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Immobilization of CMP-Sialic Acid Synthetase and α2,3-Sialyltransferase for Cascade Synthesis of 3′-Sialyl β-D-Galactoside with Enzyme Reuse

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Sialo-oligosaccharides are often synthesized via cascade reaction of CMP-sialic acid synthetase (CSS) and sialyltransferase (SiaT). Here, we studied individual enzyme immobilization to develop solid-supported preparations of the CSS from *Neisseria meningitis* (NmCSS) and the α2,3-SiaT from *Pasteurella dagmatis* (PdSiaT). Oriented immobilization via N-terminal His-tag as well as "random" (orientationally uncontrolled) immobilization via multipoint covalent coupling gave catalyst preparations of each enzyme with low activity and effectiveness factor (η). We therefore constructed N-terminal fusions of NmCSS and PdSiaT with the cationic binding module Z_{basic2} and show individual immobilization of even the unpurified enzymes on sulfonate carrier (ReliSorb SP400) in excellent yields ($\geq 95\%$) and binding selectivities. For both enzymes in individual reactions, the initial η (Z-NmCSS: 90%; Z-PdSiaT: 25%) declined sharply with increasing enzyme loading and the maximum immobilized activity was 110 U/g carrier ($\eta = 27\%$) for Z-NmCSS, 7.5 U/g ($\eta \leq 5\%$) for Z-PdSiaT. Supplied with neuraminic acid and cytidine 5′-triphosphate (20 mM each), the immobilized enzymes promoted α2,3-sialylation of the model substrate 4-nitrophenyl β-D-galactoside (20 mM) in 85% yield and could be recycled 5 times with only small loss in their overall synthetic activity ($\leq 10\%$).

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Introduction

Sialo-oligosaccharides are important carbohydrate structures of human glycobiology.[1] They occur naturally on glycoconjugates (e.g., glycoproteins, glycolipids)[2,3] but also as free oligosaccharides, like the ones found in breast milk.[4,5] The synthesis of defined sialo-oligosaccharide structures is broadly important for the study of their biological function.[6] Due to increasing interest in sialo-oligosaccharides as specialized food ingredients, precision synthesis also receives considerable attention for use in production.[7,8]

Installation of sialic acids (most commonly neuraminic acid; Neu5Ac) on oligosaccharides must proceed with suitable regio- and stereo-control. Enzyme-catalyzed synthesis presents an attractive solution.[9] Sialyltransferases (SiaT; EC 2.4.99.-) use cytidine 5′-monophosphate (CMP)-activated Neu5Ac as the donor substrate for sialylation (Figure 1).[10] For sialylation of D-galactoside acceptors, SiaTs with specificity for formation of α2,3-, α2,6- and mixed α2,3/α2,6-glycosidic linkages are known.[10] In synthetic reactions of these different SiaTs, the CMP-Neu5Ac substrate is generally prepared *in situ* from Neu5Ac and cytidine 5′-triphosphate (CTP)[11] through the reaction of CMP-Neu5Ac synthetase (CSS; EC 2.7.7.43) (Figure 1).[12] Diversity-oriented chemo-enzymatic synthesis of sialo-oligosaccharides has often relied on the cascade reaction of CSS and SiaT.[13–15]

An important strategy that has been largely overlooked to increase the overall enzyme performance (i.e., activity, stability, usability) in sialo-oligosaccharide synthesis is immobilization. Solid-supported preparations of the CSS and SiaT would facilitate recycling of the enzymes and help paving a way towards a continuous (flow) synthesis of sialo-

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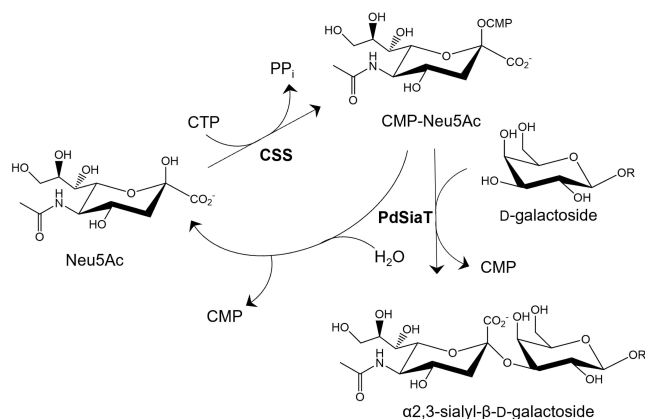**Chemistry
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Figure 1. Enzymatic cascade transformation for α2,3-sialyl-β-D-galactoside synthesis from Neu5Ac and CTP. For abbreviations, see the text. Hydrolysis of CMP-Neu5Ac by PdSiaT is also shown.

oligosaccharides.[16,17] Additionally, the enzymes can acquire enhanced stability through immobilization.[16,18,19] Structural stabilization can be significant especially with multimeric enzymes which upon immobilization dissociate less readily into subunits than in solution.[16] A well-designed immobilization can exploit binding to carrier in order to capture the enzyme from a complex protein matrix.[20] Partial purification and concentration may thus be achieved and can be relevant in particular for enzymes not highly overexpressed in the used host cell. Lastly, immobilized preparations can differ from the soluble enzyme in important catalytic properties (e.g., selectivity) which may be beneficial for the synthesis.[21,22] An example discussed later in this work is hydrolytic side reaction of SiaT (See Figure 1).[23–25]

In this study, therefore, we have explored major principles of enzyme immobilization on porous carriers in application to the CSS from *Neisseria meningitis* (NmCSS)[26] and the α2,3-SiaT from *Pasteurella dagmatis* (PdSiaT).[24] Unsurprisingly considering the large body of literature on enzyme immobilization,[27] the approaches used here are only a small selection of the broad variety of methods known. They are however somewhat representative in that they include non-covalent adsorption and covalent binding as well as tethering to the solid surface in variable degree of control of molecular orientation. It is convenient to discuss aspects of pro and con of each immobilization method not in general here, but later under Results based on direct evidence. For both enzymes, affinity-like oriented immobilization mediated by an oligohistidine peptide tag, or a partner binding module in a fusion protein, was superior in efficiency to an immobilization based on non-specific protein-carrier interactions enabled by the native enzyme.

Based on an approach of protein fusion to the cationic binding module Zbasic2[28,29] we have developed a convenient method for the immobilization of NmCSS and PdSiaT on readily available support material (ReliSorb SP400), validated from our earlier studies of enzyme immobilization mediated by Zbasic2.[30–32] The carrier features anionic sulfonate groups on the surface and Zbasic2-enzyme fusions (Z-NmCSS, Z-PdSiaT) are

immobilized in excellent yield ($\geq 95\%$) and high selectivity ($\geq 90\%$) directly from cell extract without requirement for prior purification of the target protein. The single-step purification and immobilization yields solid catalysts of Z-NmCSS and Z-PdSiaT with an activity of ∼100 U/g carrier and ∼7.5 U/g carrier, respectively. With both enzymes but especially PdSiaT, low effectiveness of the immobilized enzyme at high protein loading remains a challenge.

We have also demonstrated use of the immobilized enzymes in a repeated batchwise synthesis of a α2,3-sialoside model product (α2,3-sialyl β-D-galactosyl 4-nitrophenol) that involved 5-fold recycling of the solid catalysts. To be sure, technologically other products (e.g., α2,3-sialyl lactose) would be more relevant. We chose 4-nitrophenyl β-D-galactoside as model substrate for sialylation in this study because the PdSiaT shows good activity and substrate consumption and product formation are conveniently monitored by HPLC.[24] The literature offers only a few occasional studies of CSS[33,34] or SiaT immobilization[35–39] that were highly specific in approach (e.g., site-specific covalent coupling of enzyme on magnetic nano-particles) or limited in their applied scope. The current study therefore presents progress towards CSS and SiaT cascade reactions on solid support for the practical synthesis of sialo-oligosaccharides. The results can have relevance for cascade syntheses of oligosaccharides and sugar nucleotides showing potential for production.[40–44]

Results and Discussion

Fusion proteins of NmCSS and PdSiaT with Zbasic2. Zbasic2 is a small protein module (7 kDa; 58 amino acids) that folds into a three α-helical bundle.[28] Zbasic2 has an isoelectric point of 10.5 and is strongly positively charged (total net charge: $+10$; charge/residue: $+0.58$).[29] Arginine residues clustered on one side of the Zbasic2 contribute to positive surface charge density. Fusion proteins harboring Zbasic2 bind to anionic supports via their cationic binding module.[30,31,45,46] Zbasic2 facilitates immobilization of enzymes on anionic surfaces in controllable molecular orientation.[28,45]

Fusion constructs of NmCSS and PdSiaT feature the Zbasic2 module appended to the N-terminus of the enzyme. The PdSiaT used was the ∇3 variant (elongation of the N-terminus by 3 amino acids Lys-Thr-Ile) that was earlier shown to yield enhanced expression compared to the full-length enzyme[24]. Z-NmCSS and Z-PdSiaT were produced in *E. coli* BL21(DE3) to an estimated level of ∼20 mg/L and ∼60 mg/L of the microbial culture, respectively. The enzymes were purified by cation exchange chromatography to apparent homogeneity by the criterion of single protein band on SDS polyacrylamide gel (Figure S1). The Z-NmCSS showed a specific activity of 38 U/mg protein ($\pm 7\%$; N = 3) which can be compared to a specific activity of 36 U/mg for the His-tagged NmCSS determined previously[26] and confirmed in this study. The Z-PdSiaT showed a specific activity of 2.9 U/mg protein ($\pm 12\%$; N = 4) which is about half (56%) of specific activity of the His-tagged PdSiaT (5.2 U/mg).[26] We also measured the hydrolase activity of Z-

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PdSiaT against the CMP-Neu5Ac substrate when no acceptor substrate was present. The specific activity was 1.9 U/mg protein ($\pm 9\%$; N = 3) which was lowered by the same factor (0.54-fold) compared to the activity of His-tagged PdSiaT (3.5 U/mg) as was the sialyltransferase activity.[26] We note that fusion to the Z_{basic2} module results in relatively small changes in molecular mass of the enzyme subunit for PdSiaT (19%; with Z_{basic2}: 58 300 Da) and NmCSS (30%; with Z_{basic2}: 37 000 Da). The observed differences in specific activity must arise from non-trivial factors which excludes molecular mass change. In summary, fusion to the Z_{basic2} module was without effect on enzyme activity in NmCSS and it was reasonably tolerated in PdSiaT. It is worth noting that NmCSS is a functional dimer[47] whereas PdSiaT is a monomer.[24] The crystal structure of NmCSS shows that the N-termini are positioned relatively exposed on the same side of the enzyme dimer.[47] Structurally, therefore, N-terminal attachment of the Z_{basic2} seems well possible without interference to the enzyme function. Binding of Z-NmCSS to surfaces may involve multivalent interaction of both Z_{basic2} modules. For enzyme storage (50 mM potassium phosphate buffer, pH 7.0; ~20 mg protein/mL), both Z-NmCSS and Z-PdSiaT required the addition of NaCl (0.2 M) for stability at $-20\,°C$. The purified His-tagged enzymes are henceforth referred to His-NmCSS and His-PdSiaT. In both enzymes, the His-tag is placed at the N-terminus.

Immobilization of His-NmCSS and His-PdSiaT. Immobilization via covalent coupling to epoxy group-activated carrier was analyzed for His-NmCSS and His-PdSiaT and compared with immobilization via affinity-like noncovalent binding through the His-tag to iminodiacetic acid-chelated metal ions on the carrier surface. The carriers used were all of porous methacrylate material but differed in the particle size and the pore diameter. Carrier properties are summarized in Table S1. Purified His-NmCSS and His-PdSiaT were used. The immobilization strategies also differed in the degree of control they can possibly achieve over the molecular orientation of the enzyme with respect to the solid surface. Coupling via epoxy groups can occur in numerous protein orientations with no immediate control from the experimental conditions. Binding to metal chelate carriers is expected to involve a preferred mode of protein-surface interaction via the His-tag. Although multiple conformations may still be accessible to an enzyme bound in that way, the overall immobilization can nonetheless be considered as broadly oriented. It is distinguished from the immobilization on the epoxy group carrier which lacks the element of controlled orientation entirely.

Using an enzyme loading of 5 mg/g carrier (His-NmCSS: 150 U/g; His-PdSiaT: 29 U/g) we characterized immobilization on epoxy carriers in terms of yield, activity and effectiveness of immobilized enzyme. For His-PdSiaT, we obtained just 0.04 U/g carrier with an effectiveness factor (η) of below 1%. The yield was low (25%), irrespective of the carrier used and changes in conditions tried (temperature: 4°C or 25°C; addition of 0.1 M NaCl). For His-NmCSS, the yield was higher, with the Sepabeads and ReliZyme carriers (~65%) surpassing the Lifetech carrier (~47%). The larger pore size of the Lifetech carrier was not beneficial for the yield. Activities and their associated η were in

the range 1–10 U/g carrier and 1–10%, respectively. Tentatively, we can use structural information on NmCSS[34] to try to rationalize the low value of η. Out of 19 lysine and arginine residues on the protein surface, 5 are located near the active site.[34] Involvement of these lysines and arginines in reaction with the carrier epoxy groups would likely result in loss of activity by steric hindrance of the active site. Other effects common for these types of carrier (e.g., conformational distortion due to enzyme interaction with the hydrophobic surface) are also possible.[18,48] Overall, immobilization on epoxy carriers did not seem to be promising for both enzymes (Table 1).

To explore immobilization via the His-tag, the three epoxy carriers were derivatized with iminodiacetic acid and then loaded with Ni^{2+}, Co^{2+} or Cu^{2+}. This gave rise to a panel of 9 metal chelate carriers on which immobilization of His-NmCSS and His-PdSiaT was analyzed.

For comparison with the plain epoxy carriers, the same enzyme loading (5 mg/g carrier) was used. Results are summarized in Figure 2 and selected parameters are shown in Table 1. Immobilization parameters are dependent on enzyme, carrier and metal used, suggesting complex interplay of factors in determining immobilization performance. Considering that activity of the immobilized catalyst preparation is prime, only the Lifetech carrier in Ni^{2+} (38 U/g carrier) and Co^{2+} form (34 U/g carrier) was suitable for the immobilization of His-NmCSS (Figure 2B). Cu^{2+} was not usable irrespective of the carrier used. The activity of NmCSS was never studied in the presence of Cu^{2+}. However, the CSS from *C. thermocellum* is inhibited by Cu^{2+},[49] providing a plausible explanation for the lack of activity of the immobilized His-NmCSS. The value of η (52%; Lifetech carrier in Ni^{2+} form) follows a similar trend (Figure 2C). The immobilization yield was less dependent on the carrier and the metal used than was the activity. Within their varied ranges, particle and pore size of carrier were not major factors of His-NmCSS binding.

Compared to His-NmCSS, PdSiaT was more challenging to immobilize using His-tag binding. Activity was rather low (0.52 U/g carrier) and its associated η did not exceed 6.0% (at an enzyme loading of ~30 U/g carrier). Co^{2+}-loaded carriers are

Table 1. Summary of immobilization parameters for His-CSS and His-PdSiaT.

immobilization method	His-CSS		His-PdSiaT	
	random covalent binding[a]	oriented via tag[b]	random covalent binding[c]	oriented via tag[d]
immobilization yield [%][e]	65 ± 5.0	73 ± 2.0	25 ± 7.0	74 ± 5.0
observable activity [U/g carrier]	9.5 ± 0.9	38 ± 5.0	0.1 ± 0.07	1.0 ± 0.8
η [%]	9.7 ± 0.2	35 ± 2.0	1.4 ± 0.4	4.7 ± 3.3

[a] immobilized on carrier ReliZyme at 25°C, [b] immobilized on carrier Lifetech-IDA-Ni^{2+} at 25°C, [c] immobilized on carrier Sepabeads at 25°C, [d] immobilized on carrier Lifetech-IDA-Ni^{2+} at 4°C, [e] results are for an enzyme loading of 150 U His-CSS/g carrier and 28.5 U His-PdSiaT/g carrier. S.D. is from 3 replicate experiments.
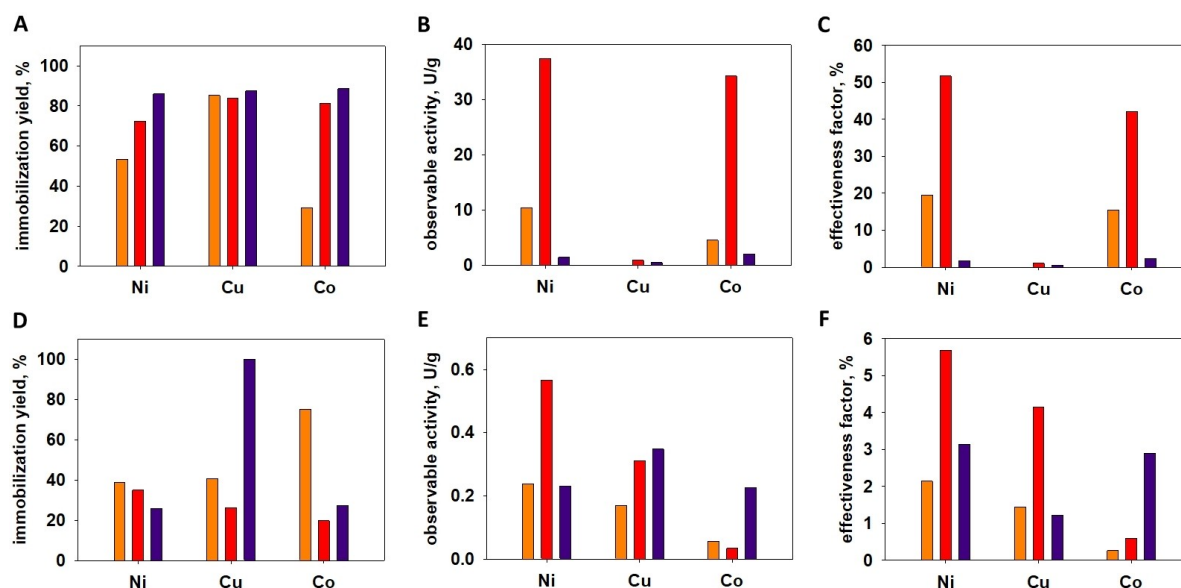
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Figure 2. Immobilization of His-CSS (A, B, C) and His-PdST (D, E, F) on different carriers (ReliZyme, orange; Lifetech, red; Sepabeads, blue) and functionalized with different metal ions (Ni^{2+}, Co^{2+}, Cu^{2+}). The enzyme loading was 150 U/g carrier and 28.5 U/g carrier for His-CSS and His-PdST, respectively. All immobilizations were performed at room temperature without the addition of salt.

not suitable. The pattern of immobilization parameters (Figure 2D–F) supports the idea that low loading benefits the activity: Ni^{2+}-loaded carriers, in particular Lifetech, show the lowest immobilization yield but give the highest activity and η. The Cu^{2+}-loaded Sepabeads give excellent immobilization yield but activity and η are comparably low.

To further enhance the immobilization of His-PdSiaT, we explored temperature (4°C or 25°C) and ionic strength (0.1 M NaCl) as process variables on Lifetech-Ni^{2+}, ReliZyme-Ni^{2+} and Lifetech-Cu^{2+}. As shown in Table S2, the addition of salt increased the immobilization yield at 25°C on all carriers, typically by ~2.6-fold. However, the η went down by an even larger factor of about 3–4 in correspondence to the increased yield, except for the Lifetech-Cu^{2+} carrier that already started out at a very low value of η ($= 1.8\%$) when no extra NaCl was present (Table S2). Catalyst activity (U/g carrier) hardly benefited from the enhanced yield. Lowering the temperature to 4°C improved the yield on Lifetech-Ni^{2+} and ReliZyme-Ni^{2+} and boosted the activity up to 3.5-fold, without compromising the η substantially (Lifetech-Ni^{2+}) or even enhancing it (ReliZyme-Ni^{2+}). However, the activity was limited to ~1 U/g carrier or lower. There exist numerous possible reasons for low activity in metal chelate immobilization of His-tagged enzymes.[18] Effect of the metal on enzyme activity and stability was mentioned above discussing the NmCSS immobilization results. Additionally, the enzyme interaction with the carrier may lack the desired control of molecular orientation in two distinct ways. Firstly, residues on the protein surface (e.g., dyads or larger groups of histidines in suitable spatial arrangement) contribute to binding to the chelated metals additionally to the His-tag.[50] Secondly, although binding to the chelated metals is effectively promoted by the His-tag, there are additional, possibly non-

specific interactions from the enzyme with the carrier surface. In both cases, the immobilized enzyme may lose activity in substantial amount. Since focus of this study was on development of a practical immobilization for NmCSS and PdSiaT, we did not pursue in depth analysis of the enzyme interaction with the metal-loaded carriers in an effort at mechanistic clarification.

Immobilization of Z-NmCSS and Z-PdSiaT. We initially used purified enzymes (Figure S1) and examined activity loadings of 100–700 U/g carrier for Z-NmCSS and 20–400 U/g carrier for Z-PdSiaT. The immobilization yield on ReliSorb SP400 was excellent, with close to 100% of the offered activity bound in the full range of activities used. Note: protein measurement confirmed the binding of the Z-enzymes and control incubations done in the absence of carrier showed the activity in the supernatant to be stable during the 1 h time of experiment.

Encouraged by these results we moved to immobilization of Z-NmCSS and Z-PdSiaT directly from the cell extract of the *E. coli* strain used for enzyme production. The immobilization conditions were the same, except for the use of 0.2 M NaCl that was added to decrease non-specific protein binding from the cell extract. The immobilization yield was lowered dramatically (up to 10-fold) when cell extract was used. For Z-NmCSS, only 10% of the offered activity were bound when the immobilization was done at 720 U/g carrier. Building on earlier evidence on the immobilization of Z_{basic2} fusions of other enzymes (e.g., sucrose phosphorylase,[51] D-amino acid oxidase[45]), we explored the combination of high salt and non-ionic detergent (Tween 20) to decrease the competition from other proteins on the binding of Z-NmCSS and Z-PdSiaT.

The results shown in Table 2 indicate a substantial improvement of yield under conditions resulting from enzyme-specific

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Table 2. Fine tuning of conditions for high-yield immobilization of Z-CSS and Z-PdSiaT at elevated protein loading from *E. coli* cell extract.

additive[a]	Z-CSS immobilization yield [%][b]	Z-PdSiaT immobilization yield [%][b]
0.2 M NaCl	10 ± 4.0	24 ± 10
0.4 M NaCl	88 ± 2.0	30 ± 13
0.5 % Tween 20	60 ± 7.0	47 ± 3.0
0.4 M NaCl + 0.5 % Tween 20	–	82 ± 6.0

[a] Other immobilization conditions: 50 mM potassium phosphate buffer (pH 7.0), 1 h, room temperature (~22 °C), [b] The enzyme loadings were 720 U Z-CSS/g carrier and 310 U Z-PdSiaT/g carrier, corresponding to a total protein loading of 19 mg/g carrier and 106 mg/g carrier, respectively. The S.D. is from 3 replicate experiments.

Figure 3. Immobilization of (A) Z-CSS and (B) Z-PdSiaT on ReliSorb SP400. Carrier loading of 100 mg/mL was used for immobilization. Immobilized yield (diamonds, pink), observable activity (circles, blue) and effectiveness factor (squares, black) are shown. Error bars show S.D. from 3 replicate experiments.

optimization. While for the Z-NmCSS the increase in NaCl to 0.4 M was sufficient, the Z-PdSiaT required additional Tween 20 for high-yield immobilization. While the expression yield of Z-PdSiaT was 3-times higher than that of Z-NmCSS (60 to 20 mg/L culture, respectively), the specific activity of Z-NmCSS was about 13-fold higher than that of Z-PdSiaT. It is therefore understood that loading of the same activity of both enzymes requires a correspondingly larger amount of total protein loaded from the cell extract when Z-PdSiaT is immobilized. Stringent conditions of binding must therefore be used with Z-PdSiaT. We used analysis by SDS PAGE (Figure S2) to demonstrate specificity of binding of Z-NmCSS and Z-PdSiaT during their immobilization on ReliSorb SP400.

In Figure 3, we show immobilization parameters of Z-NmCSS and Z-PdSiaT obtained in dependence of the activity loading and determined under optimized process conditions. At low activity loading, the effectiveness factor of immobilized Z-NmCSS was close to 100%. This result is consistent with expectation for a perfectly oriented binding via the Z_{basic2} module(s) that succeeds in preventing any loss of activity due to unfavorable protein-carrier interactions. The catalyst activity and the effectiveness factor show opposing trends dependent on loading up to 380 U/g carrier (Figure 3A).

At higher loadings (720 U/g carrier), both activity and η drop significantly. A maximum activity of ~110 U/g carrier was found. The corresponding value of η was ~30%. The yield decreased slightly dependent on loading but was still at 85% at highest loading used.

In terms of the yield, the Z-PdSiaT showed a behavior (Figure 3B) similar to that of the Z-NmCSS. The decrease in η in dependence of the activity loading was quite pronounced. At low loading (10 U/g carrier), the η was ~25%. It decreased to less than 5% when loading was increased to just 100 U/g carrier. A 20-fold increase in loading (30→630 U/g carrier) gave only a moderate (2.3-fold) increase in catalyst activity (3.0→7.0 U/g carrier). These results support the earlier notion from the study of His-tag immobilization of the PdSiaT that only sparse enzyme loading is compatible with reasonable values of η. The protein loading at the lower end of the used range (≤ 20 mg/g carrier) was certainly not enough to overcrowd the available carrier surface with Z-PdSiaT. The enzyme behavior in terms of η might be explained if activity was only shown by the
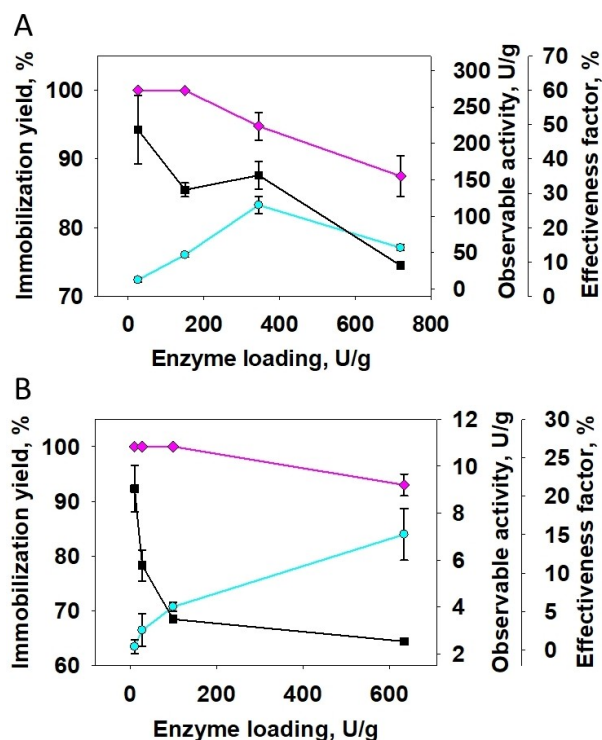
Z-PdSiaT, or His-PdSiaT, bound on the outer surface of the carrier particles. Possibilities are that PdSiaT cannot enter the carrier pores for reasons other than size exclusion (the carrier pores are big enough for PdSiaT), possibly leading to surface crowding and aggregation; or the enzyme is not active under confinement of the pore, possibly because of restrictions on protein conformational flexibility linked to enzymatic catalysis. Low effectiveness of Z-PdSiaT might also result from diffusional restrictions. The K_m of PdSiaT was determined as 1.1 mM in earlier work[24] and enzyme assays used CMP-Neu5Ac at non-saturating concentration (1.0 mM). Repulsion of like negative charges in CMP-Neu5Ac and on the carrier surface can additionally decrease substrate availability to the immobilized enzyme (for a discussion of relevant charge effects, see Liu and Nidetzky[32]). However, the immobilized enzyme activity is very low and it is debatable whether diffusion can thus present a limiting factor. More research is needed to distinguish between these different possibilities. Lastly, evidence of η higher for Z-NmCSS than Z-PdSiaT might be explained by enzyme oligomeric structure and multivalent protein-surface interactions enabled by it.

Due to its monomeric structure,[24] binding of Z-PdSiaT is limited to a strictly monovalent interaction from a single Z_{basic2} module. By contrast, the Z-NmCSS dimer might benefit from multivalency effects on carrier binding made possible by two suitably positioned Z_{basic2} modules.[12]

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Earlier studies of the immobilization of other Z-enzymes revealed a similar trend, connecting high values of η to the presence of two Z_{basic2} modules. This was seen in comparing naturally dimeric and monomeric sucrose phosphorylases as well as in monomeric sucrose phosphorylases equipped with two instead of just one Z_{basic2} module.[51] D-amino acid oxidase is a functional dimer and its Z_{basic2} fusion can be immobilized with excellent η.[52] However, not all multimeric enzymes can exploit multivalency effects in the immobilization of their Z_{basic2} fusion proteins. This was shown for sucrose synthase which is a natural homo-tetramer, but the protein subunits are arranged in a way not favorable for multivalent protein-surface interaction by the Z_{basic2} modules.[31]

Cascade synthesis of sialo-oligosaccharide with reuse of immobilized enzymes. As proof of principle for the synthetic application of immobilized Z-NmCSS and Z-PdSiaT, we examined the conversion of 4-nitrophenyl β-D-galactoside (4NPβGal) 20 mM). Neu5Ac and CTP (each 20 mM) were used to form CMP-Neu5Ac *in situ*. Enzymes were added at 0.5 U/mL, using immobilized preparations on ReliSorb SP400 with an activity of 45 U/g carrier (Z-NmCSS) and 7 U/g carrier (Z-PdSiaT). Fusion to Z_{basic2} presents an excellent strategy for the co-immobilization of multiple enzymes in order to promote biocatalytic cascade reactions on solid support, as shown with different enzyme systems before.[30,31,53] However, considering that individual immobilization of Z-NmCSS and Z-PdSiaT involved substantial optimization of conditions, we were content here to use separately immobilized enzyme preparations (on common issues involved in enzyme co-immobilization, see the review of Fernandez-Lafuente and co-workers[54]). More research beyond the immediate reach of the current study is warranted to develop a co-immobilized preparation of the two enzymes.

Time course of α2,3-sialylation of 4NPβGal is shown in Figure 4A. Substrate consumption and product formation was monitored by HPLC using authentic standards of all compounds, including that of the product α2,3-sialyl 4-nitro-phenyl β-D-galactoside (α2,3Neu5Ac-4NPβGal). From the volumetric enzyme activities added, we expected the overall reaction to take approximately 80 min to complete. The net rate (r_{net}) of the linear cascade reaction was 0.25 mM/min, calculated from the individual rates r_{CSS} and r_{SiaT} as $r_{net} = r_{CSS} \times r_{SiaT}/(r_{CSS} + r_{SiaT})$. The results confirm the expected r_{net}. There was close balance between substrate consumed and products formed, as seen in Figure 4A. The α2,3Neu5Ac-4NPβGal product was formed in amounts corresponding to the CMP released. This indicated that hydrolysis of CMP-Neu5Ac catalyzed by the Z-PdSiaT occurred to a negligible degree. The product yield was ~85%. The intermediate CMP-Neu5Ac accumulated only in small amount (≤ 2 mM) during the reaction course (Figure 4A). One can conclude from this result that the reaction of the immobilized Z-PdSiaT was not completely rate-determining for the overall cascade conversion. This in turn presents indirect evidence that the Z-PdSiaT preparation used was sufficiently stable. Additionally, it suggests that physical transport of CMP-Neu5Ac between solid catalysts of Z-NmCSS and Z-PdSiaT was not a main factor of the conversion rate.
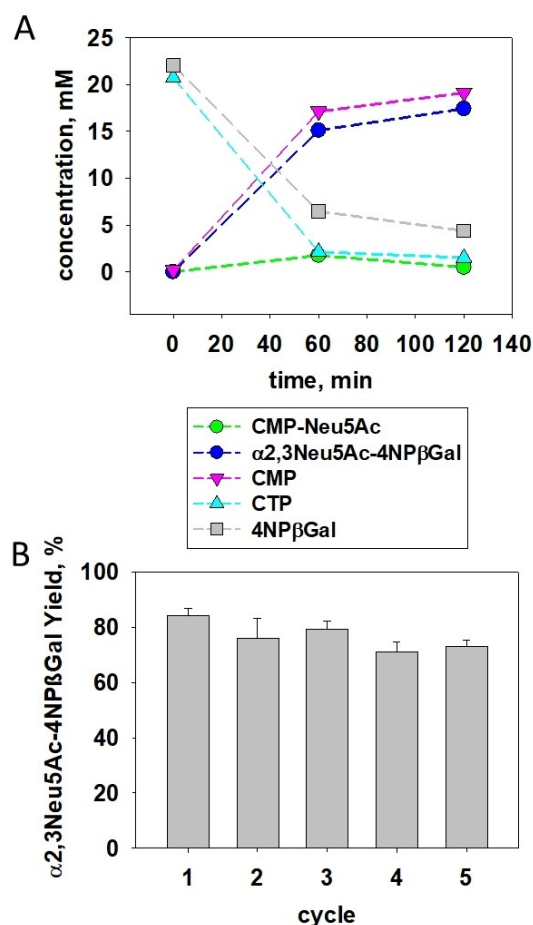

Figure 4. Synthesis of α2,3Neu5Ac-4NPβGal with immobilized preparations of Z-CSS and Z-PdSiaT. (A) Reaction time course for the first cycle. (B) Repeated reaction as in panel A with reuse of the solid catalysts. Each reaction was performed for 120 min. Conditions: 100 mM Tris/HCl (pH 8.0), 0.25 M NaCl, 37°C, 0.2 mM cysteine, 20 mM MgCl₂, 20 mM CTP, 20 mM Neu5Ac, 20 mM 4NPβGal. The starting activity of both catalysts was 0.5 U/mL. The solid catalysts were used at 11 and 71 mg/mL for Z-CSS and Z-PdSiaT, respectively. Error bars show S.D. from 3 replicate experiments.

Recycling of the immobilized enzymes was examined in 5 successive rounds of batch conversion. At the end of each batch lasting 120 min, the solid catalyst was centrifuged off and the supernatant was replaced by the same volume of fresh substrate. Figure 4B shows that the conversion efficiency was upheld well over the 5 batches performed. The product yield was decreased only slightly by ~10% between the first and the last reaction. The result implies that loss of immobilized enzyme activity during the reactions must have been small. Requirement for operational stability is twofold: first, enzyme inactivation occurs at a low rate; and second, Z-enzyme binding to the carrier is strong enough to prevent lixiviation. Supernatants recovered from the reactions were analyzed for released protein, and none was found. The immobilized enzyme was analyzed with SDS PAGE. Samples at reaction start and end showed a similar amount, and a comparable pattern, of protein bound to the solid carriers. The results suggest a stable immobilization of both Z-NmCSS

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and Z-PdSiaT. From the mass amount of immobilized Z-NmCSS (0.1 mg/mL) and Z-PdSiaT (1.7 mg/mL) supplied to the reaction with the ReliSorb SP400 carrier, we calculate a total turnover number of 3 mg α2,3Neu5Ac-4NPβGal product/mg total enzyme protein used. The number of recycles may not be limited to 5, as suggested from Figure 4B. In any case, mechanical stability of the particles was not an issue, as revealed by visual inspection and analysis under the light microscope. Although not examined here, it was shown earlier that inactive Z-enzymes can be eluted conveniently and the carriers be re-used for a new immobilization.[55]

Progress in biocatalyst development for sialo-oligosaccharide synthesis. The development of multi-enzyme catalysts for sialo-oligosaccharide synthesis involves the fundamental decision of whether whole-cell or cell-free preparations of the enzymes should be used.[8,10,56] Each approach has its specific advantages, like the greater flexibility offered by cell-free preparations in diversity-oriented syntheses.[15,57–60] The applied scope of the two approaches is largely complementary. Live whole cells are preferred in large-scale production.[61,62] The remarks are made to suggest explanation for the relative paucity of literature on the immobilization of CSS and SiaT. Whole cell-based catalysts involve immobilization of the relevant enzymes by encapsulation and are attractive in presenting an apparently simple solution.[63,64] However, the different options for biocatalyst preparation notwithstanding, immobilization on solid carrier remains an important goal of the development and a dedicated strategy to achieve it is currently not available.

Immobilization of NmCSS was only studied in context of specialized procedures and applications. N-terminal fusion with hydrophobic peptides (e.g., L protein from bacteriophage MS2) was used to anchor the enzyme in synthetic vesicles referred to as polymersomes.[33] In another example, CSS was immobilized onto magnetic nano particles by native chemical ligation.[34] Activity compared to the free enzyme was given at ~70%, while the immobilization yield was not reported. In the here discussed study, the enzyme activity could be retained for several reaction cycles of synthesizing CMP-Neu5Ac.

Affinity-like immobilization of SiaT was reported for enzyme fusions with tamavidin2, a fungal avidin-binding protein (α2,6SiaT from *Photobacterium* sp. JT-ISH-224),[35,65] the maltose-binding protein (α2,6SiaT from unknown origin),[38] or just the His-tag (α2,3SiaT from rat liver).[36,37] The His-tag SiaT was expressed in insect cell cultures and directly immobilized from the culture medium with an immobilization yield varying in a broad range from ~5% to 60% (optimization focused on ratio between enzyme concentration and carrier ratio). The His-tag immobilization was done on Ni^{2+}-NTA-Agarose. Complex sialo-oligosaccharides (e.g., [α-Neu5Ac-(2→3)-D-Gal*p*-(1→4)-β-$_D$-Glc*p*NAc—O—CH$_2$]$_2$-C-(CH$_2$OBn)$_2$) were obtained in yields of ~70%. About 75% of initial activity was retained in the immobilized enzyme after 40 h, but catalyst recycling was not shown.[36] Despite these interesting findings, applicability of the rat liver SiaT to synthesis appears limited by the difficult expression of the enzyme. A specialized expression host is required[10] and the expression yield was very low (5 U/L insect

culture).[36] This can be compared to expression of the bacterial SiaTs in microbial hosts such as *E. coli* giving 1,000–50,000 U/L microbial culture.[10]

Yu et al.[39] show enzyme immobilization for cascade synthesis of sialo-oligosaccharides. NmCSS and α2,3SiaT from *Pasteurella multocida* were used. Method of site-specific covalent coupling of enzymes on magnetic nanoparticles was developed. The particles were coated with cysteine-poly(ethylene glycol), enabling enzyme attachment via intein-based native chemical ligation.[39] The immobilized enzymes showed excellent effectiveness (NmCSS: ~80%; SiaT: 30%–200%, depending on the acceptor substrate used). Synthesis of a fluorophore-tagged ganglioside product was shown in one-pot cascade reaction that was repeated 10 times with recycle of immobilized CSS and SiaT preparations. Activity loss over all cycles was just ~50%. Yu et al.[39] demonstrate the potential of enzyme immobilization for sialo-oligosaccharide synthesis. Considering limitations on practical applicability due a rather complex method used, their study supports the importance of facile and efficient procedures for CSS and SiaT immobilization.

Conclusion

Major working principles of immobilization were explored with the aim of developing solid-supported, recyclable preparations of NmCSS and PdSiaT for cascade synthesis of sialo-oligosaccharides. Using methacrylate carriers of variable diameter (75–1000 μm) and pore size (10 – 65 nm), we showed that the immobilized enzyme activity, and the corresponding effectiveness factor, benefited strongly (up to 10-fold) from oriented immobilization via the His-tag compared to random immobilization via multipoint covalent coupling with surface epoxy-groups. However, parameters (yield, activity, η) of His-tag immobilization of the PdSiaT showed complex dependence on the carrier used and the metal (Ni^{2+}, Co^{2+}, Cu^{2+}) loaded on it. Oriented immobilization via the Z_{basic2} module in Z-NmCSS and Z-PdSiaT fusion protein presented a major advance. The Z-enzymes retained almost the full specific activity of the His-enzymes. After fine tuning of conditions for each Z-enzyme, Z-NmCSS and Z-PdSiaT were each immobilized on ReliSorb SP400 carrier in a single-step, highly efficient and selective purification-immobilization procedure directly from the corresponding *E. coli* cell lysate. The immobilized Z-enzymes were used over five cycles of α2,3Neu5Ac-4NpβGal synthesis (85% yield, 17 mM) while showing only low (~10%) loss of activity. Immobilization of CSS and SiaT can facilitate the (chemo)enzymatic synthesis of sialo-oligosaccharides as an important class of complex carbohydrate-based products.

Experimental Section

Chemicals and materials. CTP (95% purity; 5% CDP) and CMP (both disodium salts), CMP-Neu5Ac and Neu5Ac were from

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Carbosynth (Compton, Berkshire, UK). 4-Nitrophenyl β-D-galacto-side (4NPβGal) and lactose (monohydrate) were from Sigma Aldrich/Fluka (Vienna, Austria). All other chemicals were of reagent grade from Sigma Aldrich/Fluka or Roth (Karlsruhe, Germany). The carriers for immobilization are described in Table S1 of the Supporting Information.

Strains, plasmids and media. *E. coli* BL21 (DE3) was used for protein production. N-terminally His-tagged NmCSS was expressed from a pC21e1_CSS plasmid vector.[26] PdSiaT, also N-terminally His-tagged, was expressed from a pET23a(+)_∇3PdSiaT plasmid vector.[24] ∇3PdST is an elongated form of the native PdSiaT in which three amino acids (Lys-Thr-Ile-) are added to the N-terminal Met for improved expression.[24] To construct N-terminal fusion with the module Z_{basic2}, both genes without the His-tag were cloned into the plasmid vector pT7Zblinker_BM3.[66] Sequences of the Z-enzyme fusion proteins are given in the Supporting Information. *E. coli* strains were cultured in LB broth and agar plates.

Expression and purification of enzymes. His-CSS and His-PdSiaT were obtained as described previously.[26] Z-CSS and Z-PdSiaT were expressed at the same conditions. Purification of the His- and Z-enzymes is described in the Supporting Information. Protein was determined with Roti-Quant reagent (Roth) referenced to BSA. Purified enzymes were stored in 50 μl aliquots at −20 °C. For His-enzymes (~10 mg/mL), 50 mM Tris/HCl (pH 7.5) was used. For Z-enzymes (~5 mg/mL), potassium phosphate (pH 7.5) supplemented with 0.2 M NaCl was used. Enzymes were stored for 4 weeks without loss in activity. Enzyme stocks were frozen only once.

Activity assays. Assays were performed as previously described.[26] The CSS reaction contained 20 mM Neu5Ac, 20 mM CTP, 20 mM $MgCl_2$, 0.2 mM L-cysteine, and was started with 0.1 μM soluble enzyme (His- or Z-enzyme), 0.5–1.0 mg/mL cell-free extract (CFE, Z-enzyme) or 2–5 mg solid immobilizate (20–50 μl of mixed carrier and supernatant). The PdSiaT reaction contained 1.0 mM CMP-Neu5Ac, 1.0 mM 4NPβGal, and was started with 0.1 μM soluble enzyme (His- or Z-enzyme), 0.5–2.0 mg/mL CFE (Z-enzyme) or 5–10 mg solid immobilizate. For determination of the Z-PdSiaT hydrolase activity, the assay was performed in the absence of 4NPβGal and using the soluble enzyme. In all assays, the enzymatic reaction was stopped by mixing 20 μl of sample with ice-cold acetonitrile (20 μl). The mixture was left on ice for 15 min and was subsequently analyzed by HPLC.

HPLC analysis. CTP, CMP-Neu5Ac, CMP, 4NPβGal and α2,3Neu5Ac-4NPβGal were analyzed on a Shimadzu SPD-20A system equipped with a Kinetex® 5 μm C-18 (100 Å; 50×4.6 mm) column. A 20 mM potassium phosphate buffer (pH 5.9), supplemented with 40 mM tetra-*n*-butylammonium bromide as ion-pairing reagent, was used. Gradient from 6.5% to 25% acetonitrile over 4.5 min was used for elution. The flow rate was 1.5 mL/min at 40 °C and detection was at 254 nm.

General enzyme immobilization. Carriers washed with the suitable buffer were used. Their loading was based on dry matter determined from the as-supplied commercial materials. Unless stated, mass of carrier refers to dry matter. Immobilization was done at the stated temperature and buffer conditions using gentle mixing in an end-over-end rotator (20 rpm). Eppendorf tubes were used. The liquid volume was 1 mL and the carrier loading was ~100 mg based on dry matter. Samples (20 μl) were taken at certain times. Protein concentration and activity were measured. After the immobilization, carriers were washed twice with 1 mL of 50 mM potassium phosphate buffer (pH 7.0) and stored in the same buffer at 4 °C until further use. pH of supernatant was controlled

before and after the immobilization and was found unchanged in all experiments.

The immobilization parameters used were as follows. The immobilization yield for protein (Y_P, %) is given by Equation (1) and the yield for activity (Y_A, %) is defined analogously.

$$Y_P = (P_0\text{-}P_L/P_0) \times 100\% \qquad (1)$$

P_0 is the initial protein concentration and P_L is the protein concentration in the supernatant remaining in supernatant after the immobilization. A_0 and A_L are the corresponding volumetric activities.

The observable activity of the immobilized enzyme preparation (a_I) was measured directly using the activity assays described above. It is expressed as U/g dry carrier.

The effectiveness factor (η, %) was determined by Equation (2).

$$\eta = [a_I/(a_0\text{-}a_L)] \times 100\% \qquad (2)$$

a_0 and a_L are the volumetric activities normalized on the carrier used. They have dimensions of U/g dry carrier.

His-enzyme immobilization on epoxy carriers. Immobilization of purified His-CSS and His-PdSiaT on polymethacrylate carriers harboring epoxide groups (ReliZyme, Lifetech or Sepabeads; for further characterization see Table S1) was performed following a well-established three-step protocol.[67] In the first step, purified His-enzyme (stock: 10–20 mg enzyme/mL) was prepared with a final concentration of 1.0 M potassium phosphate (pH 7.0) and 0.5 mg enzyme/mL. The prepared enzyme loading was incubated with the respective carrier for 4 h at 4 °C or 25 °C. Note: the same temperature was used for all 3 steps. In the second step, the buffer was exchanged to 50 mM potassium phosphate (pH 8.0) and incubation continued for another 24 h. 3 M glycine (pH 8.0) was used to block residual functional groups on the carrier surface overnight in a final step.

His-enzyme immobilization on metal-loaded carriers. Immobilization carriers were chemically functionalized with iminodiacetic acid (IDA) groups and loaded with Ni^{2+}, Cu^{2+} or Co^{2+}. Protocol for functionalization is given in detail in the Supporting Information along with a summary of the physical characteristics of the carriers used (Table S1). Activated carriers were loaded with 0.5–1.0 mg/mL purified enzyme in 50 mM potassium phosphate (pH 7.0) at room temperature and incubated for up to 2 h. For optimization of the immobilization process, the temperature was lowered to 4 °C and 0.1 M NaCl was added to the enzyme loading buffer.

Z-enzyme immobilization. ReliSorb SP400 was used. Carrier (~ 100 mg dry matter) was washed with 1 mL of 50 mM potassium phosphate (pH 7.0) and incubated with 1 mL of enzyme solution containing 0.5–2.0 mg purified Z-enzyme or up to 10 mg protein from cell lysate. The activity of Z-PdSiaT offered was 1.5–40 U, that of Z-CSS was 19–76 U. The enzyme solution was prepared in 50 mM potassium phosphate (pH 7.0) containing 0.25 M NaCl. To optimize the immobilization from the cell lysate, the NaCl concentration was changed and Tween 20 was added, as described under Results and discussion. The suspension was incubated at 25 °C for 1 h.

Cascade reaction for synthesis of α2,3Neu5Ac-4NPβGal. Reactions were performed at 37 °C and 450 rpm in 1 mL 100 mM Tris/HCl buffer (pH 8.0), supplemented with 20 mM $MgCl_2$ and 0.2 mM L-cysteine. CTP (20 mM), Neu5Ac (20 mM) and 4NpβGal (20 mM) were used as substrates. The reaction was started by adding immobilized

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Z-CSS and Z-PdSiaT to an activity of 0.5 U/mL each. At certain times homogeneous sample (20 μl) was taken and processed as described under Activity assays above. After each reaction cycle (2 h), the solid enzymes were centrifuged off (13,500 rpm, 5 min) and washed once with 1 mL of 50 mM potassium phosphate (pH 7.0). Fresh substrate (1 mL) was added and the synthesis repeated. This was performed 5 times.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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