to that observed for CMP [32;33], indicating that Neu5Cyclo does not make a strong contribution to binding affinity. However, since this substrate is unable to be transferred to polySia, it serves as a useful lead compound in the search for new anti-cancer therapeutics. In combination with the recent structural information for ST8Sia-family member, oligosialyltransferase ST8SiaIII [37], the development of potent ST8SiaII inhibitors as cancer therapeutics can be further advanced.

Conclusion

In this study we evaluated the acceptor length preference for priming the two human polySTs ST8SiaII and ST8SiaIV. While ST8SiaII could be assayed using the DMB-DP3 acceptor, ST8SiaIV activity with this acceptor was too low for reliable evaluation of donor sugar analogs. The longer DMB-DP12 acceptor is the first fluorescent acceptor substrate for investigating inhibition of ST8SiaIV activity.

Using the optimized assay conditions, we studied a set of CMP-Sia derivatives that have previously been used for metabolic engineering of tumor cells. The CMP-activated Sia-derivatives with linear acyl substituents, from *N*-propanoyl to *N*-pentanoyl in position five, were all substrates for both of the human polySTs, and were transferred at a rate which is 50 % - 70 % slower than unmodified Sia. The absence of a significant reduction in activity with longer *N*-substituents indicates that the human polySTs do not discriminate between these Sia-derivatives. Our results provide strong support for the proposition that observed differences in the activity of the two human polySTs is not due to differences in their donor sugar binding but due to dissimilarities in their acceptor binding properties [36].

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Neither of the human polySTs were able to transfer the Sia-derivative CMP-Neu5Cyclo. Inhibition kinetics for this derivative indicated weak, competitive inhibition of ST8SiaII. This compound can serve as a lead structure for the future development of ST8SiaII inhibitors for cancer therapy.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contribution

J.E. designed and performed the experiments, analyzed the data and prepared the manuscript. C.M. and S.W. synthesized the CMP-activated sialic acid derivatives; T.G.K initiated the study of mammalian polySTs using DMB-labeled acceptors and edited the manuscript. M.S. measured the inhibitor kinetics. R.G.S and R.A.F. provided guidance on experimental design, data interpretation and manuscript preparation.

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Figure Legends

Scheme 1: Chemical structures of the donor and acceptor substrates utilized. (A) PolyST acceptor DMB-DP3. (B) PolyST acceptor DMB-DP12.

Figure 1: Time course of the polymerisation reaction obtained for ST8SiaII (A) and ST8SiaIV (B) with DMB-DP3 and CMP-Neu5Ac. Measurements were performed in triplicate. Depicted are the transfers in µM / µg enzyme against the reaction time.

Figure 2: Activity comparison for the two acceptors DMB-DP3 and DMB-DP12 with the human polySTs ST8SiaII and ST8SiaIV. (A) Depiction of the transfers of CMP-Neu5Ac onto DMB-DP3 or DMB-DP12 by the human polySTs ST8SiaII and ST8SiaIV after 4h of reaction time. (Significance ** $p < 0.01$).

Scheme 2: CMP-activated 5*N*-functionalised sialic acid derivatives .

Figure 3: Comparison of transfers of the respective donor sugar derivatives (CMP-Neu5Prop, CMP-Neu5But, CMP-Neu5Pent and CMP-Neu5Cyclo) onto one of the two acceptors DMB-DP3 and DMB-DP12 by the human polySTs ST8SiaII and ST8SiaIV after 4h reaction.

Figure 4: Enzyme kinetics of ST8SiaII with the inhibitor CMP-Neu5Cyclo (A) Lineweaver-Burk plot for different donor sugar concentrations. Linear regression resulted in the equation y = 120.9 x +1.95. From the x-intercept the K_M of ST8SiaII for CMP-Neu5Ac was calculated to be 62 µM. (B) Dixon plot for reactions of ST8SiaII with 50, 100 and 150 µM donor sugar and a series of CMP-Neu5Cyclo concentrations. The linear regression of each series of data is shown as line and follows the in Table 1 noted formula. The equation of the linear regression resulted in a K_i of 25.9 µM ± 0.4 µM.

Table-of-Contents

Two polysialyltransferases (polySTs; ST8SiaII & IV) control polysialylation in humans, but ST8SiaII is predominant in tumors. Here we exploited differences in the preference for priming polysialic acid to develop a fluorescent assay with which polySTs can be differentiated in inhibitor screens. We describe CMP-Neu5Cyclo as a first hit, competitive polyST inhibitor.

Tables

Table 1: Formulae of the linear regression curves of the Dixon plot.

Donor sugar concentration	Equation of the linear regression curve
50 µM	y = 0.1524 x + 4.317
100 µM	y = 0.1204 x +3.472
150 µM	y = 0.08462 x + 2.566

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10.1002/cbic.201700157

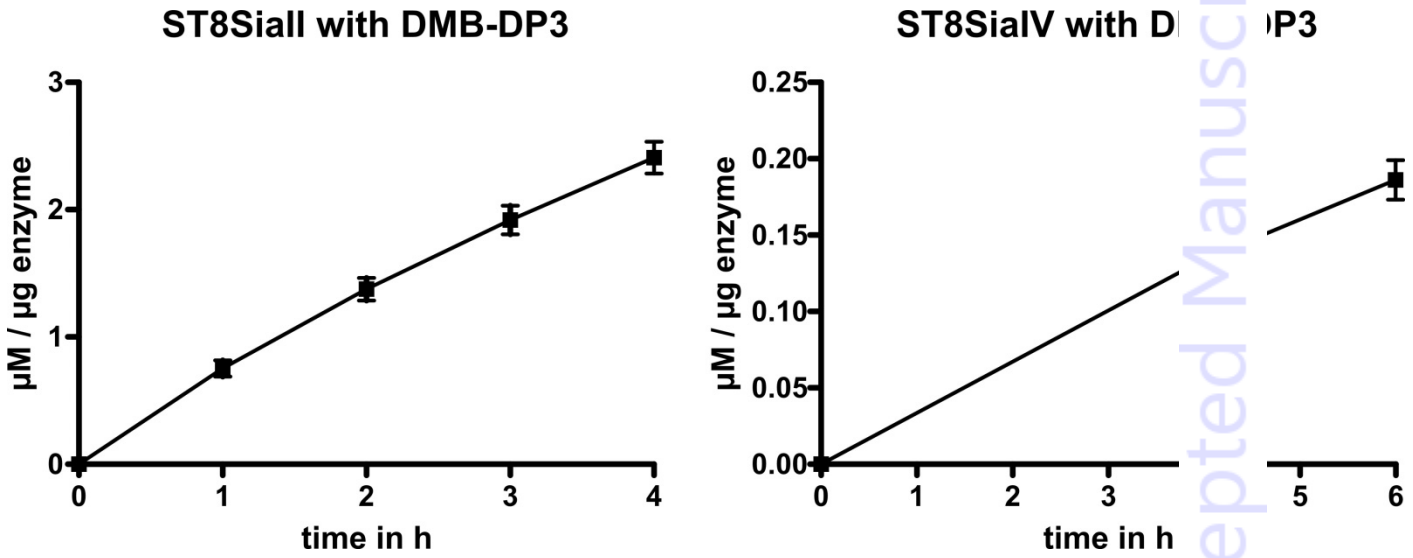

Figure 1_Ehrit et al.

10.1002/cbic.201700157

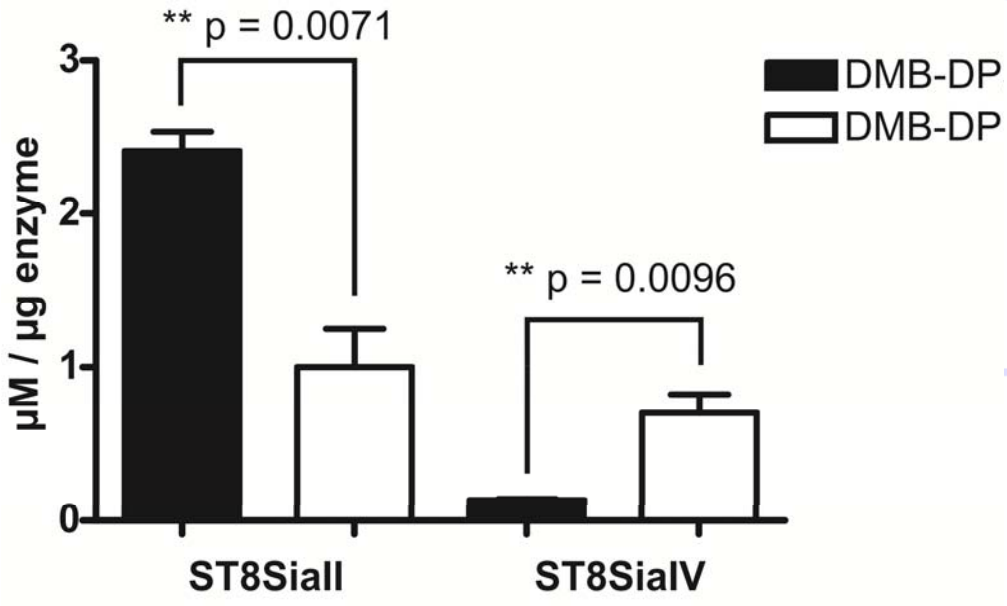

Figure 2_Ehrit et al.

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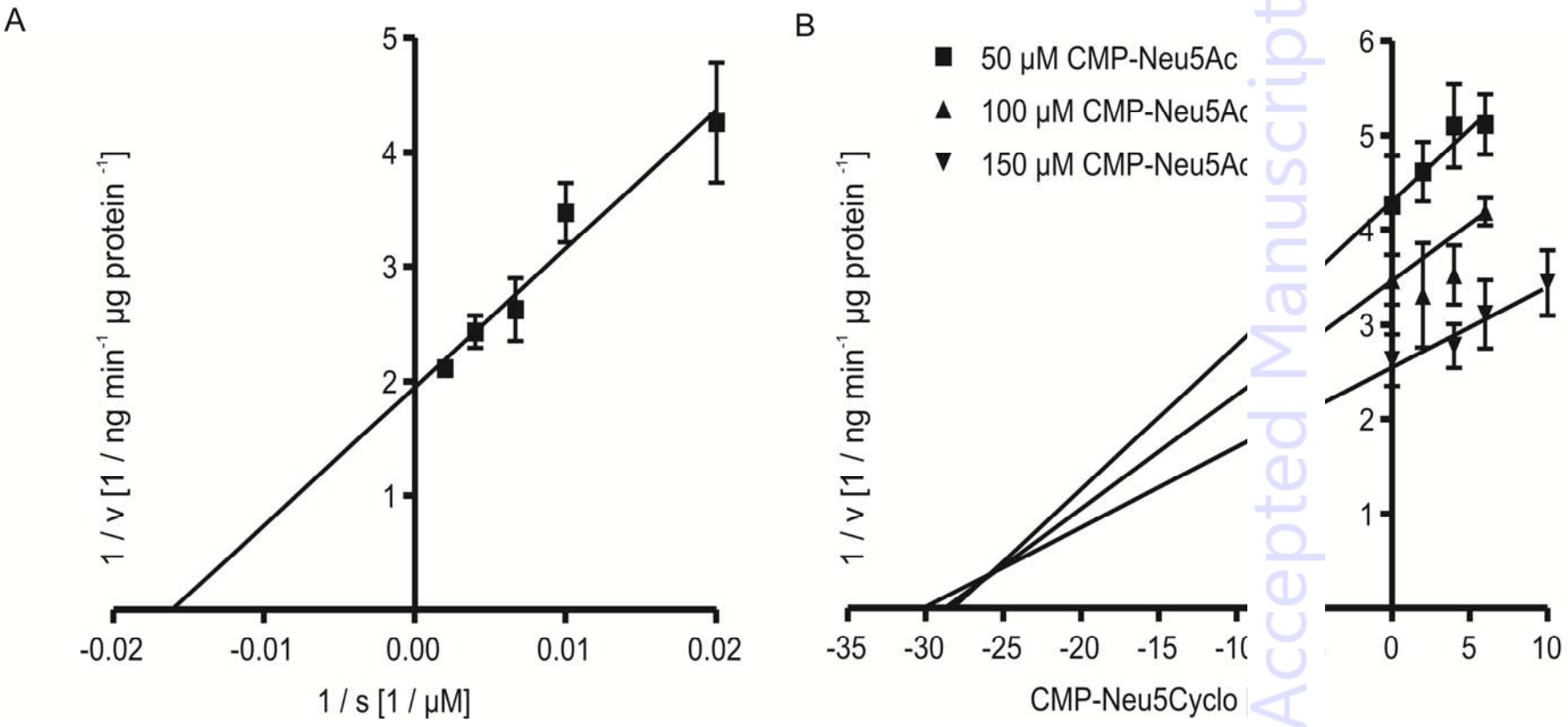
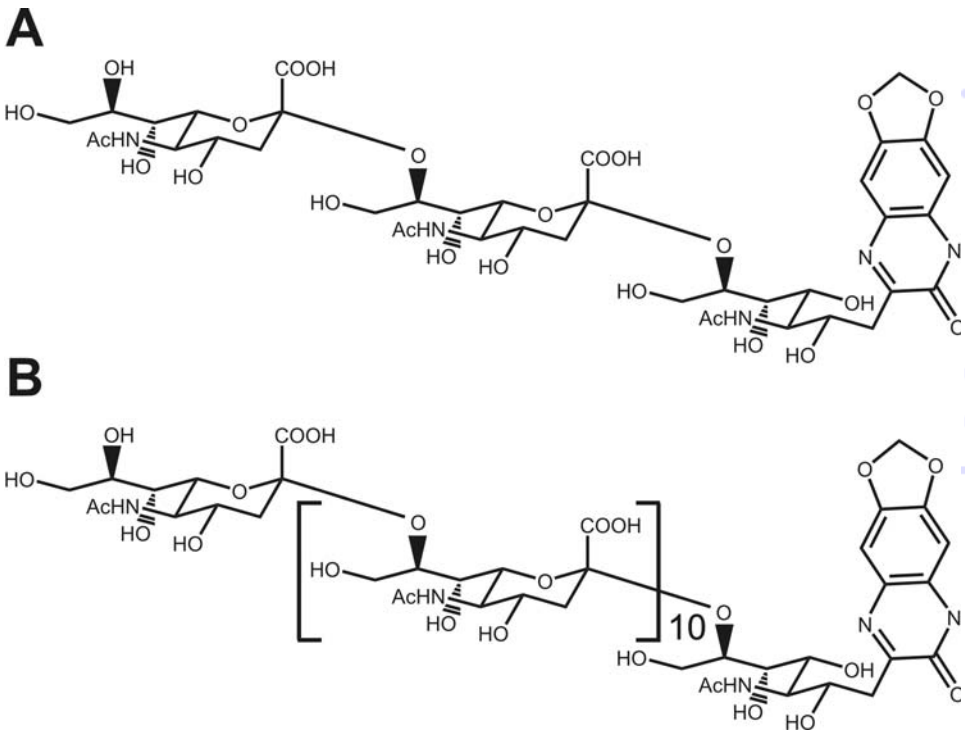


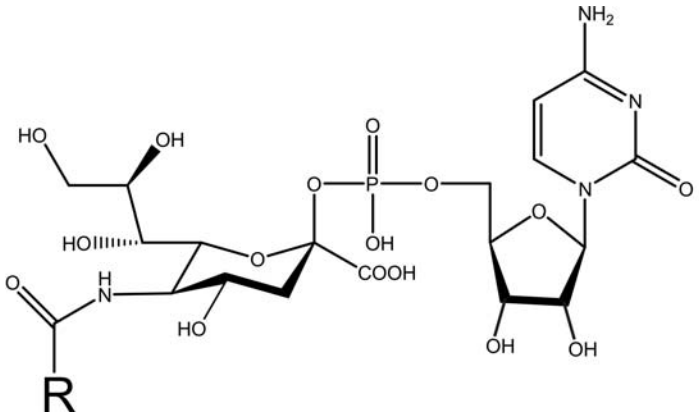
Figure 4_Ehrit et al.

10.1002/cbic.201700157



Scheme 1_Ehrit et al.

10.1002/cbic.201700157



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C_4H_9: CMP-N ent
: CMP-N yclo

Scheme 2_Ehrit et al.