Chemoenzymatic Skeletal Editing: P450-Controlled Site-Selective Ring Expansion of Natural Product Scaffolds at Aliphatic C—H Sites

John M. Bennett[1,‡], Andrew R. Bortz[1,‡], and Rudi Fasan[1,2,*]

[1] *Department of Chemistry, University of Rochester; Rochester, NY, 14627, USA.*

[2] *Current affiliation: Department of Chemistry and Biochemistry, University of Texas at Dallas, 800 W. Campbell Road, Richardson, TX 75080, United States*

‡ These authors contributed equally to this work.

**Corresponding author. Email: rudi.fasan@utdallas.edu*

Abstract

Methods for introducing subtle modifications at the level of single atoms/bonds ('skeletal editing') are highly desirable in organic and medicinal chemistry, owing to their potential for fine-tuning the structure and biological activity of organic molecules. While contemporary methods for skeletal editing of organic molecules largely rely on modification of pre-existing functional groups, opportunities for executing these transformations at ubiquitous yet unreactive aliphatic C(sp^3)—H sites are currently unavailable. Here, we report a chemoenzymatic strategy for enabling skeletal editing via ring expansion with high site-selectivity at the level of one or more aliphatic C—H sites in complex molecules. By combining cytochrome P450-catalyzed C—H oxidation with chemical oxidation and subsequent Baeyer-Villiger rearrangement or ketone homologation,

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a panel of structurally and functionally diverse natural products were edited by inserting a lactone or carbonylmethylene moiety into aliphatic regions of their carbocyclic skeletons. Using engineered P450 catalysts with divergent regioselectivity, a set of different ring-expanded products could be readily obtained from a single parent molecule, highlighting the potential of this approach for skeletal edit scanning and/or library generation. By enabling the targeting of aliphatic C—H sites with tunable site-selectivity, this strategy provides a powerful tool to rapidly access skeletally edited derivatives of natural products and other bioactive molecules that would be hard to attain by purely chemical means. We envision this approach can also enable the device of non-traditional retrosynthetic disconnections for the synthesis of complex molecules.

Introduction

Strategies for the chemo- and regioselective functionalization of C—H bonds, which are ubiquitous in both natural and anthropogenic compounds, constitute powerful tools for streamlining the functionalization of organic molecules and/or enabling their construction through new disconnections.[1-9] Methodologies for 'late-stage' C—H functionalization, in particular, are of considerable value in organic and medicinal chemistry, due to their value toward enabling the rapid functionalization or diversification of complex bioactive molecules, such as natural products and synthetic drugs.[3-6] Notable strategies in this area, in particular targeted toward C(sp^2)—H bonds, include the use of metal-catalyzed C—H activation, visible-light-induced photocatalysis, and electrosynthesis, which have often provided complementary regioselectivity toward targeting different aromatic C—H bonds in a target molecule (**Figure 1A**).[3-6, 9] In comparison, the selective functionalization of aliphatic C—H bonds, especially at the level of stereoelectronically unactivated C(sp^3)—H sites, has represented a major challenge.[7, 8]

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Whereas late-stage C—H functionalization methods provide a powerful means for decorating the core structure of an organic molecule with new functionalities (also referred to as 'peripheral editing') (**Figure 1A**), there has been a growing interest in strategies for altering the molecular skeleton of these molecules at the level of single atoms/bonds, also referred to as 'skeletal editing'.[10] Complementing the former methods, skeletal editing strategies offer an attractive avenue toward fine-tuning the core structure of a molecule of interest, but they have been comparatively less explored, in part due to the challenge of accomplishing these transformations with high efficiency and/or selectively.[10]

Important methods for skeletal editing of carbonyl-containing compounds include the Favorskii, Baeyer-Villiger (BV), and Beckmann rearrangements.[11-13] More recently, creative methodologies have been introduced to enable ring contraction or expansion in heterocycles,[10] including transformations involving nitrogen atom deletion[14-18], ring expansion of benzylic alcohols and amines, [19, 20], boron insertion into ethers,[21] and carbon atom insertion into azoles[22, 23], as exemplified by the transformation of the antichlolesterolemic drug atorvastatin into a pyridine-based analog (**Figure 1A**). Despite this progress, these skeletal editing strategies are restricted to molecules that contain the required, reactive functional group (e.g., carbonyl, amine) within their core structure. As a result, and combined with the challenges inherent to the functionalization of unactivated C(sp^3)—H bonds mentioned above, the possibility of executing skeletal editing transformations at the level of 'remote' aliphatic C—H bonds in complex molecules has so far remained elusive.

Motivated by these challenges, we report here the development of a chemoenzymatic strategy for skeletal editing—via ring expansion—of complex molecular scaffolds at the level of both single and multiple aliphatic C—H sites (**Figure 1B**). By integrating P450-mediated C—H

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oxyfunctionalization with BV rearrangement or ketone homologation, this strategy offers several unique advantages for skeletal editing, namely (a) the possibility to edit a carbon skeleton at the level of methylene C—H bonds, including remote and unactivated C(sp^3)—H sites; (b) tunability in terms of site-selectivity, providing access to multiple ring-expanded products from the same parent molecule, and (c) compatibility with complex molecular settings, providing access to structurally diverse and stereochemically rich new natural product-like scaffolds. Because the C—H functionalization step is enzymatically controlled and can be directed toward remote C(sp^3)—H sites in the substrate of interest, this approach enables ring expansion within regions of the molecule that are typically hard to access or inaccessible by chemical means alone. As shown, this approach can be applied to generate collections of ring-expanded analogs of a single parent molecule, and it can bfurther extended to introduce a single oxygen atom insertion into a carbocyclic scaffold.

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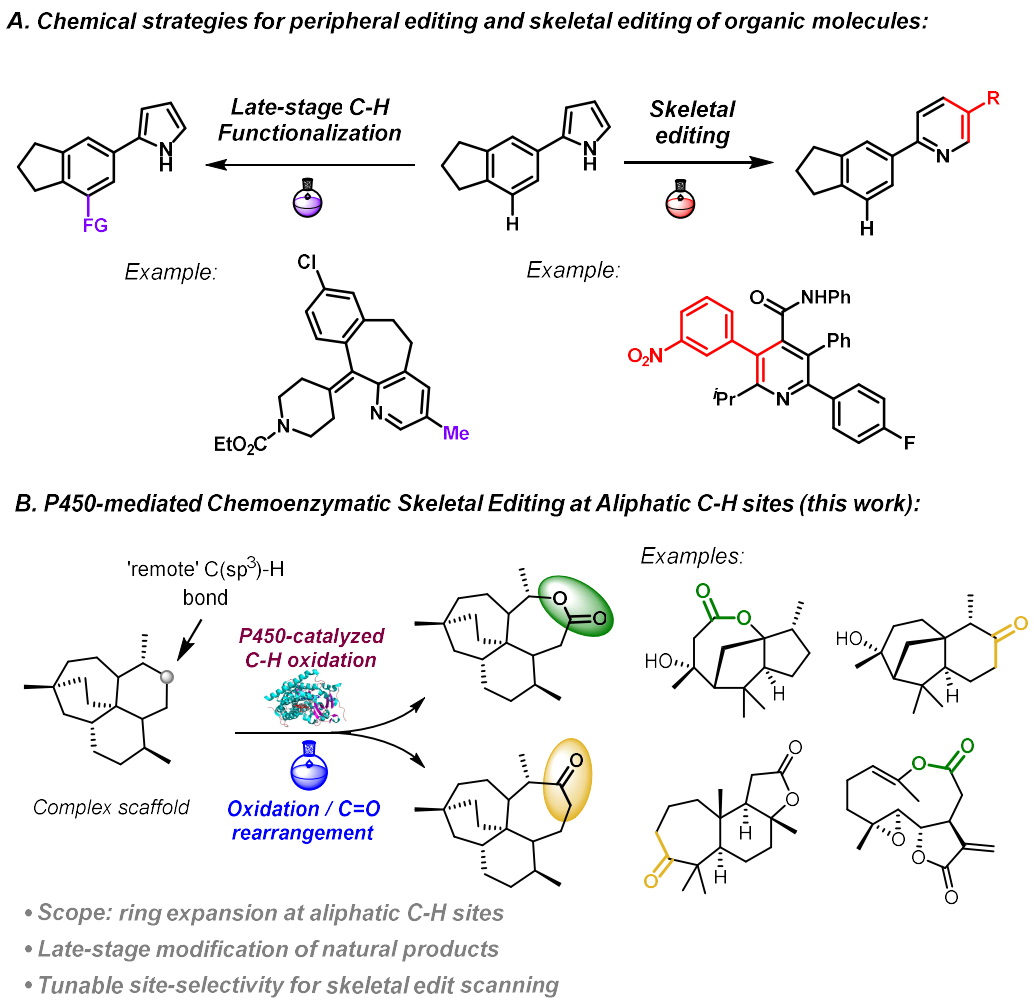

Figure 1. Late-stage molecular editing strategies. (**A**) Representative example of peripheral and skeletal editing of drug molecules via late-stage C—H functionalization [24] and carbon atom insertion into pyrroles[22], respectively. (**B**) **This work**: Skeletal editing of complex scaffolds via P450-catalyzed chemoenzymatic ring expansion at aliphatic C—H sites.

Results and Discussion

To implement the present strategy, we envisioned the possibility of employing an engineered P450 to catalyze a site-selective hydroxylation on a target molecule. Following oxidation, the resulting ketone would be primed for ring-expansion at the level of the oxyfunctionalized C—H site via BV

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rearrangement. By leveraging enzyme-controlled regioselectivity in the initial C—H hydroxylation step, we further envisioned the possibility of directing these skeletal editing modifications to different C—H sites in the target molecule. To explore the viability of this approach, we selected a panel of biologically active natural products (**Figure 2A**) whose structures encompass a diverse range of complex molecular scaffolds and biological activities. These compounds include sclareolide (SCL, **1**), a terpene natural product with antifungal properties,[25] artemisinin (ART, **2**), a drug used for the treatment of human malaria in combination therapies,[26] and the sesquiterpene lactones parthenolide (PTL, **3**) and micheliolide (MCL, **4**), which possess potent antileukemic and anticancer activity, in particular against cancer stem cells.[27, 28]

Various studies has demonstrated the utility of natural and engineered P450 enzymes for the oxidation of drugs and natural products.[29-48] In previous work, we developed engineered variants of the fatty acid monooxygenase $P450_{BM3}$ (*Bacillus megaterium*) that are capable of hydroxylating **1-4** under mild conditions,[31-33, 49] thus providing an initial enzyme set for testing our chemoenzymatic skeletal editing strategy. Briefly, these engineered P450s were generated via active site mutagenesis starting from a promiscuous $P450_{BM}$ variant, called FL#62 (**Table S1**), followed by screening of the resulting libraries via high-throughput P450 49

using a set of chromogenic substrates.[31-33, 49] Fingerprint-guided reactivity predictions were then applied to identify variants with high activity and site-selectivity toward oxidation of the target substrate under investigation, namely sclareolide, artemisinin, parthenolide or micheliolide.[31-33, 49] Ultimately, these variants were determined to contain 14-16 mutations compared to wild-type $P450_{BM3}$ (**Table S1**).

Using $P450_{BM3}$ variant II-B1, selective hydroxylation of sclareolide (**1**) at the C3 position could be accomplished on a preparative scale (0.2 g) to afford 3(*S*)-hydroxy-SCL (**10, Figure 2B**)

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in 64% yield. Similarly, P450-catalyzed methylene hydroxylation of C7 in artemisinin (**2**), C9 in parthenolide (**3**), and C2 in micheliolide (**4**) could be accomplished using engineered $P450_{BM3}$ variants IV-H4,[32] II-C5,[33] and V-F10,[31] respectively, with high regioselectivity (68-99%) (**Figure 2B**). These enzymatic transformations afforded the oxyfunctionalized products 7(*S*)-hydroxy-ART (**10**), 9(*S*)-hydroxy-PTL (**11**), and 2(*R*)-hydroxy-MCL (**12**), respectively, in good yields (31-55%) (**Figure 2C**) and on a preparative scale (0.1-0.2 g), which were used as precursors for the subsequent skeletal editing steps. To explore the scope of this methodology across other complex scaffolds, we then targeted other medicinally or biotechnologically important natural products, namely (-)-bornyl acetate (**5**), (-)-menthyl acetate (**6**), (+)-totarol (**7**), and 11,12-dihydronootkatone (**8**) (**Figure 2A**). To identify viable P450 catalysts for the selective hydroxylation of these substrates, we screened a library of engineered $P450_{BM3}$ variants previously identified as functionally diverse based on "P450 fingerprints" acquired using a set of chromogenic probes encompassing different molecular scaffolds.[32, 49] Using this approach, the broadly active P450 variant FL#62[50] was found to be useful for realizing the hydroxylation of (-)-bornyl acetate at the C5 position with high regioselectivity (>99%) and activity (>5,000 turnovers or TON) to afford 5(*S*)-hydroxy-bornyl acetate (**14, Figure 2B**) in 58% yield (**Figure 2C**). Using $P450_{BM3}$ variant II-E1 (**Figure S1**), on the other hand, diterpene (+)-totarol (**7**) could be hydroxylated at the C2 site with excellent regioselectivity to obtain 6(*R*)-hydroxy-(+)-totarol (**16, Figure 2B**) in 39% yield (**Figure 2C**). Lastly, a highly regio- and stereoselective P450 catalyst (I-D3, **Figure S1**) was identified for the C9 hydroxylation of **8**, enabling the preparative-scale synthesis of 9(*R*)-hydroxy-11,12-dihydronootkatone (**18**) in 42% isolated yield (**Figure 2B/2C**). While each of the aforementioned target molecules could be oxidized at a single methylene site within their core structure, functionalization of multiple secondary C—H sites was possible for (-)-menthyl acetate

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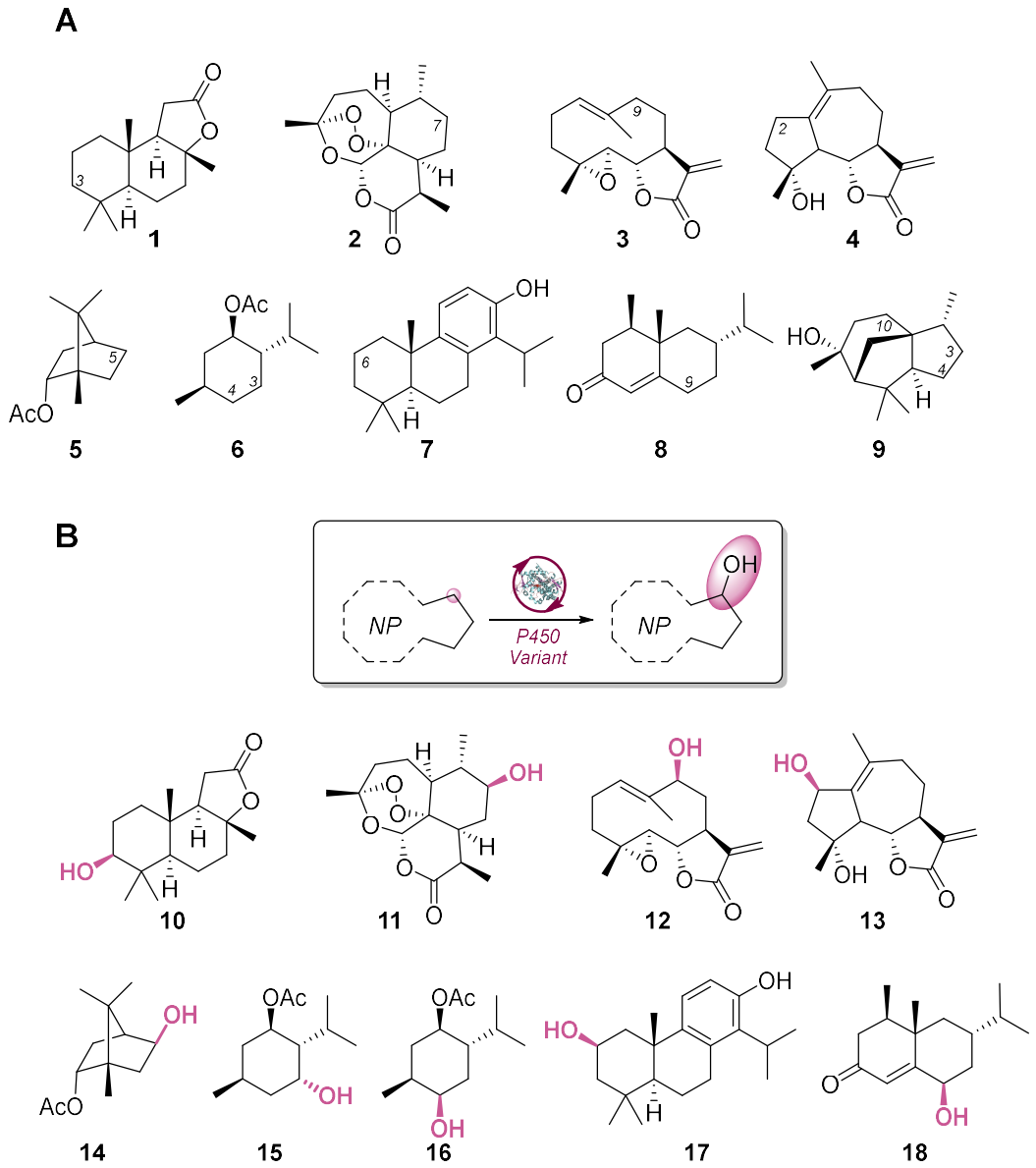
(**6**). Specifically, two sites could be hydroxylated in **6** using the $P450_{BM3}$ variant I-D3 (**Figure S1**), enabling the isolation of 3(*R*)-hydroxy-menthyl acetate and 4(*R*)-hydroxy-menthyl acetate (**15** and **16**, respectively, **Figure 2B**) in moderate yields (**Figure 2C**).



C

Substrate	P450 Variant	P450 mol %	Site Selectivity	Regio-selectivity	Yield
Sclareolide (**1**)	II-B1	0.2	C3(*S*)	>99%	62%
Artemisinin (**2**)	IV-H4	0.2	C7(*S*)	>99%	31%
Parthenolide (**3**)	II-C5	0.2	C9(*S*)	68%	44%

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Micheliolide (**4**)	V-F10	0.08	C2(*R*)	97%	55%
Bornyl Acetate (**5**)	FL#62	0.01	C5 (*S*)	>99%	58%
Menthyl Acetate (**6**)	I-D3	0.02	C3 (*R*)	22%	10%
			C4 (*R*)	27%	12%
Totarol (**7**)	II-E1	0.3	C6 (*R*)	N.D.	39%
Dihydronootkatone (**8**)	I-D3	0.1	C9(*R*)	80%	42%

Figure 2. Selective P450-catalyzed C(sp^3)—H oxidation of natural product scaffolds. **(A)** Structures of the target natural products (NPs) investigated in this study and (**B**) hydroxylation products obtained via P450-catalyzed C—H oxidation. **C**) Activity, regioselectivity, and isolated yields of the enzymatic reactions. Reaction conditions: 1-2 mM substrate, 2% v/v DMSO or 5% v/v EtOH cosolvent, 0.25–6 μM P450, 2 μM phosphite dehydrogenase (PTDH), 150 μM $NADP^+$, 50 mM potassium phosphite, in potassium phosphate (KPi) buffer (50 mM, pH 8). The selectivity for C2 oxidation of **7** could not determined due to instability of other oxidation products under the applied reaction or analytical (GC) conditions. The amino acid mutations of the $P450_{BM3}$ variants are reported in **Table S1**.

To set the stage for their ring expansion through a BV rearrangement, the enzymatic products from the reactions described above were subjected to oxidation in the presence of pyridinium chlorochromate (PCC) or Dess-Martin periodinane to afford the corresponding ketone derivatives (**39-46, Figure S1**) in good to high yields (32-90%). Notably, all products could be converted to the desired intermediates containing a keto functionality embedded in a carbocylic region of the molecule. The BV rearrangement involves attack of a peracid on a carbonyl group, followed by migration of one of the substituents in α to the ketone group to produce an ester.[51] Generally, the migratory aptitude of these substituents increases with the degree of substitution,

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although other factors, such as steric bulk, can influence migratory affinity.[12] Gratifyingly, when the keto-functionalized derivatives of sclareolide (**39**), artemisinin (**40**), and parthenolide (**41**) were subjected to reaction with *m*-chloroperoxybenzoic acid (*m*CPBA), they all underwent efficient BV rearrangement to furnish the desired ring-expanded lactone products **19-21** (**Figure 3**).

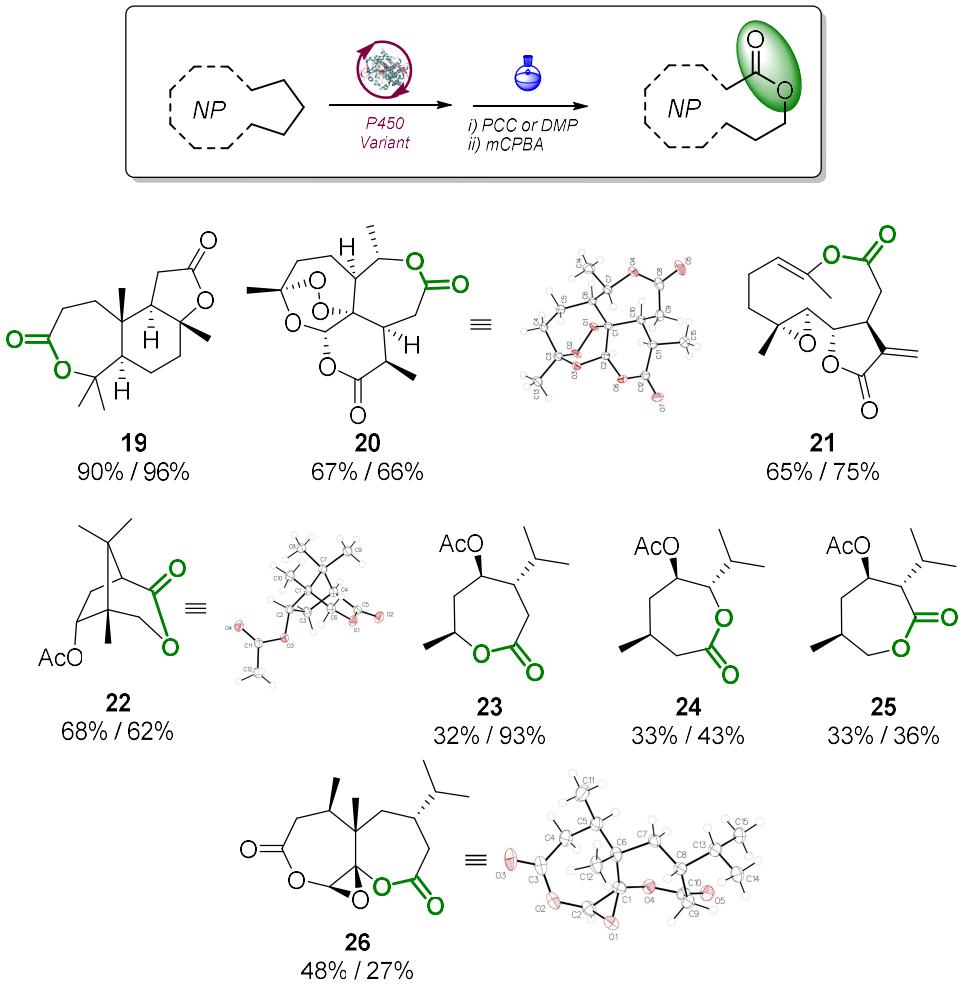

Figure 3. Chemoenzymatic ring expansion of natural product scaffolds at C(sp^3)—H sites through oxidation / BV rearrangement. Reaction conditions: (i) pyridinium chlorochromate (PCC) (1.5 eq.) or Dess-Martin periodinane (DMP) (1.5 eq.) in anhydrous dichloromethane. (ii) *meta*-chloroperoxybenzoic acid (*m*CPBA) (20 eq.) in anhydrous dichloromethane.

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In each case, the regioselectivity of the oxidative ring expansion reaction followed the expected migratory aptitude of α substituents in BV reactions,[12] with the reaction favoring migration of the tertiary (**19**), secondary (**20**), or vinylic alkyl group (**21**), respectively, over a primary alkyl group. It is also worth noting that the chemoenzymatic ring-expansion was successful even in the presence of potentially sensitive functional groups, such as the endoperoxide bridge in **40** and the α-methylene-γ-lactone moiety in **41**, which are essential for the biological activity of this natural product.[52] Notably, **41** also contains an internal double bond which is reactive toward epoxidation,[53] yet the chemoenzymatic ring-expansion reaction could be accomplished despite this potential liability.

Ring expansion of the bornyl acetate derivative **43** was also successful resulting in the exclusive formation of the ring-expanded lactone **22** (**Figure 3**), which derives from migration of the methylene group rather the more substituted bridgehead position. The latter result can be rationalized based on prior observations in the BV rearrangement of camphor[12] and considerations regarding the transition states involved in the two possible rearrangement pathways of this molecule.[54] Due to the steric bulk of the bridgehead position, attack by the peracid is favored on the least hindered side of the ketone group. The subsequent, regiochemistry-determining step can proceed either via a boat-like transition state for migration of the bridgehead group, or via a chair-like transition state from migration of the methylene group. Due to the chair-like transition state being much lower in energy, the latter pathway is favored in the BV rearrangement of camphor. We hypothesize that a similar mechanistic scenario explains the exclusive formation of **22** from the peracid-induced rearrangement of **43 (Figure 3**).

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Upon subjecting 4-keto menthyl acetate (**45**) to the same BV oxidation conditions, this compound underwent clean rearrangement to form the ring-expanded product **23**, as derived from migration of the more substituted C—C bond **(Figure 3**). In contrast, BV oxidation of **44** was found to yield the two regioisomeric products **24** and **25** in a 1.2:1 ratio (**Figure 3**), both of which could be isolated in reasonable yields (36-43%).

Some substrate-specific limitations were also noted. While the present chemoenzymatic ring expansion could be realized in parthenolide as noted above, epoxidation was found to accompany or compete with ring expansion in the case of other substrates containing olefinic groups such as 9-keto-DNK (**47**) and 2-keto-micheliolide (**42**), which were included to probe the boundaries of the method. In the case of **47**, the desired ring expansion transformation could still be achieved resulting in the formation of the highly modified scaffold **26**, which features a dual BV rearrangement, along with epoxidation of the 1,10-alkene (**Figure 3**). For micheliolide-derived **42**, however, epoxidation outcompeted the desired ring expansion in the presence of *m*CPBA or other oxidants (e.g., dibenzyl diselenide), affording epoxide **48** or the elimination product **49**, respectively (**Scheme S1**). Molecules with oxidation sensitive functional groups also fall beyond the scope of the present method. For **46** derived from totarol (**7**), the phenol moiety was indeed found to decompose upon exposure to BV oxidation conditions, even after protection of the phenolic OH group via silylation (e.g., TMS or TBDMS) or acetylation.

Despite these substrate-specific idiosyncrasies, the present chemoenzymatic strategy could be applied to the majority of the of the tested natural product substrates (7/9 = 77%), thereby providing efficient access to a total of eight, unprecedented skeletally edited analogs of these molecules. Importantly, the results with **6** show how three different, regioisomeric lactone products, i.e., **23, 24,** and **25**, could be obtained from a single parent molecule, highlighting the

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potential utility of this method for 'skeletal edit scanning' or diversity-oriented synthesis applications.

Based on the success of the chemoenzymatic ring-expansion strategy described above, we sought to demonstrate the capability of this method to enable other types of ring-expansion transformations targeted at aliphatic C—H bonds. Accordingly, we envisioned subjecting the chemoenzymatically produced ketone intermediates to homologation in the presence of diazo compounds.[55, 56] Initial attempts to perform the target homologation reaction with diazomethane were unsuccessful, resulting in either degradation of the substrate or no reactivity. Gratifyingly, further attempts using trimethylsilyl diazomethane[57] were more fruitful and multiple substrates, namely **39**, **40**, and **43**, were successfully transformed using this protocol to afford another series of skeletally edited derivatives of sclareolide, artemisinin, and bornyl acetate (**27**-**30**, **Figure 4**). Consistent with previous observations on other substrates,[55, 56] methylene insertion in these homologation reactions was found to prefer the less substituted group in α to the ketone, resulting in a generation of a single homologation product in the case of **27** and **28**. Interestingly, starting from 7-oxo-artemisin **40**, formation of the target ring expansion product **28** was accompanied by formation of the spiro-epoxide **50** (**Figure 4**), which could be isolated and characterized by X-ray crystallography. Thus, this chemoenzymatic route overall enabled transformation of a C(sp^3)–H site into a spiro-epoxide group, which is known to have peculiar reactivity compared to equivalent disubstituted epoxides[58] and which is found in various bioactive natural products (e.g., fumagillin, trichothecenes).[59, 60] Starting from 4-oxo-bornyl acetate **43**, on the other hand, two ring-expanded regioisomers **29** and **30** were obtained, with the homologation product derived from migration of more substituted bridgehead position (**29**) predominating over **30** in a ratio of 5.2:1 (**Figure 4**). As for the BV rearrangement of this scaffold, stereoelectronic effects likely affect the regiopreference

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of the rearrangement step. Overall, these results demonstrated the functionality of the approach of **Figure 4** for the ring expansion of various complex scaffolds via an overall methylene to –CH_2CO– substitution.

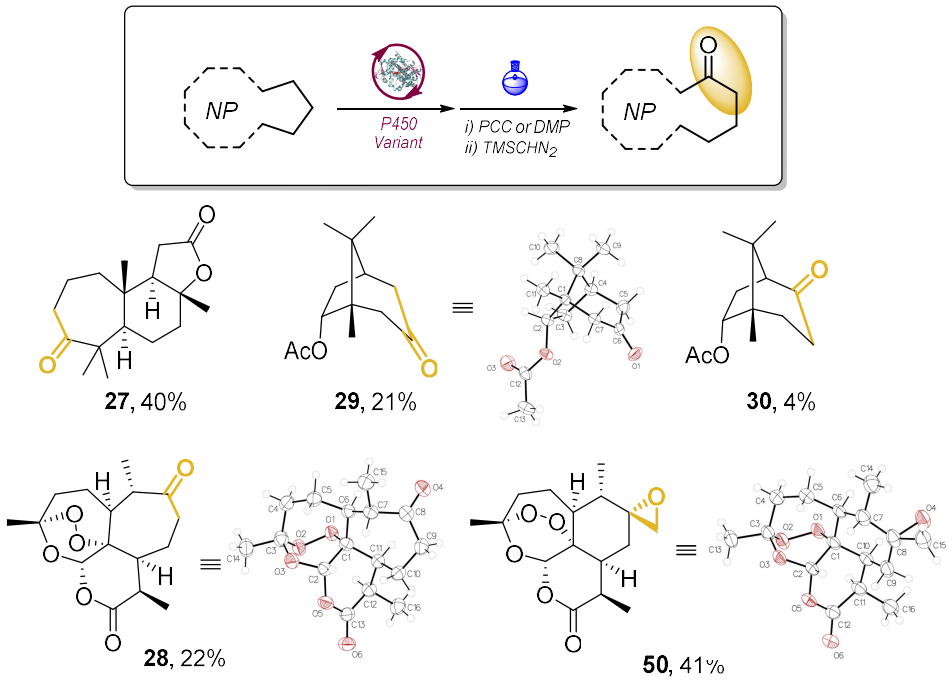

Figure 4. Chemoenzymatic ring expansion of natural product scaffolds at C(sp^3)—H sites through oxidation / ketone homologation. Reaction conditions: (i) pyridinium chlorochromate (PCC) (1.5 eq.) or Dess-Martin periodinane (DMP) (1.5 eq.) in anhydrous dichloromethane. (ii) trimethylsilyldiazomethane (3-20 eq.) and boron trifluoride diethyletherate (1.5-20 eq.) in anhydrous dichloromethane.

To explore the potential of this methodology for *regiodivergent* skeletal editing at multiple aliphatic C—H positions in the same molecule, the terpene natural product cedrol (**9**), which features a complex fused ring bicyclic scaffold with five methylene C—H sites was chosen as the target substrate. To identify P450 catalysts for regiodivergent C—H oxyfunctionalization of this

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compound, cedrol was screened against an in-house collection of functionally diverse P450 catalysts obtained via fingerprinting of active-site libraries of engineered $P450_{BM3}$ variants as described above. From this screening, three different P450 variants, namely VI-D11, I-D3, and II-H10, were isolated for executing the selective hydroxylation of position C3, C4, or C10, respectively, with up to 81% regioselectivity (**Figure 5**). Each of the corresponding enzymatic products, i.e., 3(*S*)-hydroxy-cedrol (**35**), 4(*R*)-hydroxy-cedrol (**33**), and 10(*R*)-hydroxy-cedrol (**31**), could be then subjected to the tandem DMP/*m*CPBA-mediated oxidation to afford the ring-expanded products **36**, **34**, and **32**, respectively, thus providing a divergent path to editing of the carbocyclic backbone of this molecule. Furthermore, 3-hydroxy-cedrol **35** could be subjected to the oxidation/$TMSCHN_2$-mediated homologation protocol to form the alternative, ring-expanded product **37** (**Figure 5**). Following another pathway, lactone **36** was reduced using diisobutylaluminum hydride (DIBAL), followed by treatment with triisopropylsilane (TIPS) and boron trifluoride diethyletherate ($BF_3 \cdot OEt_2$), to furnish the ring=expanded ether **38**, in which a single oxygen atom is inserted into the 5-membered carbocyclic ring of the parent molecule (**Figure 5**). Altogether, using the present strategy, a panel of five ring-expanded derivatives were rapidly obtained starting from a single parent scaffold.

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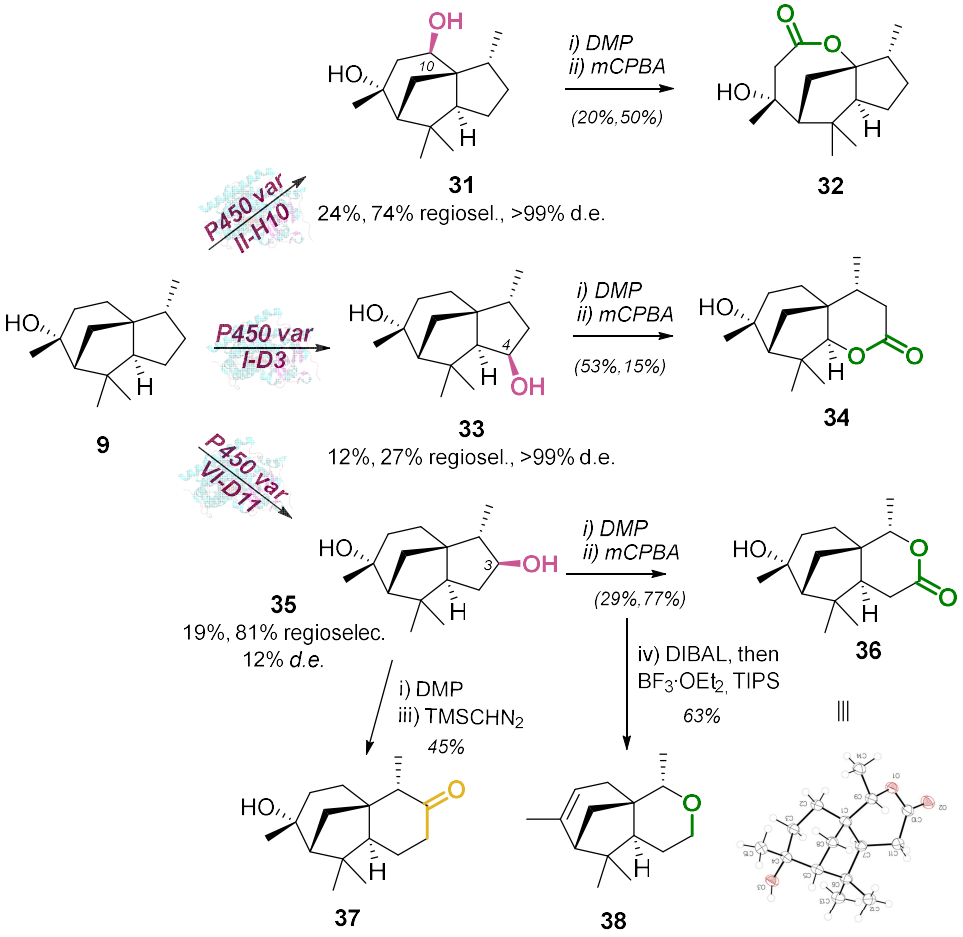

Figure 5. Regiodivergent chemoenzymatic skeletal editing of cedrol (9). Enzymatic reactions: 2 mM cedrol, 2 % v/v DMSO cosolvent, 0.3 mol% P450, 2 µM phosphite dehydrogenase (PTDH), 150 µM $NADP^+$, 50 mM potassium phosphite, in potassium phosphate (KPi) buffer (50 mM, pH 8). Mutations of the P450 variants are described in **Table S1**. Reaction conditions: (*i*) 1.5 eq. Dess-Martin periodinane (DMP) in anhydrous DCM. (*ii*) *meta*-chloroperoxybenzoic acid (*m*CPBA) (20 eq.) in anhydrous dichloromethane. (*iii*) trimethylsilyldiazomethane (2 eq.) and boron trifluoride diethyletherate (1.5 eq.) in anhydrous dichloromethane. (*iv*) Diisobutylaluminium hydride (DIBAL) (2.0 eq.) then triisopropylsilane (4 eq) and boron trifluoride diethyletherate (4 eq.) in anhydrous dichloromethane.

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Conclusions

In summary, we have developed a chemoenzymatic methodology to execute subtle skeletal modifications targeted at aliphatic C—H sites in complex molecules. Complementing the capabilities of current skeletal editing strategies,[10] this approach leverages P450-mediated molecular recognition to access and activate a C(sp^3)—H site in the target molecule for subsequent formation of a ring-expanded lactone, ketone, or ether derivative. Using this strategy, a total of 17 new skeletally edited derivatives of structurally diverse natural products such as parthenolide, artemisinin, sclareolide, and cedrol were obtained. As exemplified with cedrol, the tunable site-selectivity of the P450 catalyst can be leveraged to target multiple unactivated and remote C(sp^3)—H sites in a late-stage and regiodivergent manner, thus providing a unique opportunity for 'skeletal edit scanning' and the generation of libraries of core edited analogs derived from a single parent molecule. As demonstrated here, this approach is applicable in the context of natural products, which are notoriously challenging to manipulate due to their stereochemical and functional complexity.[3-9] As such, this approach can provide access to and enable the rapid exploration of novel, complex natural product-like compounds of potential value for chemical biology or drug discovery campaigns. The present strategy should also enable the design of non-traditional retrosynthetic disconnections for the synthesis of complex molecules. Finally, we envision further extension of this general approach in the future to other types of skeletal editing transformations targeted at aliphatic C—H sites.

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ASSOCIATED CONTENT

Supporting Information

Supporting information includes supplementary Tables, Figures and Schemes, experimental procedures, synthetic procedures, compound characterization data, NMR spectra, crystallographic data.

AUTHOR INFORMATION

Corresponding Authors:

* rudi.fasan@utdallas.edu.

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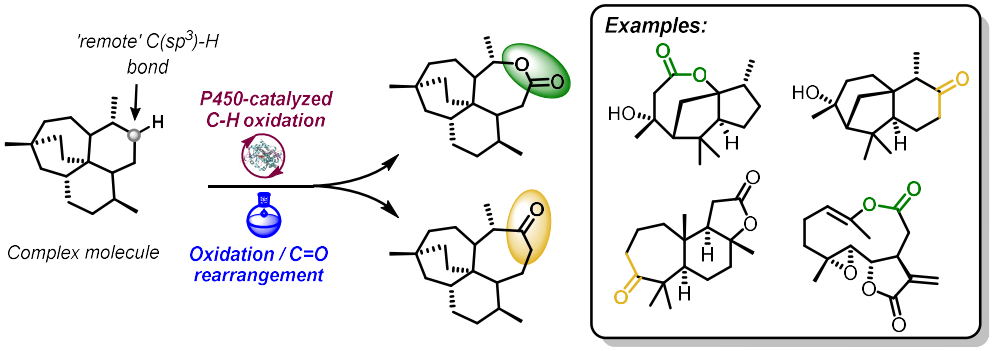
Graphical Abstract



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