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Positioning-Group-Enabled Biocatalytic Oxidative Dearomatization

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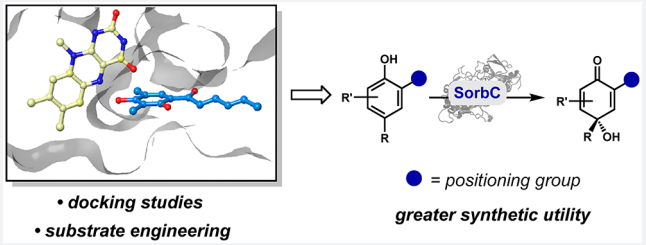
Supporting Information

ABSTRACT: Biocatalysts have the potential to perform reactions with exceptional selectivity and high catalytic efficiency while utilizing safe and sustainable reagents. Despite these positive attributes, the utility of a biocatalyst can be limited by the breadth of substrates that can be accommodated in the active site in a reactive pose. Proven strategies exist for optimizing the performance of a biocatalyst toward unnatural substrates, including protein engineering; however, these methods can be time intensive and require specialized equipment that renders these approaches inaccessible to synthetic chemists. Strategies accessible to chemists for the expansion of a natural enzyme's substrate scope, while maintaining high levels of site- and stereoselectivity, remain elusive. Here, we employ a computationally guided substrate engineering strategy to expand the synthetic utility of a flavin-dependent monooxygenase. Specifically, experimental observations and computational modeling led to the identification of a critical interaction between the substrate and protein which is responsible for orienting the substrate in a pose productive for catalysis. The fundamental hypothesis for this positioning group strategy is supported by binding and kinetic assays as well as computational studies with a panel of compounds. Further, incorporation of this positioning group into substrates through a cleavable ester linkage transformed compounds not oxidized by the biocatalyst SorbC into substrates efficiently oxidatively dearomatized by the wild-type enzyme with the highest levels of site- and stereoselectivity known for this transformation.



INTRODUCTION

Biocatalytic transformations have the potential to be exceptionally efficient, exquisitely selective, and highly sustainable, positioning biocatalysis as an attractive option for synthetic chemists.[1] The chemo-, site-, and stereoselectivity possible with a biocatalyst can translate to high yields of the desired product without the need to employ protecting groups.[2] Additionally, the use of protein catalysts under mild reaction conditions gives these processes excellent safety and sustainability profiles.[3] Even with these advantages, biocatalytic conditions are often not employed due to limitations in substrate scope, which can be more narrow for enzymes in comparison to small molecule reagents and catalysts. Protein engineering is a proven approach to alter the substrate scope of a given biocatalyst, in which the structure of the catalyst is altered to accommodate a targeted non-native substrate;[4,5] however, this approach requires expertise and equipment not uniformly available in synthetic chemistry laboratories. Classical chemical methods rely on not only tuning the catalysts but also an orthogonal strategy to engineer interactions between a catalyst and substrate by altering the substrates to contain a functional group that possesses a high affinity for the catalyst. Using this approach, high levels of site- and stereoselectivity have been obtained in a wide variety of chemical transformations (Figure 1A).[6,7]

In contrast to the widespread use of directing groups in organic synthesis with small molecule reagents and catalysts, the analogous strategy of substrate engineering has remained underutilized in biocatalysis. Reported examples of substrate engineering for the purpose of synthetic chemistry have been restricted to a single class of enzymes, cytochromes P450.[8−15] However, we anticipate that substrate engineering can radically expand the utility of a range of enzyme classes in biocatalysis. Substrate engineering can impact various aspects of a biocatalytic reaction. For example, approaches to substrate engineering have included the installation of a directing group to anchor a substrate through a specific protein−substrate interaction (Figure 1B).[9,11−13] Using this strategy, substrates with low affinity for binding within a biocatalyst's active site are transformed into substrates with an increased affinity for the catalyst. Both Auclair and Sherman have demonstrated this anchoring group approach, identifying heterocycles that can enhance the binding of non-native substrates with P450s and allow for predictable hydroxylation of engineered substrates.[9,12,13] Substrate engineering can also be targeted toward inducing a conformational change upon binding to shift from an inactive protein conformation to a catalytically productive conformation, thus initiating substrate turnover.[16] Herein, we demonstrate a strategy in which a tightly bound but unreactive substrate is engineered to include a functional group that poses the substrate within the enzyme active site for a productive

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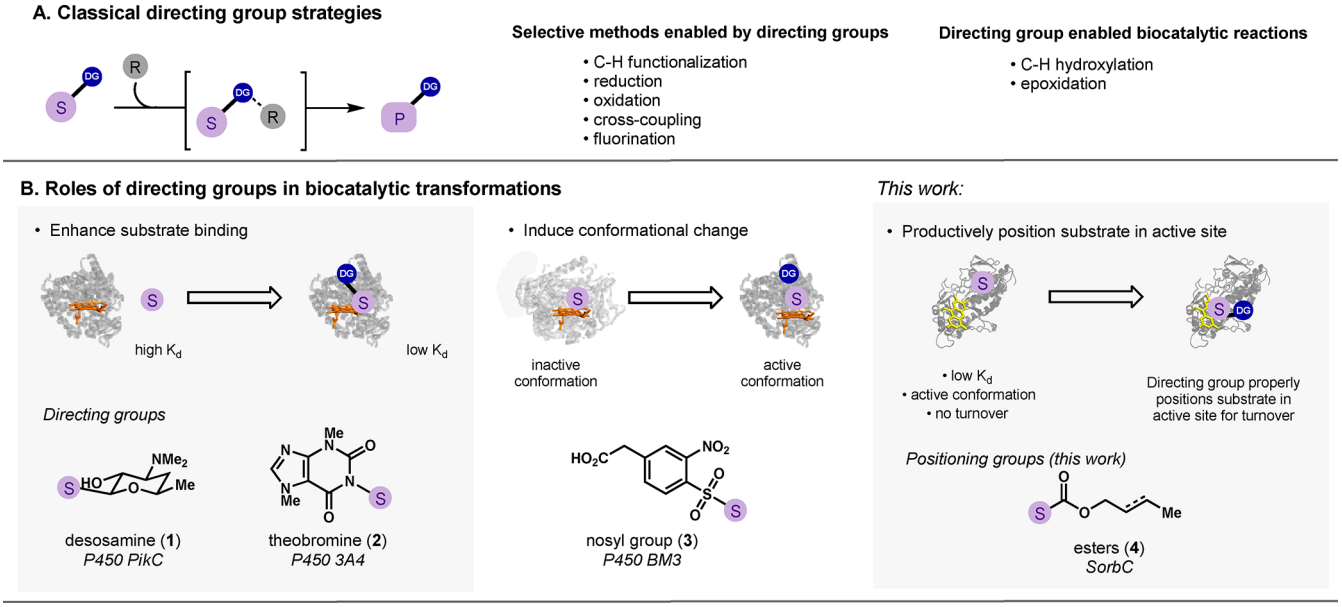

Figure 1. (A) Classical and biocatalytic methods enabled by directing groups. S = substrate, R = chemical reagent, DG = directing group. (B) Roles of directing groups in biocatalytic transformations including enhanced substrate binding, impact on conformation, and substrate positioning within the active site.

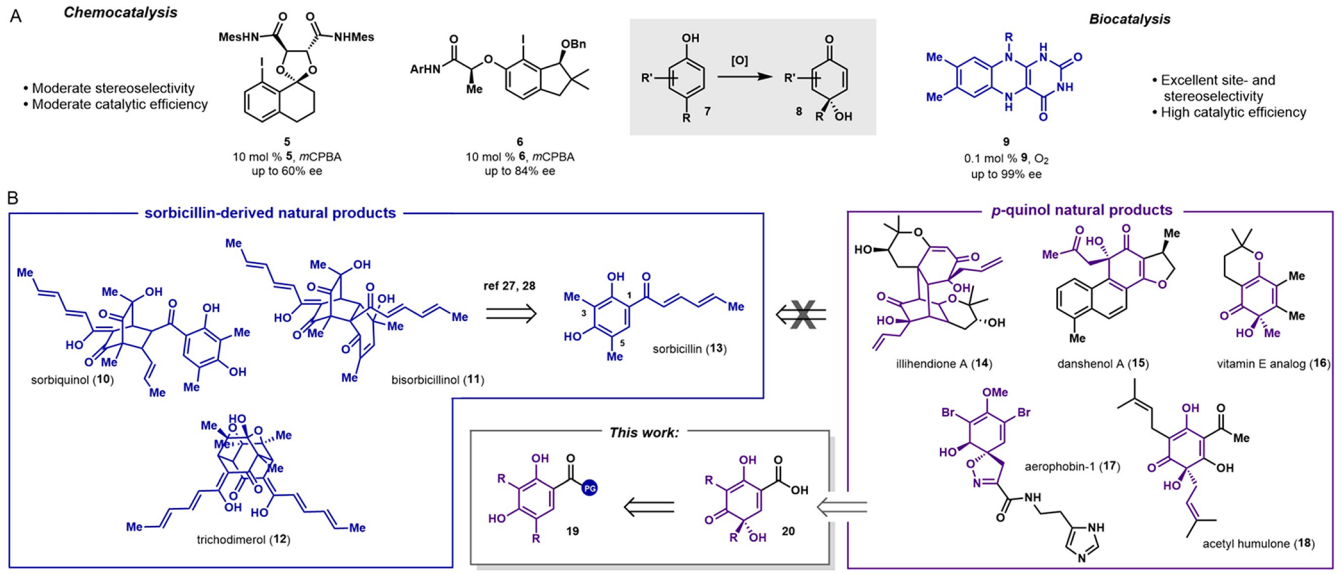

Figure 2. (A) Chemo- and biocatalytic methods for asymmetric oxidative dearomatization. (B) Sorbicillin derived natural products accessible by previous methodology and examples of *p*-quinol natural products that can be accessed via a directing-group strategy. PG = positioning group.

reaction (Figure 1B). This approach addresses a problem distinct from substrate binding, which we have shown occurs with substrates which lack the positioning group but do not undergo conversion. We have applied this positioning group strategy to expand the synthetic utility of a flavin-dependent monooxygenase, which mediates a powerful transformation and operates with site- and stereoselectivity which exceed existing chemical methods.

Like cytochromes P450, flavin-dependent enzymes mediate a broad array of chemical transformations encompassing both reductive and oxidative processes.[17] Within the category of oxidative transformations, flavin-dependent enzymes are capable of catalyzing highly selective hydroxylation,[18] epoxidation,[19] halogenation,[20] and Baeyer−Villiger[21] reactions among others and have found application in a number of commercial processes.[22] Recently, we explored the synthetic potential of a panel of flavin-dependent monooxygenases which mediate the highly site- and stereoselective oxidative dearomatization of phenolic substrates.[23] While several wild-type biocatalysts investigated demonstrated an impressive substrate scope, an enzyme with unique site-selectivity, SorbC, operated on a limited range of substrates.[23] The site-selectivity achieved in reactions with SorbC is difficult to access with chemical reagents and catalysts, often requiring specific substitution patterns to bias the site of hydroxylation. Controlling the stereochemical outcome in this transformation has also challenged chemists with state-of-the-art methods employing hypervalent iodine catalysts providing products in

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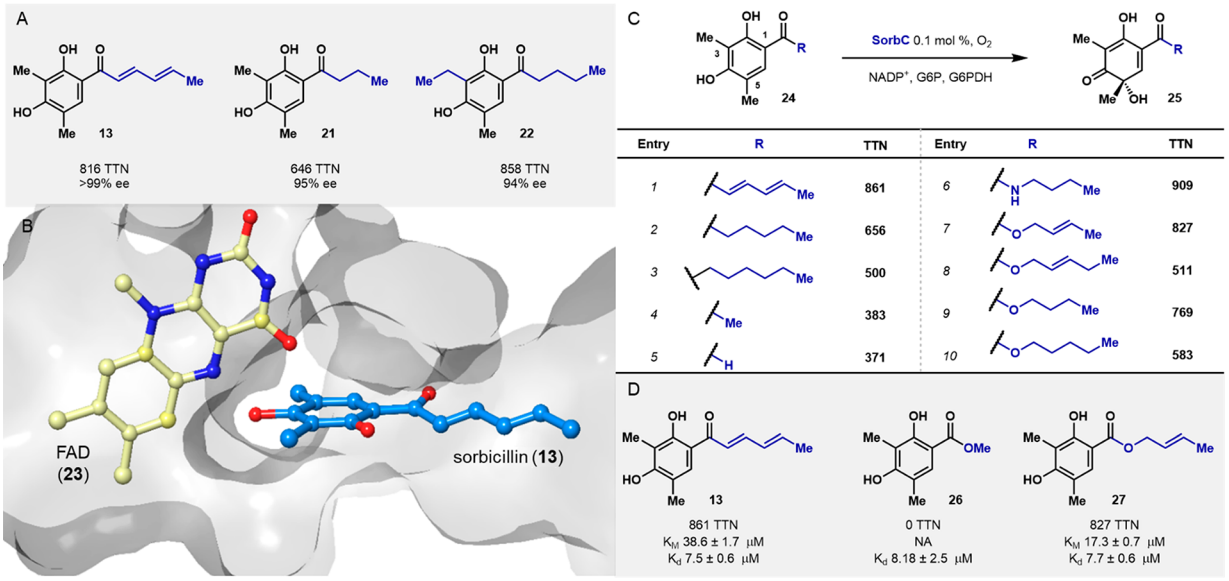

Figure 3. (A) SorbC substrates including native substrate **13** and substrates that are dearomatized with decreased stereoselectivity **21** and **22**. (B) Homology model of SorbC (gray surface) with FAD (yellow sticks), and sorbicillin (**13**, blue) in the major pose observed in favorable docking solutions. (C) Impact of C1 substituent on SorbC activity. (D) Analysis of reactivity, kinetics, and binding of sorbicillin (**13**), methyl ester **26**, and crotyl ester **27** with SorbC.

60−84% ee (Figure 2A).[24,25] In the biocatalytic mechanism, molecular oxygen reacts with reduced flavin adenine dinucleotide ($FADH_2$, **9**) to form C4a-hydroperoxyflavin, an electrophilic source of oxygen.[26] The site- and stereoselectivity of hydroxylation arise from the pose of the substrate in the enzyme active site relative to the flavin cofactor (**9**). This strategy for stereocontrol allows for perfect selectivity in the native reaction.

Our initial investigations indicated that SorbC is capable of mediating oxidative dearomatization with exceptional stereoselectivity, generating products such as **8** in >99% ee, solidifying SorbC as the catalyst with the highest reported enantioselectivity for this transformation (Figure 2A).[23] However, the limited substrate scope demonstrated by SorbC presents a challenge in applying this biocatalyst to the synthesis of structurally diverse target molecules. For example, profiling the substrate promiscuity of SorbC revealed the requirement of an alkyl chain extending from the C1 position (see **13**, Figure 2B). Thus, while SorbC has been used in the synthesis of complex molecules derived from sorbicillin (**13**),[23,27,28] it is limited to the synthesis of compounds containing long alkyl chains (compounds in blue, Figure 2B) and does not provide access to the full breadth of biologically active molecules that could arise from *para*-quinol precursors (see compounds in purple, Figure 2B). For example, the native substrate of SorbC, **13**, bears a linear six-carbon chain at this position with a carbonyl at the benzylic position and one or two carbon−carbon double bonds. Truncation of this six-carbon chain resulted in lower conversion to dearomatized products in reactions with SorbC (Figure 3, entries 4 and 5). Additionally, an erosion in the enantioenrichment of the product was observed as this C1 chain was truncated, or the C3 methyl group was extended to an ethyl substituent (see **21** and **22**, respectively). On the basis of these data, we hypothesized that the six-carbon chain plays a critical role in substrate binding and orientation within SorbC's active site. Herein, we demonstrate how this finding can be leveraged in a structure-guided substrate engineering approach to expand the synthetic utility of SorbC in the enantioselective oxidative dearomatization of phenolic substrates to enable access to a much broader array of quinol-containing natural products (see Figure 2B). Ultimately, this work will enable the syntheses of a variety of complex targets employing wild-type SorbC in the enantiodetermining step.

RESULTS AND DISCUSSION

Toward expanding the substrate scope of SorbC through a substrate engineering strategy, we aimed first to evaluate the structural role of the substrate C1 alkyl substituent in the SorbC−substrate interactions computationally and experimentally. A homology model of SorbC was generated on the basis of a crystal structure that we recently obtained of TropB,[29] a homologous protein with 34% sequence identity to SorbC. Computational docking[30,31] of the native substrate to the SorbC-FAD model revealed that all the structures with favorable interaction energies and with C5 positioned to react with the C4a-hydroperoxyflavin cofactor to afford the observed stereoselectivity also placed the C1 alkyl substituent along the substrate entryway (Figure 3B). This pose places the six-carbon chain in a hydrophobic tunnel at the mouth of the SorbC active site. This favorable pose of the substrate positions the aromatic ring of the substrate proximal to the hydroperoxyflavin cofactor in an orientation primed to afford a product with the experimentally observed site- and stereoselectivity.

With computational support for the importance of the C1 chain in substrate positioning, we sought to experimentally probe the impact of this structural motif on binding and reactivity with SorbC. Several analogues of the native substrate were synthesized and evaluated in reactions containing 0.1 mol % enzyme. First, the role of the double bonds and carbonyl group in the native substrate's C1 substituent was gauged by comparing the total turnover number (TTN) of SorbC with the native substrate, a substrate bearing a saturated C1 substituent, and a compound in which the carbonyl group was reduced to a methylene group (Figure 3C, entries 1−3). Each

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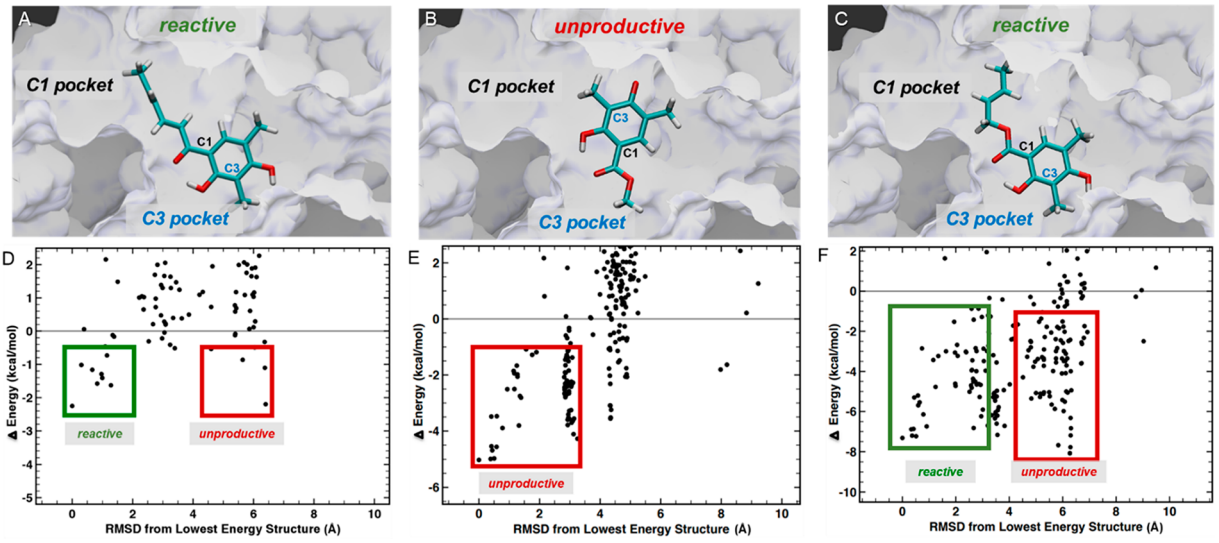

Figure 4. Top row: homology model of SorbC (gray surface) with (A) sorbicillin (**13**), cyan sticks; (B) methyl ester **26**; and (C) crotyl ester **27** in the major reactive pose observed in docking studies. Bottom row: plots of the change in energy of poses relative to the lowest-energy structure with (D) sorbicillin (**13**), (E) methyl ester **26**, or (F) crotyl ester **27**.

of these substrates was dearomatized by SorbC with the native substrate (**13**) having the highest TTN, 861, and the further reduced compounds exhibiting decreased TTNs (656 and 500, respectively). In reactions with substrates possessing a truncated C1 substituent, such as a methyl ketone or aldehyde, a further decrease in TTN was observed (383 and 371, entries 4 and 5). With evidence supporting the importance of a lipophilic linear chain for achieving a productive reaction, the ketone of the native substrate **13** was replaced with groups that meet the length requirement but are synthetically more tractable for further elaboration toward target molecules (see Figure 3C, entries 6−10). This panel of amide and ester substrates was productively dearomatized by SorbC with TTNs ranging from 500 to 909. The amide substrate was converted to product most efficiently with a slightly higher TTN (909) than that observed for the native substrate, sorbicillin (**13**). This could be due to the increased nucleophilicity of the phenol due to the replacement of the electron-withdrawing ketone with the less electron-withdrawing amide. Of the ester substrates tested (entries 7−10), crotyl ester **27** exhibited the greatest TTN in reactions with SorbC, nearly matching the reactivity observed with SorbC's native substrate, **13**. In contrast, reactions with SorbC and methyl ester **26** afforded no detectable product.

To further probe the role of the lipophilic linear chain in substrate binding and reactivity, we performed binding assays and carried out Michaelis−Menten kinetic analysis of SorbC with three test substrates; the native substrate (sorbicillin, **13**), methyl ester **26**, and the crotyl ester **27**. Methyl ester **26** was chosen as a substrate mimic, which lacked the long carbon chain at C1 present in **13** and **27**, but with similar electronics to **27** to probe the role of the long chain in binding and turnover. Interestingly, despite the differences in reactivity, the dissociation constants for SorbC with each substrate indicated similar binding affinity across the three substrates (Figure 3D). This suggests that reactivity does not correlate with substrate binding and that this group is not acting as an anchoring group (Figure 1B). Additionally, steady-state kinetic analysis of SorbC with this set of substrates reveals the K_M for sorbicillin (**13**) with SorbC was determined to be 38.6 ± 1.7 μM, and crotyl ester **27** has a lower K_M than the native substrate, 17.3 ± 0.7 μM (Figure 3D). For methyl ester **26**, no reaction was observed.

As binding experiments suggested that the role of the C1 substituent is not important for increasing the concentration of substrate in the active site, we explored the influence of this group on substrate orientation computationally. Docking studies revealed that substrates **13**, **26**, and **27** can each achieve favorable binding interactions; however, only substrates with a C1 substituent of sufficient length are poised to undergo a productive reaction with the C4a-hydroperoxyflavin. The lowest-energy poses identified through docking each of these three substrates (**13**, **26**, and **27**) with SorbC are illustrated in Figure 4A−C. Notably, while the carbonyl group of **13** and crotyl ester moiety of **27** occupy the hydrophobic substrate entryway (C1 pocket, Figure 4A,C), the unreactive substrate **26** adopts a flipped orientation (Figure 4B), positioning the C1 methyl ester moiety of **19** in the binding pocket occupied by the C3 substituent of the native substrate **13** (C3 pocket). The root-mean-square deviation (RMSD) values from the lowest-energy structures of each of these three substrates were then plotted against the change in energy from the lowest-energy structures (Figure 4D−F). This allowed us to rapidly identify clusters of favorable binding poses for the three substrates. The majority of the energetically favorable docking solutions for the native substrate place the C1 substituent in the hydrophobic entryway (boxed in green), and a smaller set of energetically favorable docking solutions place the C3 substituent in the hydrophobic entryway (boxed in red), which correspond with unreactive poses (FAD-C4a—C5 distances above 5 Å). For methyl ester substrate **26**, most energetically favorable docking poses position the substrate in a flipped orientation compared to the dominant binding mode for SorbC's native substrate **13**. This flipped orientation is anticipated to be unproductive (Figure 4E). Finally, crotyl ester **27** had numerous favorable docking solutions with SorbC in which the crotyl ester group occupied the hydrophobic entryway in the same fashion as the native substrate **13** (boxed in green, Figure 4F), and like **13** a number of favorable poses

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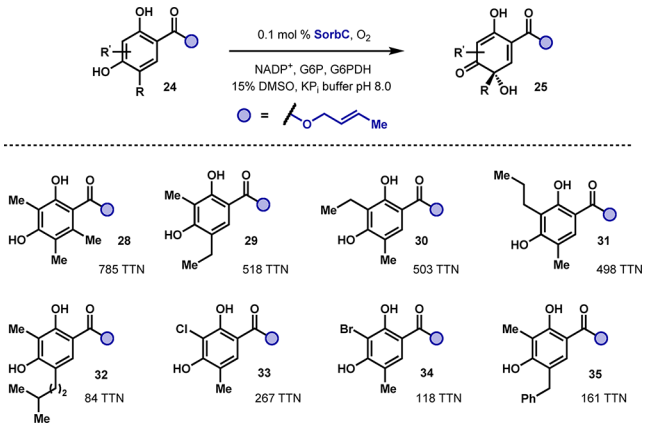
were also obtained with the C1 substituent in the C3 binding site in an unreactive pose (boxed in red, Figure 4F).

Taken together, these computational and experimental results indicate the necessity of the C1 substituent as a critical structural feature, not for lowering the K_d of a substrate, but for appropriately positioning the substrate within the active site for turnover. We envisioned exploiting this structural requirement for productive catalysis to expand the substrate scope of SorbC through a novel substrate engineering strategy. To execute a synthetically useful substrate engineering approach, the directing group must be both easily introduced prior to the biocatalytic step and readily removed or converted into a range of versatile handles following the targeted transformation. In addition to facilitating a high conversion to product, we recognized the synthetic advantages of the crotyl ester directing group, which could easily be installed from a carboxylic acid precursor and removed following the biocatalytic reaction. A number of substrates bearing a crotyl ester directing group were synthesized and evaluated for reactivity with SorbC. This substrate engineering strategy proved successful for resorcinol scaffolds with differing steric and electronic properties (Table 1). For example, hexasubstituted substrate **28** was efficiently converted to dearomatized product with a TTN close to that of the native scaffold. Additionally, halogen substituents were tolerated at the C3 position (see **33** and **34**). Docking studies indicated that the C3 pocket would have sufficient space to accommodate up to an *n*-propyl group. Substrates with increasing steric bulk at the C3 position, from a methyl group to an ethyl substituent and further to an *n*-propyl group, were efficiently turned over with only a minor decrease in activity (see **27**, **30**, and **31**), as predicted (Figure S30, Supporting Information). Substrates with additional steric bulk including a benzyl substituent at the C5 position (see **35**) or an isopentyl group (see **32**) were also tolerated. With a subset of substrates a second peak was visible by LC-MS analysis of crude reaction mixtures. The material corresponding to this peak was not isolable under a variety of conditions and did not match the products generated in chemical methods for oxidative dearomatization (see Figure S23).

Table 1. Total Turnover Numbers (TTNs) of Each Enzyme/Substrate Pair[a]



[a]Reaction conditions: 2.5 mM substrate, 2.5 μM SorbC, 1 mM $NADP^+$, 5 mM glucose-6-phosphate (G6P), 1 U mL^{-1} glucose-6-phosphate dehydrogenase (G6PDH), 50 mM potassium phosphate buffer, pH 8.0, 30 °C, 15% v/v DMSO, 1 h.

To evaluate the stereoselectivity achieved using this substrate engineering approach, the enantioenrichment of dearomatized products was measured by comparison of products generated enzymatically to racemic material obtained in oxidative dearomatization reactions mediated by lead tetraacetate (LTA, yields shown in gray, Figure 5). Gratifyingly, all products examined were obtained in good enantiopurity, supporting our hypothesis that the lipophilic carbonyl is critical for proper substrate positioning. The crotyl ester with the native substitution pattern was obtained in a 72% isolated yield and 98% ee. The C3 and C5 ethyl products were obtained in 98% and 96% ee, respectively. Notably, the *n*-propyl product **41** was also obtained in 98% ee, indicating that the crotyl group is recognized in preference to the C3 alkyl chain which, if recognized in the same manner as the C1 substituent, would result in diminished facial selectivity and ultimately eroded enantiopurity of the product. This is in contrast to the diminished enantiopurity observed in the ketone substrate class wherein the C3 ethyl product was obtained in 94% ee.

This substrate engineering strategy allowed us to expand the range of highly enantioenriched quinol products accessible through SorbC catalysis. To assess the utility of quinol products containing the crotyl ester positioning group, we explored conditions for removing the crotyl group as well as the innate reactivity of the ester-containing products. Removal of the crotyl group proved to be facile under Pd-catalyzed decrotylation conditions. By employing 7 mol % $Pd(PPh_3)_4$ and morpholine, the crotyl group of **37** was removed to unveil the carboxylic acid present in **42** (Figure 5B).[32] To further explore the innate reactivity of ester-containing products, we sought to transform these directly into analogues of the natural product urea sorbicillinoid (**45**).[33] Although previous reports have described ester derivatives of sorbicillinol as unreactive in the dimerization to afford the bisorbicillinol core,[34] we have demonstrated that esters undergo facile *in situ* [4 + 2] cycloaddition with maleimide to afford the [2,2,2] tricycle **45** in 23% yield over two steps (Figure 5C). On the basis of these results, we anticipate that this positioning-group-based substrate engineering strategy will expand the utility of SorbC from a biocatalyst that is restricted to sorbicillinol derived natural products to a breadth of natural and unnatural compounds accessible through the dearomatization of structurally diverse resorcinol derivatives to give products such as **45**.

Due to the high synthetic value of products obtained from the biocatalytic oxidative dearomatization catalyzed by SorbC, we endeavored to employ a substrate engineering strategy to overcome the limited product profile of this transformation. Initial investigations into the substrate scope of SorbC led us to hypothesize that the lipophilic carbon chain was critical for productive substrate binding in the active site. Further analysis of substrate binding experimentally and computationally revealed that the role of the C1 substituent is not in enhancing the binding affinity of the substrate for the protein, but rather, the C1 substituent is critical for positioning the substrate in a reactive pose. Optimization of the positioning group led us to identify the crotyl ester as an ideal group to attain high TTNs on scaffolds which are otherwise not competent substrates in the enzymatic reaction. This ester group required for productive biocatalysis can be transformed into the corresponding carboxylic acid, a versatile handle for further chemical functionalization, or the ester-containing dearomat-

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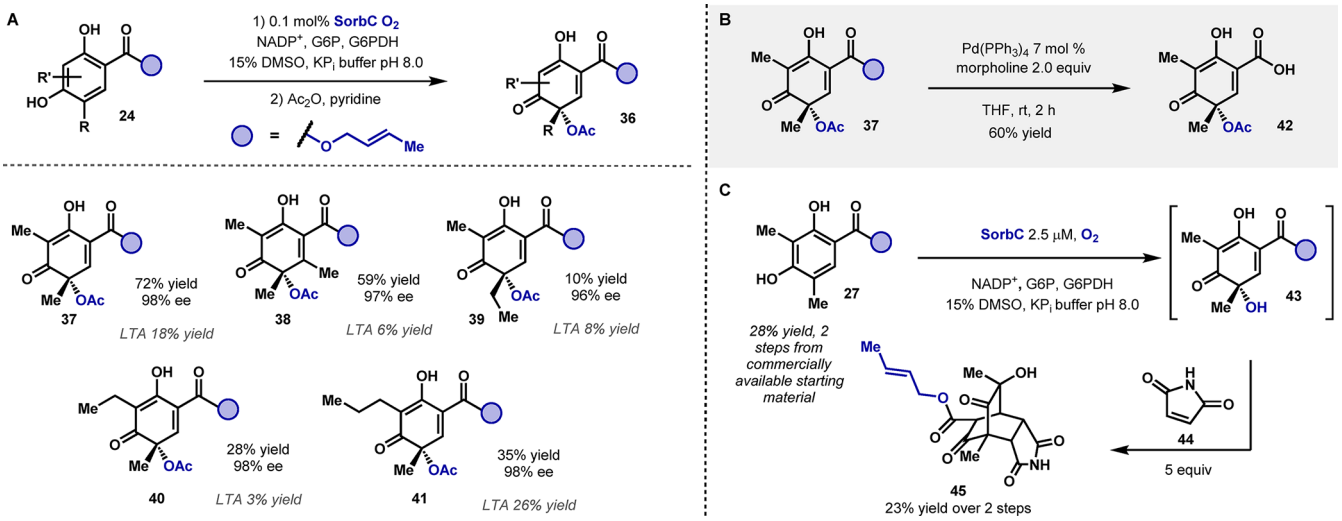

Figure 5. Application of directing-group strategy for preparative-scale oxidative dearomatization. (A) Evaluation of substrate scope and reaction enantioselectivities. Yields for racemic standard synthesis in gray. LTA = lead tetraacetate. (B) Removal of directing group through palladium-catalyzed decrotylation. (C) Elaboration of dearomatized product through [4 + 2] cycloaddition.

ized products can be used directly in subsequent complexity-generating transformations. The discovery of this new directing group demonstrates how substrate engineering can be leveraged to expand the utility of a biocatalyst, and we anticipate that our findings will increase the accessibility of this biocatalytic transformation to synthetic chemists. Currently we are engaged in employing this substrate engineering approach with homologues of SorbC as well as other flavin-dependent monooxygenases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.9b00163.

Detailed experimental procedures, spectroscopic data for all new compounds, UPLC-PDA and SFC traces, and details for molecular modeling simulations (PDF)

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Author Contributions

S.A.B.D. and C.E.S. synthesized substrates and carried out biocatalytic reactions. S.A.B.D. and A.R.B. conducted protein expressions and purifications as well as binding and kinetic assays. T.W. and C.L.B. carried out and analyzed computational work. S.A.B.D. and A.R.H.N. conceived the project and wrote the manuscript with feedback from all authors.

Notes

The authors declare no competing financial interest.

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