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Communication

Enantioselective Hydroxylation of Benzylic C(sp^3)−H Bonds by an Artificial Iron Hydroxylase Based on the Biotin−Streptavidin Technology

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ABSTRACT: The selective hydroxylation of C−H bonds is of great interest to the synthetic community. Both homogeneous catalysts and enzymes offer complementary means to tackle this challenge. Herein, we show that biotinylated Fe(TAML)-complexes (TAML = Tetra Amido Macrocyclic Ligand) can be used as cofactors for incorporation into streptavidin to assemble artificial hydroxylases. Chemo-genetic optimization of both cofactor and streptavidin allowed optimizing the performance of the hydroxylase. Using H_2O_2 as oxidant, up to ∼300 turnovers for the oxidation of benzylic C−H bonds were obtained. Upgrading the ee was achieved by kinetic resolution of the resulting benzylic alcohol to afford up to >98% ee for (*R*)-tetralol. X-ray analysis of artificial hydroxylases highlights critical details of the second coordination sphere around the Fe(TAML) cofactor.

The selective functionalization of C−H bonds represents one of the frontiers in synthetic methodology.[1−7] To address this challenge, homogeneous catalysis often relies on directing groups present on the substrate that coordinate to the metal center, thus allowing distinguishing between equally reactive C−H bonds.[7] Enzymes have been optimized thanks to evolution to differentiate C−H bonds with exquisite selectivity: The active site around the cofactor is tailored to ensure proper orientation of the substrate.

For the hydroxylation of inert C−H bonds, iron-containing enzymes and iron-based homogeneous catalysts occupy a place of choice. They are complementary in many respects. While the former operate under physiological conditions, homogeneous catalysts perform best at low temperature in organic solvents. The reactivity of homogeneous catalysts is often tuned via first-coordination sphere modifications, whereas enzymes rely on secondary sphere interactions.

Iron metalloenzymes catalyze the C−H oxyfunctionalization of hydrocarbons via iron−oxygen species resulting from activation of O_2.[8−17] The selective hydroxylation of C−H bonds using homogeneous catalysts has been achieved by designing structurally elaborated ligands that provide a tailored cavity around the metal center.[18−33]

To complement homogeneous catalysts and enzymes, artificial metalloenzymes (ArMs), that result from anchoring an abiotic cofactor within a macromolecular scaffold, have attracted increasing interest in the past years. The well-defined secondary coordination sphere around the cofactor provided by the protein offers fascinating perspectives to optimize both activity and selectivity of the ArMs.[34−39] In this context several protein scaffolds have proven versatile.[34] These include carbonic anhydrase,[40] hemoproteins,[41,42] proline oligopeptidase,[43] lactococcal multiresistance regulator,[44] four helix bundles,[45,46] nitrobindin,[47] (strept)avidin,[48−50] etc. In the context of asymmetric C−H hydroxylation, introduction of a Mn-porphycene cofactor within myoglobin afforded promising ArMs[51] that complement evolved cytochrome P450 enzymes.[52−54]

Fe(TAML) complexes are a versatile family of iron complexes that typically contain a ferric center tightly bound to a tetraamido macrocyclic ligand.[55,56] Their reactivity as peroxidase mimics has been extensively studied.[55,57,58] Some Fe(TAML) complexes hydroxylate hydrocarbons in aqueous media using oxidants such as *t*BuOOH or *m*-CPBA[56,59−61] or electrochemically.[62] Thanks to their stability in water, we surmised that Fe(TAML) complexes may allow assembly of an iron-based artificial hydroxylase using the biotin−streptavidin technology. The secondary coordination sphere provided by streptavidin (Sav) may enable enantioselective hydroxylation and minimize the formation of less reactive diiron dimeric species.

Initial Ligand Design and Reactivity Tests. Sav is a homotetrameric protein that displays exceptional affinity for biotinylated probes (K_d 10^{-14} M) and maintains its function and quaternary structure in the presence of various chaotropic agents (pH, temperature, cosolvent tolerance, etc.).[48,50,63] To ensure localization of the TAML cofactor within Sav, we synthesized a complex bearing a biotin anchor, **biot^{CS}−1**. The anchor was designed to bind to the Fe-TAML moiety through an "inverted" amide bond to the aromatic ring (Scheme 1a) to

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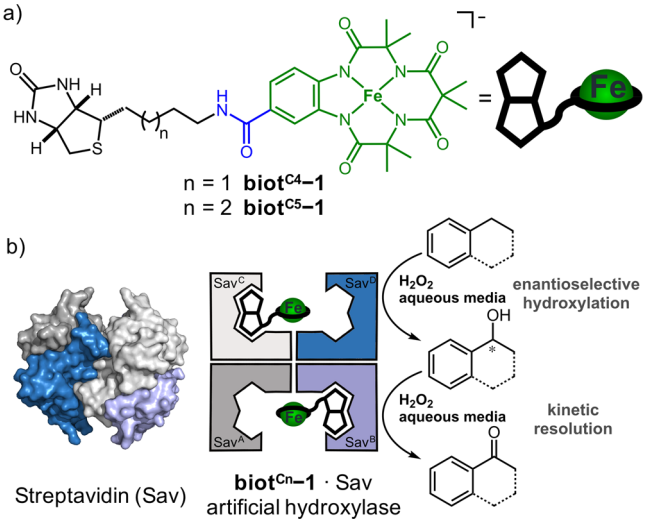
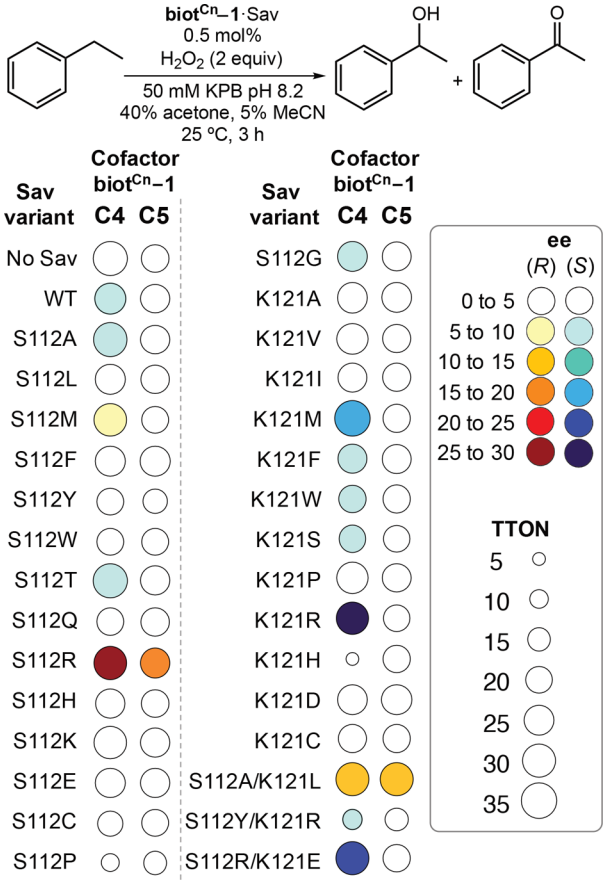
Scheme 1. Artificial C−H Hydroxylase Based on Biotin−Streptavidin: (a) Structure of Cofactors biot^{C4}−1 and biot^{C5}−1[a]; (b) Representation of the ArM Resulting from Anchoring biot^{Cn}−1 in Streptavidin



[a]To increase the electron-withdrawing property of the ligand, a biotin amine was coupled to Fe-TAML (green) bearing a carboxylic acid to afford an "inverted" amide (blue).

Scheme 2. Fingerprint Summary of the Artificial Hydroxylase Optimization with PhEt



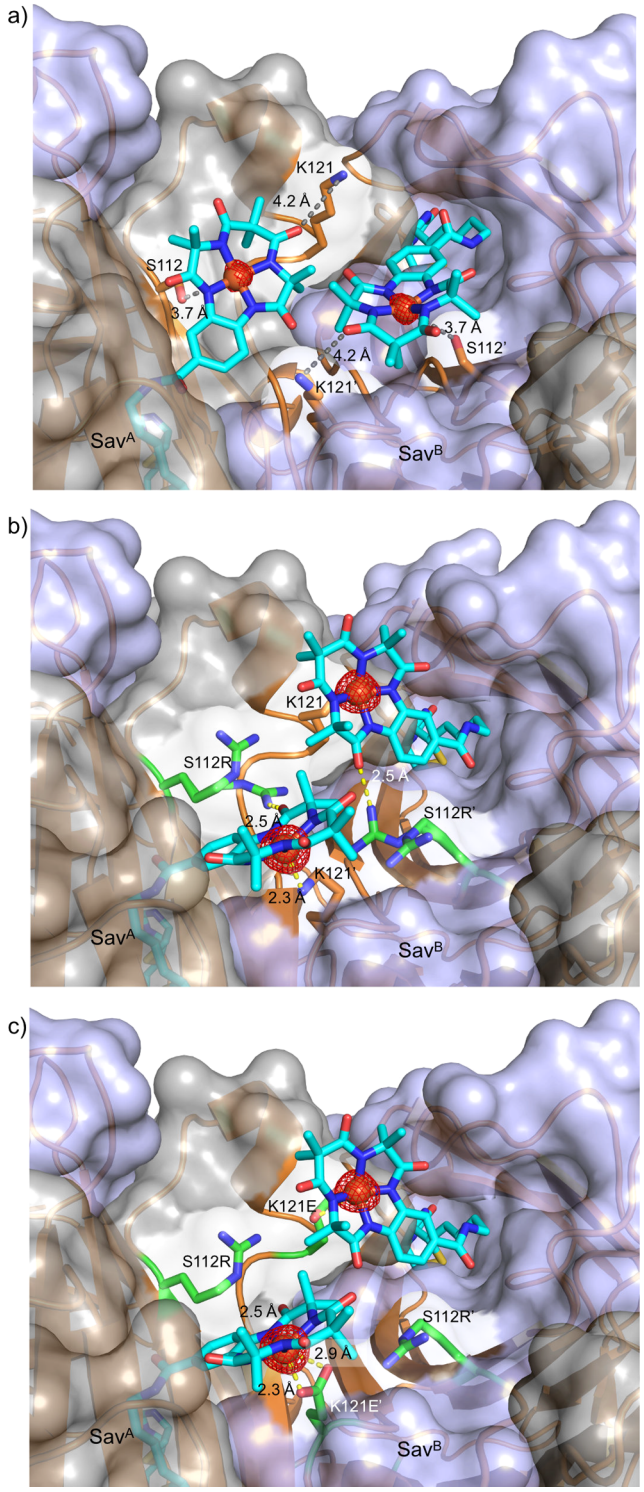


Figure 1. Crystallographic characterization of **biot^{C4}−1**·Sav WT (a, PDB: 6Y2T), **biot^{C4}−1**·Sav S112R (b, PDB: 6Y2M), and **biot^{C4}−1**·Sav S112R/K121E (c, PDB: 6Y25). Sav is depicted as orange cartoon, and its surface representation in gray and mauve (for Sav^{A} and Sav^{B} monomers, respectively). The cofactor and relevant amino acids are depicted as sticks. The Fe atoms are depicted as spheres and surrounded by their anomalous electron density (red mesh at 5 σ).

increase the electron-withdrawing effect, which has been shown to be beneficial for the reactivity of Fe-TAML complexes.[64]

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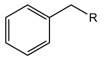
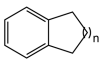
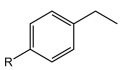
Table 1. Benzylic C−H Oxidations Catalyzed by biot^{C4}−1·Sav S112R[a]

Substrate		TTON	ee (%)	[alcohol]/[ketone]	Conversion (%) (alcohol yield (%))[c]
	R = CH_3	57	32	3.2	11.5 (8.8)
	R = CH_2CH_3	26	45	8.4	5.9 (5.3)
	R = $(CH_2)_2CH_3$	19	45	7.5	4.3 (3.8)
	R = $C(CH_3)_2$	0	-	-	-
	n = 2	316	65	5.7	60 (44.3)
		300[b]	>98	1.1	86.8 (34.3)
	n = 1	205	47	7.4	45.7 (40.2)
		173[b]	80	0.8	55.7 (24.7)
	n = 0	0	-	-	-
	R = OCH_3	120	12	5.9	26.3 (22.5)
	R = Cl	20	14	3.8	4.1 (3.3)
	R = Br	9	18	>20	2.3 (2.3)

[a]Conditions: 25 μM **biot^{C4}−1**·Sav S112R (50 μM Fe), 20 mM substrate, 20 mM H_2O_2, 50 mM KPB pH 8.5, 35% acetone, 2.5% MeCN, 3 h at 25 °C. [b]10 mM substrate, 25 mM H_2O_2, to promote alcohol overoxidation which yields increased ee. [c]See Table S6 for more details.

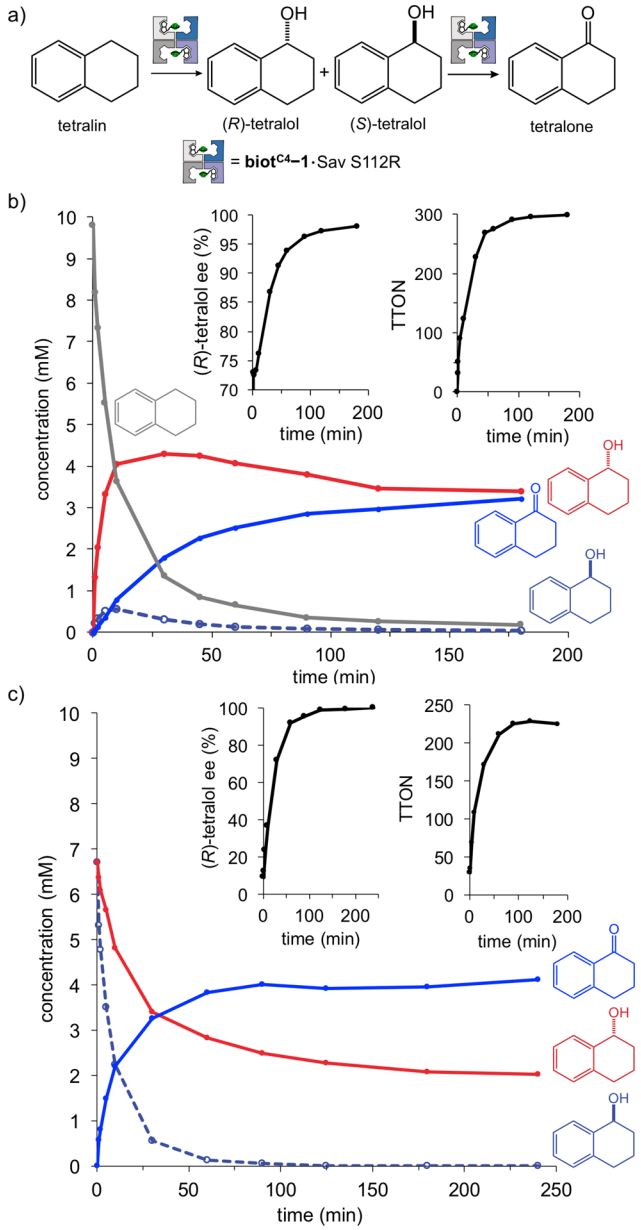
Initial reactivity tests were performed with ethylbenzene (PhEt, BDE_{C-H} = 87 kcal/mol) using 2 equivalents of H_2O_2 in phosphate buffer (KPB) at pH 8.2 and 40% acetone for 3 h.[61,65] Under these conditions, **biot^{C5}−1**·Sav WT afforded (*rac*)-1-phenylethanol ((*rac*)-PhEtOH)) and acetophenone (total turnover number, TTON,[66]= 23). Both activity and selectivity of **biot^{C5}−1**·Sav WT were comparable to the free cofactor **biot^{C5}−1** (TTON = 21, (*rac*)-PhEtOH). Next, we screened a Sav library that included mutations at positions Sav S112X and/or Sav K121X (Scheme 2 and Table S4). The TTON and enantioselectivity remained moderate (up to 16% ee (*R*)-PhEtOH and TTON = 29). We hypothesized that the moderate influence of the host protein on the catalytic performance may be due to the poor localization of the Fe-TAML within the biotin-binding vestibule. We surmised that a shorter biotin **C^n**-linker may increase the influence of Sav on the catalytic performance by positioning the metal center deeper within the binding pocket. We prepared **biot^{C4}−1** and evaluated its performance (Schemes 1, 2 and Table S4).

Shortening the **C^n**-linker positively affects the selectivity: **biot^{C4}−1**·Sav WT affords 6% ee (*R*)-PhEtOH. Screening the above Sav library with **biot^{C4}−1** reveals that close-lying amino acids influence the ee: **biot^{C4}−1**·Sav S112R yields 28% ee (*R*)-PhEtOH (TTON = 28), and **biot^{C4}−1**·Sav S112R/K121E affords 24% ee (*S*)-PhEtOH (TTON = 29).

Intrigued by these findings, the oxidation of PhEt by **biot^{C4}−1**·Sav S112R was monitored. Two consecutive oxidation steps take place. Initially, hydroxylation of the benzylic position affords (*R*)-PhEtOH with ee >40% after a few TTONs (Figure S8). As the reaction progresses, the formation of acetophenone is observed along with a gradual erosion of the ee. This suggests that the alcohol oxidation is (partially) stereospecific: (*R*)-PhEtOH is oxidized preferentially to acetophenone. Indeed, kinetic resolution of (*rac*)-PhEtOH by **biot^{C4}−1**·Sav S112R affords acetophenone (TTON = 38), leaving enantioenriched (*S*)-PhEt (20% ee after 3 h, $E = k_{(R)}/k_{(S)}$ = 3.4, Figure S9).

In contrast, product analysis after PhEt oxidation by **biot^{C4}−1**·Sav (Sav: K121R or S112R/K121E) yielded ee of (*S*)-PhEtOH (Scheme 2), the opposite enantiomer than **biot^{C4}−1**·Sav S112R. However, monitoring product formation over time reveals a similar reaction pathway for all three ArMs: The

Scheme 3. Enantioselective Hydroxylation of Tetralin and Kinetic Resolution of Tetralol by biot^{C4}−1·Sav S112R: (a) Consecutive Oxidation Scheme; (b) Time Course of Tetralin Oxidation (Inset: Kinetic Resolution Affords >98% ee (*R*)-Tetralol and TTON = 300); (c) Time Course of the Kinetic Resolution of *rac*-Tetralol by biot^{C4}−1·Sav S112R (Inset: Kinetic Resolution Yields >99% ee (*R*)-Tetralol (TTON = 220); See SI for Details)



hydroxylation of PhEt yields preferentially (*R*)-PhEtOH, which is then oxidized faster to acetophenone (Figures S10−S11). This mechanistic pathway is reflected in an erosion of ee over time, eventually affording (*S*)-PhEtOH with both Sav K121R and Sav S112R/K121E. Indeed, the ee is highly variable, depending on conversion and mutant.

The reaction conditions to improve the performance of the hydroxylase were fine-tuned for **biot^{C4}−1**·Sav S112R. A large excess of H_2O_2 favors overoxidation and erosion of ee (Figure S13). The impact of Sav on the activity is also evident at different pH's: **biot^{C4}−1**·Sav S112R displays maximum TTON

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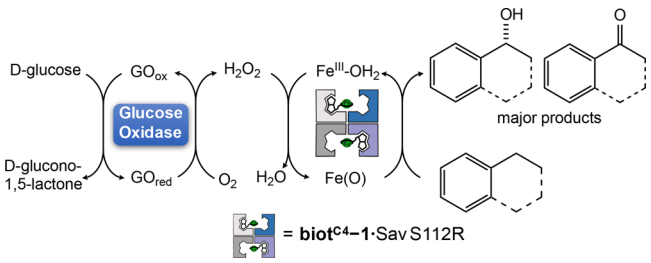
Scheme 4. Cascade with GO To Generate H_2O_2 *in Situ*, Enabling Hydroxylation Using O_2 as Oxidant[a]



[a]See SI.

and enantioselectivity at 8.2 < pH < 8.8. Outside this window, the activity decreases markedly (Figure S14). The free cofactor **biot^{C4}−1** has maximum activity at 6 < pH < 8 and is quenched at higher pH.

Structural Characterization. To scrutinize the differences in the second coordination sphere that influence the activity of the ArMs, we determined their structure by crystallography. Data sets were obtained for **biot^{C4}−1**·Sav and **biot^{C5}−1**·Sav (Sav = WT, S112R, and S112R/K121E, Tables S1 and S2).

The structures reveal the following features: all six structures are nearly superimposable, reflected by a C_α−RMSD varying between 0.038 and 0.256 Å (Table S3). The electron density of the Fe-TAML moiety is defined for **biotC4−1**; the Fe-occupancy is 60% for Sav WT and 100% for Sav S112R and S112R/K121E (Figure 1). This contrasts with **biotC5−1**, for which only the electron density of the biotin C5−linker is defined and modeled with 100% occupancy (Figures S5−S7). We tentatively trace this to the higher flexibility of the C5-linker, resulting in delocalization of the Fe-TAML moiety.

The localization of the Fe(TAML) moiety is affected by the residue at position 112 (Figures 1 and S2−S4). For **biot^{C4}−1**·Sav WT, the closest amino acids, are Sav^A S112 (3.7 Å) and Sav^B K121′ (4.2 Å). They hardly interact with Fe(TAML), resulting in a reduced occupancy of Fe(TAML). The mutation Sav S112R forces the Fe(TAML) into a fixed conformation with 100% occupancy, placing the arginine within H-bonding distance to the C═O of the Fe(TAML) (2.5 Å, in one of two conformations, Figure 1b). This alternative position of Fe(TAML) allows Sav^B K121′ to coordinate to Fe of **biot^{C4}−1**·Sav^A (2.3 Å, Figure 1b). To enable the coordination of Sav^B K121′ to the Fe of **biot^{C4}-1**, the lysine side chain adopts a compact conformation with acute dihedral (χ) angles of 54.2°, 106.9°, 80.0°, and 41.2°. We hypothesize that both the precise localization of the Fe(TAML) and its interaction with either K121′ or E121′ through an η^2-coordination (in **biot^{C4}−1**·Sav S112R/K121E, Fe···O 2.3 and 2.9 Å, Figure 1c) impact the catalysis outcome (product distribution and ee, Scheme 2).

Substrate Scope. The substrate scope for **biot^{C4}−1**·Sav S112R was expanded to substrates containing benzylic C(sp^3)−H bonds (Table 1). Propylbenzene and butylbenzene afforded the corresponding (*R*)-alcohol in 45% ee (TTON = 26 and 19, respectively). Electron-rich *p*-substituted ethylbenzenes afforded higher TTONs, highlighting the electrophilic character of the Fe(O) species (Figure S15).

A kinetic isotope effect KIE = 9.2 was determined for the oxidation of PhEt/PhEt-d_{10} by **biot^{C4}−1**·Sav S112R at 25 °C (Figure S16). This value compares well with the previously described KIE for Fe-TAML complexes and suggests that the rate-determining step of the reaction is the hydrogen abstraction.[56,60,67,68]

The oxidation of indane and tetralin (BDE$_{C-H}$ = 87 and 85.7 kcal/mol)[69] afforded high TTONs (TTON = 205 and 316, respectively) and good ee in favor of the (*R*)-alcohol (47% and 65% ee, respectively, Table 1).

Prompted by the good TTON and ee for tetralin, its oxidation by **biot^{C4}−1**·Sav S112R was scrutinized. Using 2.5 equivalents of H_2O_2, 73% ee of (*R*)-tetralol was determined at early stages (Scheme 3a). In contrast to PhEt oxidation, the ee increased with conversion, highlighting the preferential (over)-oxidation of (*S*)-tetralol. After 3 h, >98% ee (*R*)-tetralol was obtained (TTON = 300, Scheme 3b). Minimal overoxidation at the second benzylic position was also detected (Figure S17). Oxidation of (*rac*)-tetralol with **biot^{C4}−1**·Sav S112R yielded tetralone and >99% ee of (*R*)-tetralol (unreacted starting material) after ∼120 min (Scheme 3c, $E = k_{(S)}/k_{(R)} = 2.7$, and Figure S18). Similarly, a TTON of 173 was obtained for indane oxidation (80% ee (*R*)-indanol, Figure S19). Thus, (*R*)-benzyl-alcohol derivatives are preferentially overoxidized, while the (*S*)-enantiomers of the cyclic derivatives (tetralol and indanol) are oxidized faster. This phenomenon can be attributed to the 1,3-allylic strain (Scheme S2).[70,71]

Lastly, we developed an enzymatic cascade with Glucose Oxidase (GO) to enable the *in situ* production of H_2O_2, using O_2 as oxidant and glucose as reductant (Scheme 4).[72] To our delight, after combining **biot^{C4}−1**·Sav S112R and GO the oxidation reactions progressed in a similar way compared to the single batch addition of H_2O_2. A TTON of 50 was obtained for PhEt oxidation, with an initial ee of (*R*)-PhEtOH of 47%, which eroded to 37% after kinetic resolution. For tetralin, a TTON of 170 was obtained, again observing the initial formation of (*R*)-tetralol in 64% ee and posterior kinetic resolution that upgraded it to up to 95%.

Catalysts derived from earth-abundant metals are gaining attention in homogeneous catalysis. The inherent lability of most such systems however limits their use in water. In contrast to polypyridinamine-derived catalysts,[73] and thanks to its remarkable stability and catalytic activity, the Fe(TAML) system proved amenable to the design and optimization of an artificial hydroxylase based on the biotin−streptavidin technology.

Chemogenetic optimization of the catalytic performance led to the identification of **biot^{C4}−1**·Sav S112R as our best hydroxylase for the oxidation of benzylic C−H bonds. With *in vivo* applications in mind, we have shown that the activity of the artificial hydroxylase is compatible with glucose oxidase, using O_2 as the terminal oxidant.

Efforts at modulating the activity of the hydroxylase by fine-tuning the cofactors' structure, and expanding the substrate scope toward the oxidation of more complex molecules, are currently underway.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c02788.

General information, experimental section, Figures S1−S21, Tables S1−S6, Scheme S1−S2 (PDF)

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Notes

The authors declare no competing financial interest.

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