Chemistry–A European Journal
Research Article
doi.org/10.1002/chem.202404688
Chemistry Europe
European Chemical Societies Publishing
www.chemeurj.org

Improving the Long-term Enantioselectivity of a Silicon-Carbon Bond-Forming Enzyme

Annika J. Weber,[a] Celine Moser,[b] Maria Alessandra Martini,[a] Franziska J. Laß,[c] Katharina Bleher,[c] Claudia Muhle-Goll,[b] Christof M. Niemeyer,[a] and Kersten S. Rabe*[a]

Enantioselectivity is a key advantage of enzymatic catalysis. Understanding the most important factors influencing enantioselectivity necessitates thorough investigation for each specific enzyme. In this study, we explore various approaches to optimize reaction conditions for organosilicon production using an immobilized Cytochrome C recently tailored via directed evolution. Over extended reactions, this enzyme experiences a loss of enantioselectivity. Mass spectrometry (MS) revealed covalent modifications on the enzyme, but mutating the respective amino acids did not restore enantioselectivity. Nuclear magnetic resonance (NMR), along with a detailed comparison of the influence of reaction components such as cosolvents and reducing agents, indicated significant conformational changes in the presence of the diazo ester substrate. Additionally, we identified sodium ascorbate as a suitable and milder reducing agent compared to the previously used sodium dithionite, ensuring anaerobic conditions for silicon-carbon bond formation. Ultimately, maintaining a high enzyme-to-substrate ratio in the reaction was found to be crucial for achieving high enantiomeric purity of the organosilicon product over four days in sequential, repetitive batch reactions, thus improving the previously established reaction system. The methods and findings presented here are particularly valuable for addressing enantioselectivity issues in other enzymes that operate with diazo compounds as the substrates in carbene-transfer reactions.

Introduction

Biocatalysis is an emerging and crucial key technology for more sustainable industrial processes.[1] This is especially true since enzymes can be precisely tailored via enzyme engineering[2] and can be combined into reaction cascades.[3] Numerous successful examples of enzymes developed through directed evolution now enable new-to-nature reactions, significantly expanding the range of reactions accessible to biocatalysis.[4] These primarily include heme proteins, which contain a catalytically active iron complex as a cofactor and exhibit high selectivity due to the substrate-specific conformation of their apoenzymes.[5] An outstanding example is the triple mutant of the Cytochrome C from *Rhodothermus marinus*[6] (V75T M100D M103E, here abbreviated as CC) that has been engineered to catalyze the formation of silicon-carbon bonds.[7] This creates the opportunity for the production of valuable enantiomerically pure organosilicon compounds, which are interesting for the development of pharmaceuticals.[8] To enhance the overall productivity of this enzyme, we have recently established the immobilization of CC,[9] as this strategy provides benefits such as easy recovery and reuse of the catalyst and can be achieved through various approaches.[10] Covalent coupling of CC on agarose beads via the SpyTag/SpyCatcher (ST/SC) system[11] allowed its implementation in a flow reactor as well as in sequential batch reactions. Compared to previously described activities,[7] we were able to maintain formation of the expected organosilicon for several days with an increased total product yield. However, we observed a significant decrease in enantioselectivity of the reaction over time. Since enzyme catalysis can be influenced by many factors such as thermodynamic instability over time,[12] intolerance to cosolvents,[13] or detrimental influence of a substrate,[14] a holistic view of the system is necessary for the development of an effective biocatalytic process.[15] In this work, we aimed to understand and minimize the loss in enantiomeric purity of the organosilicon compound produced via CC, by evaluating a range of reaction parameters with potential influence on the enantioselectivity. To this end, we initially established sodium ascorbate (NaAsc) as an alternative reducing agent. Then, we screened the reaction components involved, whether their pre-incubation with CC affected its enantioselectivity, with a particular focus on the diazo ester substrate

[a] A. J. Weber, Dr. M. A. Martini, Prof. Dr. C. M. Niemeyer, K. S. Rabe
Institute for Biological Interfaces 1 (IBG-1)
Karlsruhe Institute of Technology (KIT)
Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen (Germany)
E-mail: kersten.rabe@kit.edu

[b] C. Moser, Dr. C. Muhle-Goll
Institute for Biological Interfaces 4 (IBG-4)
Karlsruhe Institute of Technology (KIT)
Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen (Germany)

[c] F. J. Laß, Dr. K. Bleher
Institute of Functional Interfaces (IFG)
Karlsruhe Institute of Technology (KIT)
Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen (Germany)

Supporting information for this article is available on the WWW under https://doi.org/10.1002/chem.202404688

and its impact on the enzyme. As a result, we could successfully produce the desired organosilicon compound with consistently high enantiomeric purity in a four-days sequential batch experiment.

Results and Discussion

Sodium Ascorbate as Alternative Reducing Agent for CC

Since the enzyme is initially purified and handled under aerobic conditions, the initial step in the model reaction of CC requires the activation of the enzyme by the removal of the bound molecular oxygen from the heme cofactor via reduction of the iron ion from Fe^{3+} to Fe^{2+} (Figure S1). As reported in the literature, this is carried out under anaerobic atmosphere using the reducing agent sodium dithionite (NaDT, $Na_2S_2O_4$). Cofactor activation then allows the reaction of ethyl 2-diazopropanoate (**1**) and phenyl dimethylsilane (**2**) to proceed, resulting in the organosilicon product (**3**) (Figure 1a).[7,9] NaDT-mediated reduction of metalloenzymes is commonly used thanks to its high redox potential ($E^{0\prime} = -0.66$ V vs SHE at pH 7),[16] affordability and ease of use. However, it has been reported that NaDT (or its degradation products)[17] can interact with some metalloenzymes.[18] We thus decided to investigate if the use of this reducing agent could be the source for the decreased enantioselectivity we observed. A widely used milder reducing agent is sodium ascorbate (NaAsc, $NaC_6H_7NaO_6$, redox potential $E^{0\prime} = -0.08$ V vs SHE at pH 7),[19] that is often employed for metal ion reduction in chemical synthesis, as in CuAAC reaction,[20] but also to generate the reduced state in metalloenzymes.[21] We thus exchanged NaDT for NaAsc and employed a 2000-fold excess of reducing agent compared to CC and could detect the expected color change via the specific absorption of the reduced state at a wavelength of 550 nm, proving the reducing capability of NaAsc for CC (Figure S1). The reduction occurred with a slower kinetic, probably due to the larger molecular size of NaAsc compared to the $SO_2^{\bullet}$ radical, which is the reducing species of NaDT demanding for a greater protein rearrangement to reach the cofactor as well as due to the higher redox potential. We then confirmed that NaAsc also enables the biocatalytic formation of a silicon-carbon bond between substrates **1** and **2**, albeit with a lower overall yield of **3** after 4 h when compared to the reaction with NaDT (Figure 1b). Indeed, this was to be expected, since we have recently reported[9] that substrate conversion by CC competes with the decomposition of **1** due to hydrolysis in the aqueous solution.[9] Due to the slow reduction of CC by NaAsc, there is a delay in enzyme catalysis. During this delay, **1** gradually decomposes, resulting in less availability of **1** when CC is ready to convert it. Encouragingly, the change in reducing agent also resulted in high enantioselectivity in these batch reactions. As established in previous work,[9] **3** was identified via GC and the enantiomeric purity was determined by chiral HPLC (Figure S2).

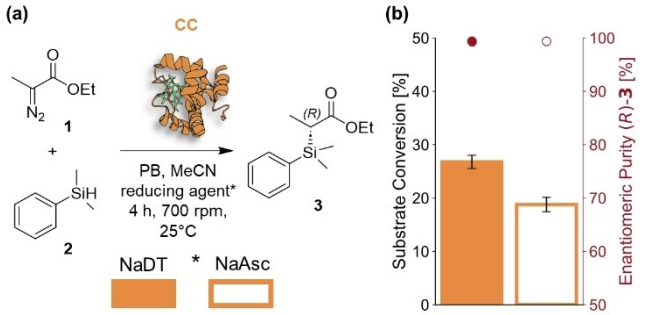

Figure 1. Sodium ascorbate (NaAsc) as alternative reducing agent for CC catalyzing silicon-carbon bond formation. (**a**) Ethyl 2-diazopropanoate (**1**) and phenyl dimethylsilane (**2**) are converted to the organosilicon (**3**). The reaction can be initiated by reducing the iron in the heme cofactor through addition of sodium dithionite (NaDT)[7,9] or alternatively by NaAsc. (**b**) Substrate conversion of CC (bars) and enantiomeric purity (circles) of product **3** formed in batch reactions with 5 μM enzyme, 10 mM NaDT (filled) or NaAsc (unfilled), 10 mM **1** and 10 mM **2** in PB (phosphate buffer, 50 mM sodium phosphates, pH 7.4) with 5% MeCN for 4 h under anaerobic conditions at room temperature. Activities are indicated as substrate conversions, which are defined as the ratio of the amount of product formed during the reaction time, measured via GC, to the amount of applied **1** following established protocols.[9] Enantiomeric purity is given as the proportion of the *(R)*-enantiomer of **3** formed as determined by chiral HPLC following established protocols.[9] Error bars were obtained from two experiments.

To account for the short lifespan of **1** when investigating the CC productivity over prolonged time periods, sequential batch reactions can be employed, which also profit from the convenient separation of immobilized catalyst from reaction solution by simple sedimentation (Figure 2a). To investigate the differences of NaDT and NaAsc on the enantioselectivity in such reactions, CC was immobilized on agarose beads (AB) via the SpyTag/SpyCatcher (ST/SC) coupling system[11] (Figure 2b) as previously reported.[9,22] CC@AB were then employed in sequential batch reactions to perform the model reaction (Figure 1a) under anaerobic conditions in PB (phosphate buffer, 50 mM sodium phosphates, pH 7.4) with 25 % MeCN, optimized for immobilized CC@AB determined in the previous work (Figure 2c).[9] As expected from our previous work, the use of NaDT as the reducing agent led to continuous production of **3** over four days (filled bars), however with decreasing enantiomeric purity (filled circles). Using NaAsc as the reducing agent also led to conversion over four days (unfilled bars), however the substrate conversion remained below that of the approach with NaDT, as expected from the experiments in solution (Figure 1b). Although the enantioselectivity was slightly increased (Figure 2c, unfilled circles), the use of NaAsc as reducing agent did not maintain enantiomeric purity of product **3** to the levels observed with free CC (Figure 1b). This observation indicated that potential soluble oxidation products of the NaDT, which are not present when NaAsc is used as a reducing agent, are not exclusively responsible for the decrease in enantioselectivity.

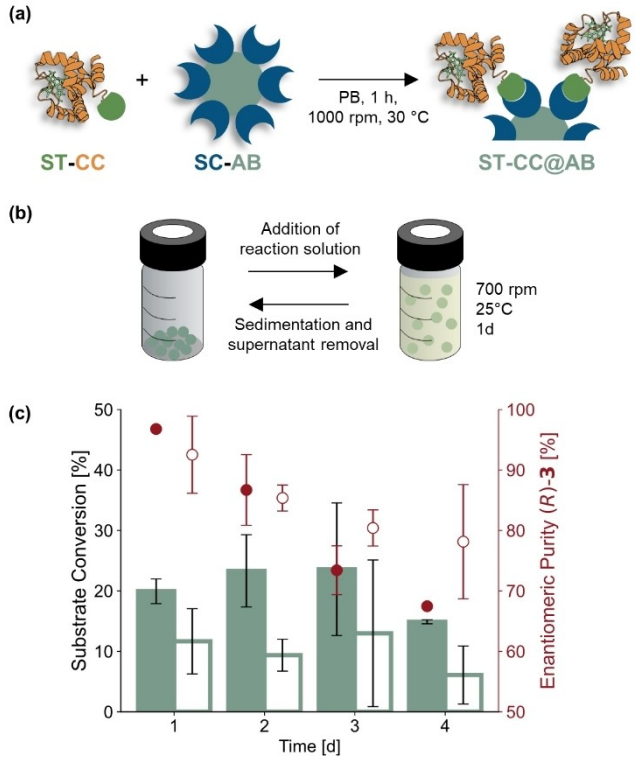

Figure 2. Sequential batch reaction to investigate long-term productivity and reusability of immobilized CC. (**a**) Immobilizing CC was achieved via the SpyTag/SpyCatcher (ST/SC) system,[11] coupling ST-modified CC (ST–CC) to SC-decorated agarose particles (SC-AB). (**b**) Suspension of heterogeneous CC catalyst was separated after one day of reaction to analyze the supernatant. Subsequently it was resuspended in fresh reaction solution. (**c**) Substrate conversion (bars) and enantiomeric purity (circles) were monitored over four days in sequential batch reactions under anaerobic conditions using 2 nmol CC@AB and reaction solutions consisting of 10 mM NaDT (filled) or NaAsc (unfilled), 10 mM **1** and 10 mM **2** in PB with 25 % MeCN. Error bars were obtained from two independent experiments.

Screening of Reaction Components

Since a simple change from NaDT to the milder reducing agent NaAsc did not maintain the enantioselectivity of CC, we decided to investigate the individual reaction components regarding their detrimental effects on the biocatalytic activity in silicon-carbon bond formation. To this end, CC was pre-incubated with the components for four days (Figure 3a, I) followed by the addition of the full reaction solution containing all reaction components (Figure 3a, II). In separate reactions, different compositions of reaction components were added during the pre-incubation for four days (Figure 3b), ranging from only CC in PB (green) to CC in PB with 25 % MeCN (purple), in PB with 25 % MeCN and NaDT (dark blue) or in PB with 25 % MeCN, NaDT and diazo ester **1** (grey) or silane substrate **2** (light blue). The influence of the different pre-incubation conditions on the substrate conversion and enantiomeric purity of product **3** was then compared to a batch reaction lacking any pre-incubation (orange). While all other five different pre-incubation conditions had no effect on substrate conversion, the pre-incubation in the presence of **1** (grey) resulted in a substantial decrease in enantiomeric purity of product **3** to 60 %. In fact, this result is in line with the results from the sequential batch reaction employing CC@AB (Figure 2c). Since this was not observed upon pre-incubation of CC for four days neither in pure PB (green) nor in PB with 25 % MeCN (purple), a time-dependent ageing process based on unfolding of the enzyme could be ruled out as the cause. Furthermore, pre-incubation in the presence of NaDT (blue) confirmed the above assumption that the use of NaDT has no adverse effects on the enzyme. Thus, as the enantiomeric purity only decreased significantly upon pre-incubation with **1**, and while the overall activity remained the same, we considered that structural changes in the protein close to the heme cofactor caused by **1** might lead to a less specific binding of the substrates to the catalytic site.

In previous work, Renata *et al.*[23] observed covalent modification of nucleophilic amino acids in a heme-containing P450 cytochrome by the reactive carbene intermediate formed from a diazo ester at the heme, in the absence of a second substrate. Although this finding, identified via mass spectrometry (MS), involved a different enzyme system, we decided to perform similar investigations for 2 nmol ST-CC that was incubated under anaerobic conditions for four days with daily addition of 100 µL PB with 25 % MeCN, 10 mM NaDT and 10 mM **1** (Figure 3c). Subsequently the treated ST-CC was purified via SDS-PAGE and fragmented by tryptic digestion. The resulting fragments were released from the gel, separated via UHPLC and analyzed via MS. We indeed found indications that two adjacent amino acids of CC (K65 and R66) were covalently modified by **1** (Figure S3). Although the two amino acids were on the opposite side of the substrate tunnel (Figure 3d),[24] a substantial influence on the overall conformation of the entire protein was conceivable. However, when exchanging these nucleophilic amino acids against more hydrophobic residues by site-directed mutagenesis, leading to the enzyme variants CC(LV) and CC(VT) (Figure S4), no improvement of the enantioselectivity could be observed when pre-incubated with **1** (Figure 3e). These findings ruled out covalent modifications as the cause of the reduced enantioselectivity.

To further investigate potential smaller perturbation of the enzyme structure around the active site, we produced ^{15}N-labeled CC to conduct nuclear magnetic resonance (NMR) studies (Figure S5). These revealed that **1** induced conformational changes for more than 40 % of the amino acids all over the structure of CC, independent of whether NaAsc or NaDT as a reducing agent was present. This conformational change of CC driven by the presence of **1** seems to be a likely cause for the reduced enantioselectivity, as it presumably facilitates multiple orientations of the two substrates in the heme pocket and enabling the formation of the *(S)*-enantiomer of product **3**.

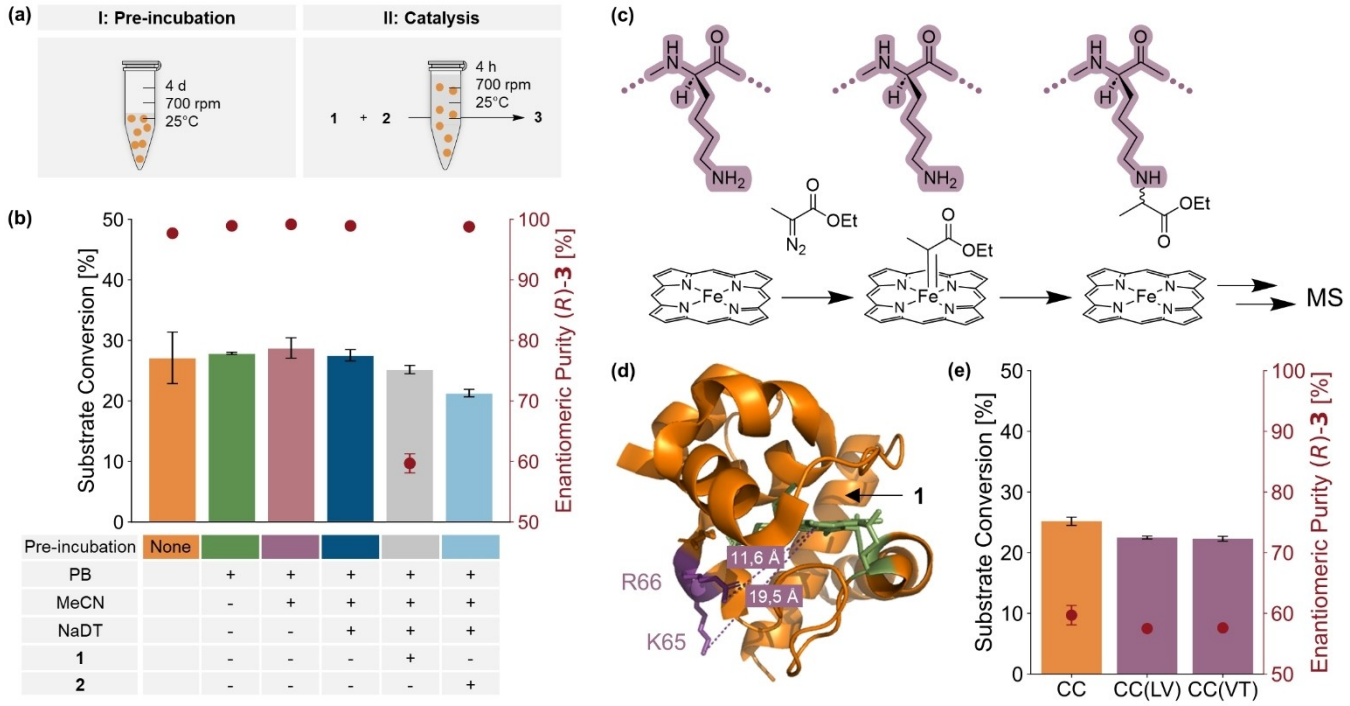

Figure 3. Influence of reaction components on CC activity and enantioselectivity. (**a**) Pre-incubation of 2 nmol CC for four days at room temperature under anaerobic conditions with different combinations of the reaction components involved (I) with subsequent addition of all reaction components and reaction for 4 h (II). (**b**) Substrate conversion (bars) and enantiomeric purity (circles) of the different pre-incubation conditions (color-coded). A reference reaction with CC under standard reaction conditions, not pre-incubated at all (orange), was compared to CC pre-incubated in PB (green), in PB with 25% MeCN (purple), in PB with 25% MeCN and 10 mM NaDT (dark blue), in PB with 25% MeCN, 10 mM NaDT and 10 mM **1** (grey) and in PB with 25% MeCN, 10 mM NaDT and 10 mM **2** (bright blue). The biocatalytic reaction was then initiated by the addition of 10 mM NaDT, 10 mM **1** and 10 mM **2** in PB with 25% MeCN. After 4 h, substrate conversion (bars) and enantiomeric purity (circles) were determined by GC and chiral HPLC, respectively. (**c**) Potential reaction product of the nucleophilic amino acid lysine modified by the reactive intermediate of **1**, as reported in the literature for a different heme-containing enzyme can be analyzed using mass spectrometry (MS).[23] (**d**) Incubation of 2 nmol ST-CC under anaerobic conditions for four days with daily addition of 100 μL PB with 25% MeCN, 10 mM NaDT and 10 mM **1** led to the transfer of the carbene to the amino acids K65 and R66 (purple, crystal structure of CC (PDB: 6CUK)[25]), as identified using MS. The modified amino acids (their distance to the heme cofactor is given) are located on the opposite side of the heme anchor points (green) and the substrate tunnel.[24] (**e**) Influence of pre-incubation of 2 nmol either CC (orange) or CC mutants (purple), where the respective amino acids had been replaced by non-nucleophilic residues, characterized by pre-incubation with **1** and subsequent biocatalytic reaction under the same conditions as in (b, grey bar). Error bars were obtained from two experiments.

Dependence of Enantioselectivity from CC and 1 Ratio

Given that the presence of **1** influences the structure of CC and thus its enantioselectivity, we next investigated the impact of the relative ratio of enzyme to substrate. To this end, the standard reaction was carried out under varying CC concentrations (0.1, 1, 5 and 25 μM) while the substrate concentration was kept constant at 10 mM (Figure 4a). The more CC is present in comparison to **1**, the faster **1** is converted to the product **3**, reducing the overall time CC is exposed to **1**. As expected, lower concentrations of CC led to reduced enantiomeric purities of product **3**. This was in line with the results from NMR analysis, wherein a 1:1 stoichiometry of CC (200 μM) to substrate **1** (200 μM) led to significantly less amino acids with chemical shifts if compared to a 1:50 ratio (200 μM CC:10 mM **1**) (Figure 4b, c).

Based on the above findings, we reasoned that performing sequential batch reactions employing CC@AB as before (Figure 2), but with a lower relative ratio of **1** to CC, would lead to improvements of the reaction outcome. As expected and desired, product **3** could be produced over a period of four days with a consistently elevated enantiomeric purity approaching 100% (Figure 4d). Hence, this process development clearly showed that substrate **1** needs to be converted fast to reduce its detrimental impact on the enzyme and to improve enantiomeric purity. This lower substrate availability leads to enhanced product purity, albeit at the expense of a lower yield unless higher enzyme concentrations are used (Figure 4a, 25 μM). As shown in previous work,[9] the availability of compound **1** can be enhanced, leading to higher substrate conversions, when the reaction is conducted in a flow reactor with a continuous feed of pure MeCN. However, the development of the process described here offers a decisive advantage by avoiding further purification procedures to separate the enantiomers of **3**, with simultaneous recovery and reuse of the catalyst. The findings also open up the possibility of technically realizing enantiomerically pure organosilicon production employing CC in fed-batch or flow reactions by adjusting the amount of substrate applied.

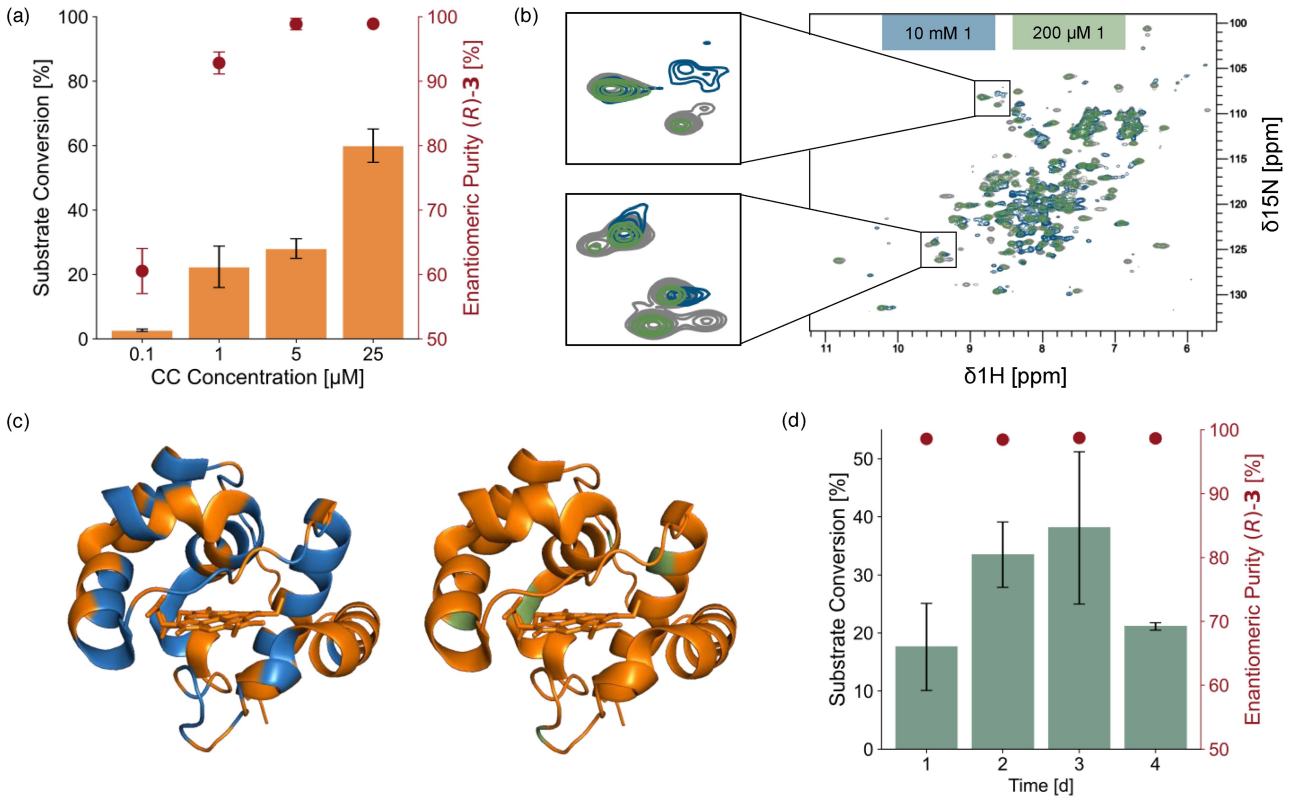

Figure 4. Dependence of enantioselectivity on the relative ratio of CC and **1**. (**a**) Substrate conversion (bars) and enantiomeric purity (circles) of product **3** formed in batch reactions employing different enzyme concentrations (0.1, 1, 5 and 25 μM) with 10 mM NaDT, 10 mM **1** and 10 mM **2** in PB with 25% MeCN for 4 h under anaerobic conditions at room temperature. (**b**) ^{1}H-^{15}N-HSQC spectrum of 200 μM ^{15}N-CC incubated with 10 mM NaDT (grey) and additional 10 mM **1** (blue) or 200 μM **1** (green), with exemplary zoomed-in images of peaks which shift upon addition of **1**. (**c**) Chemical shift perturbation was used to identify changes of CC amino acid residues positions caused by the addition of 10 mM **1** (blue) or 200 μM **1** (green). Relative chemical shifts were determined according to literature.[26] Note that due to various overlaps, unambiguous assignment of the peaks to specific amino acid residues was not always possible. Amino acids that either shifted more than 0.1 ppm or disappeared in the presence of **1** were highlighted in the crystal structure of CC (PDB: 6CUK).[25] (**d**) Substrate conversion (bars) and enantiomeric purity (circles) in sequential batch reactions over four days under anaerobic conditions using 2 nmol CC@AB and reaction solutions consisting of 10 mM NaDT, 1.25 mM **1** and 10 mM **2** in PB with 25% MeCN. Error bars were obtained from two independent experiments.

Conclusions

In summary, through extensive investigations employing a range of methodological approaches, we have developed a comprehensive understanding that is crucial for engineering and optimizing reaction systems utilizing the carbon-silicon bond forming catalyst CC. The ratio of enzyme to diazo substrate was identified to be a critical factor influencing the enantiomeric purity of the organosilicon produced. Using this knowledge, we could establish excellent enantiomeric purities in repetitive, sequential batch reactions for the first time, improving significantly over previous work.[9] Additionally, we introduced sodium ascorbate as an alternative reducing agent that facilitates the reduction of the CC catalyst and the formation of the silicon-carbon bond. This approach may also be valuable for other similar reactions where sodium dithionite needs to be replaced. Our study emphasizes that MS and NMR analysis are useful tools to investigate causes for reduced enantioselectivity. Specifically, MS revealed two covalent modifications on adjacent amino acid residues of the enzyme via a reaction intermediate, which however had no influence on the enantioselectivity. Using NMR analyses of ^{15}N-labeled CC we also gained insights into conformational changes due to non-covalent interactions induced by substrate **1**. Our study thus introduces a variety of methods to approach enzymatic enantioselectivity issues that can be useful for other enzymes facing similar challenges, particularly those that utilize diazo compounds as substrates in carbene-transfer reaction mechanisms.[5c,27] Since the high selectivity of enzymes is one of the most significant factors in biocatalytic production of pharmaceuticals, the results of this work, which demonstrates the influence of substrates, can also provide valuable insights for other syntheses that require high enantiomeric purity. Based on the findings of this study, suitable high-throughput screening methods could be developed in the further studies to generate mutants that exhibit higher tolerance to substrate **1** while maintaining enantioselectivity. In future works focusing on scaling up or numbering up the repetitive batch process or the establishment of other reaction systems such as fed-batch or flow reactions, considering the knowledge from this study can pave the way to preparative scale reactions that yield enantiomerically pure organosilicon, making industrially relevant applications of CC feasible.

Experimental Section

Further experimental details on protein analysis, activity determination as well as methods relevant for data presented in the Supporting Information are given there.

Protein Production

Standard procedure. SC was produced heterologously, as in previously described procedures.[10e] *E. coli* BL21(DE3) was transformed with the corresponding plasmid using heat shock transformation. *E. coli* cells harboring the plasmid were selected on LB/agar plates containing 100 $\mu g \cdot mL^{-1}$ ampicillin overnight at 37 °C. Liquid cultures of 100 mL TB medium containing ampicillin were generated from a single colony and cultured at 37 °C and 180 rpm for 16 h. Two flasks with 2 L of ampicillin-containing TB-medium were inoculated with 40 mL overnight culture and incubated at 37 °C and 180 rpm until an OD_{600} of 1.1 was reached. The temperature was then lowered to 25 °C, 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the cultures were incubated for an additional 20 h at 25 °C and 180 rpm. The cells were harvested by centrifugation at 10,000 rcf and 4 °C for 10 min, resuspended in 60 mL NP_i10 buffer (500 M NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0) and stored at −80 °C until further processing.

Adjustments for CC and CC variants. The heterologous expression of CC variants was also performed similar to that previously described[9] and deviations from the expression above are indicated in the following. The respective plasmid for the CC variant was co-transformed with the Cytochrome C maturation plasmid pEC86[28] into *E. coli* BL21(DE3) using electroporation. LB/agar plates for the selection of the *E. coli* cells harboring both plasmids, as well as the liquid TB cultures, contained 30 $\mu g \cdot mL^{-1}$ chloramphenicol and 100 $\mu g \cdot mL^{-1}$ ampicillin. After an OD_{600} of 1.1 was reached, the culture was then cooled on ice for 20 min. Expression was induced by adding 20 μM IPTG and 200 μM 5-aminolevulinic acid and the culture was incubated at 20 °C and 180 rpm for 20 h.

Adjustments for ^{15}N-CC. The heterologous expression of ^{15}N and ^{13}C labeled CC was also performed similar to unlabeled CC, based on an adapted protocol from the literature.[29] Cultures of *E. coli* BL21(DE3) harboring the plasmids pET22_pelB-CytC(TDE)-his_6[9] and pEC86[28] were grown in 1 L of LB media until the OD_{600} reached 1.0. The cultures were centrifuged for 30 min at 6,400 g and 4 °C. The pellet was then resuspended in 1 L M9 Minimal Media supplemented with 0.5 mg/L^{-1} $^{15}NH_4Cl$ and 2 $g \cdot L^{-1}$ of ^{13}C-glucose as sole nitrogen and carbon sources and 200 μM 5-aminolevulinic acid was added. Cultures were grown for 1 h at 37 °C and 220 rpm and inoculated with 20 μM IPTG. Cultures were shaken continuously for 20 h at 30 °C.

Protein purification. All proteins carried a his_6-tag, which enabled purification via fast protein liquid chromatography (FPLC) using its affinity to Ni, as previously described.[9] The cell suspension was thawed at 25 ° and subsequently incubated with DNaseI and lysozyme (AppliChem) for 30 min at room temperature. After ultrasonication, cell debris was removed using centrifugation for 1 h at 45,000 rcf and 4 °C and the protein-rich supernatant filtered through a 0.45 μm PVDF membrane (Durapore, Steriflip, Millipore). The lysate was applied on a Ni column (5 mL HisTrap™ FF, Cytiva) installed in an FPLC system (Äkta pure, GE Healthcare). The column was first washed with a mixture of 2% NP_i500 (500 mM NaCl, 50 mM NaH_2PO_4, 500 mM imidazole, pH 8.0) and 98% NP_i10 and subsequently the protein was eluted using a linear gradient from 2% NP_i500 and 98% NP_i10 to 100% NP_i500 over 10 column volumes. The collected fractions containing the purified protein were combined and buffer exchanged to PBS (phosphate-buffered saline, 50 mM NaCl, 8 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, pH 7.3) for SC and PB (phosphate buffer, 50 mM sodium phosphate, pH 7.4) for the CC variants using centrifugal concentrators (Vivaspin 20, 5,000 MWCO, Sartorius). The concentrated protein solution was aliquoted, frozen in liquid nitrogen and stored at −80 °C until use.

Activity Determination

Batch reactions. The activity of CC regarding the formation of silicon-carbon bonds was studied as previously described.[7,9] In a total volume of 400 μL of anaerobic PB containing 5% acetonitrile (MeCN), 5 μM of free CC were mixed with 10 mM sodium dithionite, 10 mM ethyl 2-diazopropanoate (**1**) and 10 mM phenyl dimethylsilane (**2**). The components were combined in an anaerobic vinyl chamber and incubated anaerobically for 4 h on a thermoshaker at 25 °C and 700 rpm. The reaction was stopped by adding cyclohexane and analyzed by GC and chiral HPLC.

Pre-incubation experiments. In a total volume of 200 μL 2 nmol of free CC were mixed in an anaerobic vinyl chamber in different approaches with different combinations of the reaction components and incubated anaerobically for 4 h on a thermoshaker at 25 °C and 700 rpm. CC was kept exclusively in PB, supplemented with a 25% MeCN proportion, 10 mM reducing agent and/or 10 mM **1**. After the pre-incubation 200 μL of a reaction solution consisting of 20 mM reducing agent, 20 mM **1**, 20 mM **2** in anaerobic PB with 25% MeCN was added, resulting in 10 mM reducing agent, 10 mM **1** and 10 mM **2** in the total solution as in the batch reactions. The reaction was stopped by adding cyclohexane and analyzed by GC and chiral HPLC.

Preparation of CC@AB. SC proteins were loaded on Agarose beads (AB, PureCube Epoxy Activated Agarose, Cube Biotech) following the manufacturer's instructions resulting in SC-AB as previously described.[9,22] ST-CC was bound by incubating 500 pmol enzyme per μL of SC-AB in PB for 1 h at 30 °C and 1,000 rpm (CC@AB). The amount of ST-CC bound to the AB was previously determined as 93 $pmol_{ST\text{-}CC}/\mu L_{AB}$.[9]

Sequential batch reactions. Long-term activity and reuse of immobilized CC was investigated using sequential batch reactions similarly as described previously.[9] In a total volume of 400 μL anaerobic PB with 25% MeCN, 21.5 μL CC@AB were incubated with 10 mM reducing agent, 10 or 1.25 mM **1** and 10 mM **2** anaerobically for 4 h on a thermoshaker at 25 °C and 700 rpm. Every 24 h, CC@AB were sedimented, the supernatant was removed and analyzed by GC and chiral HPLC. CC@AB were resuspended in fresh reaction solution and incubated further. This was carried out on four consecutive days.

NMR Analysis

In a glass vial in an anaerobic vinyl chamber, 200 μM ^{15}N-labeled CC were mixed with 10 mM reducing agent and/or 20 mM **1** in a total volume of 200 μL PB with 10% deuterated H_2O and 5% deuterated MeCN. The mixture was transferred to an NMR tube and analyzed with an NMR spectrometer (Avance III, 600 MHz, Bruker BioSpin) using a TXI cryoprobe. ^{1}H-^{15}N-HSQC-spectra (10 scans, 256 points in the indirect dimension) as well as standard triple resonance backbone experiments (^{1}H-^{13}C-^{15}N-HNCA, ^{1}H-^{13}C-^{15}N-HNCOCA, ^{1}H-^{13}C-^{15}N-HNCACB and ^{1}H-^{13}C-^{15}N-HNCOCACB were acquired at 301 K. Spectra were acquired and

processed using Topspin (Bruker BioSpin) and analyzed using CcpNMR Analysis.

Supporting Information

The authors have cited additional references within the Supporting Information.[30–37]

Acknowledgements

This work was supported through the Helmholtz program "Materials Systems Engineering" under the topic "Adaptive and Bioinstructive Materials Systems" (43.33.11). A.J.W. is grateful for support by a Kekulé fellowship by Fonds der Chemischen Industrie (FCI). We thank Madleen Richter for experimental support. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interests

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.

Keywords: Anaerobic synthesis · Cytochromes · enantioselectivity · organosilicon · protein NMR

[1] a) A. R. Alcántara, P. D. de María, J. A. Littlechild, M. Schürmann, R. A. Sheldon, R. Wohlgemuth, *ChemSusChem* **2022**, *15*; b) A. Kinner, P. Nerke, R. Siedentop, T. Steinmetz, T. Classen, K. Rosenthal, M. Nett, J. Pietruszka, S. Lütz, *Biomedicine* **2022**, *10*; c) S. P. France, R. D. Lewis, C. A. Martinez, *JACS Au* **2023**, *3*, 715–735.

[2] a) S. Lutz, S. M. Iamurri, *Methods Mol. Biol.* **2018**, *1685*, 1–12; b) R. Buller, S. Lutz, R. J. Kazlauskas, R. Snajdrova, J. C. Moore, U. T. Bornscheuer, *Science* **2023**, *382*, eadh8615.

[3] a) K. S. Rabe, J. Muller, M. Skoupi, C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2017**, *56*, 13574–13589; b) S. P. France, L. J. Hepworth, N. J. Turner, S. L. Flitsch, *ACS Catal.* **2017**, *7*, 710–724; c) J. A. McIntosh, A. E. Owens, *Curr. Opin. Green Sustain. Chem.* **2021**, *29*.

[4] a) F. H. Arnold, A. A. Volkov, *Curr. Opin. Chem. Biol.* **1999**, *3*, 54–59; b) F. H. Arnold, *Angew. Chem. Int. Ed.* **2018**, *57*, 4143–4148; c) K. Chen, F. H. Arnold, *Nat. Catal.* **2020**, *3*, 203–213; d) Y. J. Wang, P. Xue, M. F. Cao, T. H. Yu, S. T. Lane, H. M. Zhao, *Chem. Rev.* **2021**, *121*, 12384–12444.

[5] a) O. F. Brandenberg, R. Fasan, F. H. Arnold, *Curr. Opin. Biotechnol.* **2017**, *47*, 102–111; b) L. M. Schmitz, K. Rosenthal, S. Lütz, *Biotechnol. Bioeng.* **2019**, *116*, 3469–3475; c) P. Kaur, V. Tyagi, *Adv. Synth. Catal.* **2021**, *363*, 877–905; d) X. K. Ren, R. Fasan, *Curr. Opin. Green Sustain. Chem.* **2021**, *31*; e) G. Roelfes, *J. Inorg. Biochem.* **2021**, *222*, 111523; f) Y. Yang, F. H. Arnold, *Acc. Chem. Res.* **2021**, *54*, 1209–1225; g) I. Kalvet, M. Ortmayer, J. Zhao, R. Crawshaw, N. M. Ennist, C. Levy, A. Roy, A. P. Green, D. Baker, *J. Am. Chem. Soc.* **2023**, *145*, 14307–14315.

[6] M. Stelter, A. M. Melo, M. M. Pereira, C. M. Gomes, G. O. Hreggvidsson, S. Hjorleifsdottir, L. M. Saraiva, M. Teixeira, M. Archer, *Biochemistry* **2008**, *47*, 11953–11963.

[7] S. B. Kan, R. D. Lewis, K. Chen, F. H. Arnold, *Science* **2016**, *354*, 1048–1051.

[8] a) E. Rémond, C. Martin, J. Martinez, F. Cavelier, *Chem. Rev.* **2016**, *116*, 11654–11684; b) R. Ramesh, D. S. Reddy, *J. Med. Chem.* **2018**, *61*, 3779–3798; c) J. L. Panayides, D. L. Riley, F. Hasenmaile, W. A. L. van Otterlo, *RSC Med. Chem.* **2024**.

[9] S. Gallus, E. Mittmann, A. J. Weber, M. Peng, C. M. Niemeyer, K. S. Rabe, *ChemCatChem* **2023**, *15*.

[10] a) Y. Jiang, J. X. Zheng, M. N. Wang, W. Q. Xu, Y. Q. Wang, L. Wen, J. Dong, *Appl. Biochem. Biotechnol.* **2024**; b) R. A. Sheldon, A. Basso, D. Brady, *Chem. Soc. Rev.* **2021**, *50*, 5850–5862; c) T. Peschke, M. Skoupi, T. Burgahn, S. Gallus, I. Ahmed, K. S. Rabe, C. M. Niemeyer, *ACS Catal.* **2017**, *7*, 7866–7872; d) S. Gallus, E. Mittmann, K. S. Rabe, *ChemBioChem* **2022**, *23*, e202100472; e) T. Peschke, P. Bitterwolf, S. Gallus, Y. Hu, C. Oelschlaeger, N. Willenbacher, K. S. Rabe, C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2018**, *57*, 17028–17032; f) J. S. Hertel, P. Bitterwolf, S. Kroll, A. Winterhalter, A. J. Weber, M. Grosche, L. B. Walkowsky, S. Heissler, M. Schwotzer, C. Woll, T. van de Kamp, M. Zuber, T. Baumbach, K. S. Rabe, C. M. Niemeyer, *Adv. Mater.* **2023**, *35*, e2303952.

[11] B. Zakeri, J. O. Fierer, E. Celik, E. C. Chittock, U. Schwarz-Linek, V. T. Moy, M. Howarth, *Proc. Nat. Acad. Sci. USA* **2012**, *109*, E690–E697.

[12] a) P. V. Iyer, L. Ananthanarayan, *Process Biochem.* **2008**, *43*, 1019–1032; b) A. S. Bommarius, M. F. Paye, *Chem. Soc. Rev.* **2013**, *42*, 6534–6565; c) Q. Liu, G. Xun, Y. Feng, *Biotechnol. Adv.* **2019**, *37*, 530–537; d) M. Musil, H. Konegger, J. Hong, D. Bednar, J. Damborsky, *ACS Catal.* **2019**, *9*, 1033–1054.

[13] a) A. Wolff, A. J. J. Straathof, J. A. Jongejan, J. J. Heijnen, *Biocatal. Biotransform.* **1997**, *15*, 175–184; b) M. Persson, D. Costes, E. Wehtje, P. Adlercreutz, *Enzyme Microb. Technol.* **2002**, *30*, 916–923; c) G. de Gonzalo, G. Ottolina, F. Zambianchi, M. W. Fraaije, G. Carrea, *J. Mol. Catal. B* **2006**, *39*, 91–97.

[14] a) K. Fujiwara, D. Tsuru, *Int. J. Pept. Protein Res.* **1973**, *5*, 447–453; b) W. V. Tuomi, R. J. Kazlauskas, *J. Org. Chem.* **1999**, *64*, 2638–2647; c) A. Tafi, F. Manetti, M. Botta, S. Casati, E. Santaniello, *Tetrahedron: Asymmetry* **2004**, *15*, 2345–2350; d) K. L. Fow, L. C. H. Poon, S. T. Sim, G. K. Chuah, S. Jaenicke, *Eng. Life Sci.* **2008**, *8*, 372–380; e) A. A. Koesoema, Y. Sugiyama, K. T. Sriwong, Z. C. Xu, S. Verina, D. M. Standley, M. Senda, T. Senda, T. Matsuda, *Appl. Microbiol. Biotechnol.* **2019**, *103*, 9529–9541.

[15] a) R. A. Sheldon, P. C. Pereira, *Chem. Soc. Rev.* **2017**, *46*, 2678–2691; b) K. Y. Chook, M. K. Aroua, L. T. Gew, *Ind. Eng. Chem. Res.* **2023**, *62*, 10800–10812.

[16] a) S. G. Mayhew, *Eur. J. Biochem.* **1978**, *85*, 535–547; b) A. W. DeMartino, L. Poudel, M. R. Dent, X. Chen, Q. Xu, B. S. Gladwin, J. Tejero, S. Basu, E. Alipour, Y. Jiang, J. J. Rose, M. T. Gladwin, D. B. Kim-Shapiro, *Nat. Chem. Biol.* **2023**, *19*, 1256–1266; c) J. Huang, B. H. Ferlez, E. J. Young, C. A. Kerfeld, D. M. Kramer, D. C. Ducat, *Front. Biomed. Biotechnol.* **2019**, *7*, 432.

[17] M. S. Spencer, *Trans. Faraday Soc.* **1967**, *63*, 2510-.

[18] a) J. K. Bilyj, C. M. Gregg, C. C. Wood, T. D. Rapson, *ChemCatChem* **2024**; b) M. A. Martini, O. Rüdiger, N. Breuer, B. Nöring, S. DeBeer, P. Rodríguez-Maciá, J. A. Birrell, *J. Am. Chem. Soc.* **2021**, *143*, 18159–18171; c) K. Tanifuji, C. C. Lee, N. S. Sickerman, K. Tatsumi, Y. Ohki, Y. L. Hu, M. W. Ribbe, *Nat. Chem.* **2018**, *10*, 568–572.

[19] a) H. Borsook, G. Keighley, *Proc. Natl. Acad. Sci. USA* **1933**, *19*, 875–878; b) J. Shen, P. T. Griffiths, S. J. Campbell, B. Utinger, M. Kalberer, S. E. Paulson, *Sci. Rep.* **2021**, *11*, 7417; c) M. Senger, J. Duan, M. V. Pavliuk, U. P. Apfel, M. Haumann, S. T. Stripp, *Inorg. Chem.* **2022**, *61*, 10036–10042.

[20] a) V. Hong, S. I. Presolski, C. Ma, M. G. Finn, *Angew. Chem. Int. Ed.* **2009**, *48*, 9879–9883; b) S. I. Presolski, V. P. Hong, M. G. Finn, *Curr. Protoc. Chem. Biol.* **2011**, *3*, 153–162.

[21] a) A. I. Al-Ayash, M. T. Wilson, *Biochem. J.* **1979**, *177*, 641–648; b) D. Njus, M. Wigle, P. M. Kelley, B. H. Kipp, H. B. Schlegel, *Biochemistry* **2001**, *40*, 11905–11911.

[22] T. Peschke, P. Bitterwolf, S. Hansen, J. Gasmi, K. S. Rabe, C. M. Niemeyer, *Catalysts* **2019**, *9*.

[23] H. Renata, R. D. Lewis, M. J. Sweredoski, A. Moradian, S. Hess, Z. J. Wang, F. H. Arnold, *J. Am. Chem. Soc.* **2016**, *138*, 12527–12533.

[24] M. Garcia-Borras, S. B. J. Kan, R. D. Lewis, A. Tang, G. Jimenez-Oses, F. H. Arnold, K. N. Houk, *J. Am. Chem. Soc.* **2021**, *143*, 7114–7123.

[25] R. D. Lewis, M. Garcia-Borras, M. J. Chalkley, A. R. Buller, K. N. Houk, S. B. J. Kan, F. H. Arnold, *Proc. Nat. Acad. Sci. USA* **2018**, *115*, 7308–7313.

[26] a) M. P. Williamson, *Prog. Nucl. Magn. Reson. Spectrosc.* **2014**, *80*, 64–64; b) Z. Q. Yu, P. F. Li, K. Merz, *Biochemistry* **2017**, *56*, 2349–2362.

[27] M. J. Weissenborn, R. M. Koenigs, *ChemCatChem* **2020**, *12*, 2171–2179.

[28] E. Arslan, H. Schulz, R. Zufferey, P. Künzler, L. Thöny-Meyer, *Biochem. Biophys. Res. Commun.* **1998**, *251*, 744–747.
[29] A. P. Fernandes, I. Couto, L. Morgado, Y. Y. Londer, C. A. Salgueiro, *Protein Expression Purif.* **2008**, *59*, 182–188.
[30] M. B. Katan, *Anal. Biochem.* **1976**, *74*, 132–137.
[31] C. A. Schneider, W. S. Rasband, K. W. Eliceiri, *Nat. Methods* **2012**, *9*, 671–675.
[32] E. Mittmann, Y. L. Hu, T. Peschke, K. S. Rabe, C. M. Niemeyer, S. Bräse, *ChemCatChem* **2019**, *11*, 5519–5523.
[33] H. Keipour, T. Ollevier, *Org. Lett.* **2017**, *19*, 5736–5739.
[34] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **1990**, *215*, 403–410.
[35] F. Madeira, N. Madhusoodanan, J. H. Lee, A. Eusebi, A. Niewielska, A. R. N. Tivey, R. Lopez, S. Butcher, *Nucleic Acids Res.* **2024**, *52*, W521–W525.
[36] D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, H. O. Smith, *Nat. Methods* **2009**, *6*, 343–U341.
[37] T. Peschke, K. S. Rabe, C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2017**, *56*, 2183–2186.

Manuscript received: December 20, 2024
Accepted manuscript online: January 28, 2025
Version of record online: March 16, 2025