Diverse engineered heme proteins enable stereodivergent cyclopropanation of unactivated alkenes

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Abstract

Stereodivergent syntheses leading to the different stereoisomers of a product are useful in the discovery and testing of drugs and agrochemicals. A longstanding challenging in catalysis, developing sets of stereodivergent catalysts is often solved for enzymes by screening Nature's diversity for biocatalysts with complementary stereoselectivities. Here, Nature's protein diversity has been leveraged to develop stereodivergent catalysts for a reaction not known in biology, cyclopropanation via carbene transfer. By screening diverse native and engineered heme proteins, we identified globins and serine-ligated cytochromes P450 with promiscuous activity for cyclopropanation of unactivated alkene substrates. Their activities and stereoselectivities were enhanced by directed evolution: 1-3 rounds of site-saturation mutagenesis and screening generated enzymes that catalyze the stereodivergent cyclopropanation to form each of the four stereoisomers of unactivated alkenes and electron-deficient alkenes with up to 5,400 total turnovers and 98% enantiomeric excess. These fully genetically encoded biocatalysts function in whole *E. coli* cells in mild, aqueous conditions and provide the first example of enantioselective, intermolecular iron-catalyzed cyclopropanation of unactivated alkenes via carbene transfer.

Introduction

The biological world is a marvelous ensemble of chiral molecules. From the amino acid and nucleoside building blocks that form proteins and DNA to intricate natural products produced by living organisms, chirality dictates how molecules interact with living systems.[1] Many modern medicines draw inspiration from natural products.[2] Because alternate stereoisomers can have very different biological effects,[3] characterization of novel bioactive compounds during drug candidate screening should include testing each stereoisomer.[4] Developing stereodivergent syntheses, where a set of complementary catalysts can generate every possible stereoisomer of the product, is therefore useful and is actively sought after in catalysis.[5] Enzymes are green, sustainable options for stereoselective catalysis, and stereo-complementary enzymes can often be found in nature's diversity: lipases[6], ketoreductases[7], and transaminases[8] chosen using genome mining[9], for example, have all afforded products with different stereoselectivities. We show here that natural protein diversity can be leveraged in a similar fashion to achieve stereodivergence for a new, non-natural enzyme-catalyzed reaction, cyclopropanation of unactivated alkenes.

Previous work from this group and others has shown that iron-porphyrin (heme) proteins can be engineered to catalyze the cyclopropanation of styrenyl alkenes with ethyl diazoacetate (EDA, **1**).[10-13] This new-to-nature carbene transfer reaction has been applied in the synthesis of key pharmaceutical intermediates such as levomilnacipran[14], ticagrelor[15-16], and tasimelteon.[16] Thus far, however, alkene cyclopropanation by heme proteins with the native iron cofactor has been limited to styrenyl and other activated alkenes. Unactivated, aliphatic alkenes are attractive feedstocks for chemical synthesis, but their transformation to higher-value chiral products is challenging due to their inert nature, high degree of conformational flexibility, and limited steric and electronic bias to guide stereocontrol.[17]

State-of-the art methods for unactivated alkene cyclopropanation often rely on noble metals[18-20] (Supplemental Table S1); no iron-based catalyst for the enantioselective intermolecular cyclopropanation of unactivated alkenes has been reported. However, directed evolution of heme proteins has previously enabled biocatalysis to access reactions performed with noble-metal catalysts, such as carbon–silicon bond formation[21] and intermolecular C–H amination[22]. We therefore set out to create a genetically encoded catalyst with the native heme cofactor that could cyclopropanate unactivated alkenes. Furthermore, we wished to take advantage of the natural diversity of heme proteins to identify suitable starting points for engineering stereodivergent biocatalysts.

Results and Discussion

We collected a panel of eleven heme proteins from thermophilic and hyperthermophilic bacteria and archaea to test for unactivated alkene cyclopropanation (Supplemental Table S2). Thermostable proteins can better withstand the destabilizing effects of mutations and are therefore more 'evolvable'.[23] They are also often easier to work with and better tolerate polar organic solvents used to solubilize substrates. Wild-type *Aeropyrum pernix* protoglobin (ApePgb WT, UniProt ID: Q9YFF4) and wild-type *Rhodothermus marinus* nitric oxide dioxygenase (RmaNOD WT, UniProt ID: D0MGT2) were found to have low but measurable cyclopropanation activity on 1-octene **2a**, catalyzing the reaction with 18 and 27 total turnovers per enzyme active site (TTN). Notably, ApePgb WT and RmaNOD WT displayed complementary diastereoselectivity, preferentially producing *cis* (***1R, 2S***)-**3a** and *trans* (***1S, 2S***)-**3a**, respectively.

In addition to searching natural heme protein diversity for this novel reactivity, we also investigated heme proteins obtained in previous directed evolution studies. A panel of 36 variants of a *Bacillus megaterium* cytochrome P450 (BM3) engineered for other non-natural carbene and nitrene transfer reactions[13] was tested for the ability to cyclopropanate 1-octene **2a** and 4-phenyl-1-

butene **2b**. **2b** was chosen as a substrate for library screening, because the UV-visible phenyl group enables screening by HPLC-UV. BM3 variant P411-CIS L437F T438Q L75Y L181I (P411-UA, sequence in Supporting Information) showed significant activity and selectivity for production of *cis* (***1S***, ***2R***)-**3a**, the third of the four possible isomers. This variant of a serine-ligated "P411" (P411-CIS[24]) had been engineered for cyclopropanation reactivity on electron-rich, non-styrenyl alkenes such as *N*-vinyl amides (Brandenberg et al., unpublished results).

Site-saturation mutagenesis libraries were generated and screened to increase the activities and selectivities of the different enzymes. Because crystal structures of ApePgb and RmaNOD have not been reported, homology models were built to help us identify residues within the putative distal heme pocket, where carbenoid formation and substrate binding are predicted to take place (Supplemental Figure S2). P411-UA residues were selected based on the crystal structure of its P411-CIS predecessor (PDB ID: 4H23). Individual site-saturation libraries were screened for increased activity and diastereoselectivity using 4-phenyl-1-butene (**2b**) and EDA **1** as substrates. Variants with enhanced diastereoselectivity in the production of **3b** were regrown in larger scale, and their activities were tested in cyclopropanation of 4-phenyl-1-butene (**2b**) and 1-octene (**2a**) with EDA. Enzyme variants with the greatest overall selectivity enhancements against **3a** and **3b** were used as parents in the next rounds of site-saturation mutagenesis and screening. A single mutation (Q52V) gave RmaNOD near-perfect stereoselectivity for producing *trans* (***1S***, ***2S***)-**3a**. Three mutations (W59A Y60G F145W, or "AGW") gave ApePgb the ability to make *cis* (***1R***, ***2S***)-**3a** with 89:11 diastereomeric ratio (d.r.) and 99% enantiomeric excess (*e.e.*). During screening to increase P411-UA's *cis* diastereoselectivity, a single mutation, V87F, was found to completely invert the diastereoselectivity from 89:11 *cis* (***1S***, ***2R***)-**3a** to 4:96 *trans* (***1R***, ***2R***)-**3a**, affording the fourth and final stereoisomer we needed. Residue 87 is known to modulate the stereoselectivity of P450 BM3 for oxygenation of various substrates.[25]

With initial screening of 11 new and 36 previously engineered proteins, followed by just one to three rounds of site-saturation mutagenesis, we discovered four protein variants capable of cyclopropanating unactivated alkenes (RmaNOD Q52V, ApePgb W59A Y60G F145W (= ApePgb AGW), P411-UA-V87C, and P411-UA-V87F), each of which produced a distinct stereoisomer of the desired product **3a** with 89:11 to <1:99 d.r. and 96 to >99% *e.e.* (Figure 1). The enzyme activities against unactivated alkenes are comparable to the state-of-the-art catalysts, with 100-490 TTN for **3a** and as high as 2,400 TTN for **3b**, the substrate against which the enzymes were screened. The system is straightforward and easy to use: the protein-expressing bacterial cells need only be resuspended to the desired concentration and the alkene and diazo ester added directly under an anaerobic atmosphere. When the reaction is complete, the product is extracted into organic solvents for analysis or purification. While these enzymes were optimized for use in whole cells, they also function to some degree in lysates and as purified proteins (Supporting Information).

The four engineered biocatalysts were tested on a range of alkenes. Their activities and selectivities were high on unbranched aliphatic alkenes similar to those for which they were engineered, but their substrate scope extends to sterically hindered and electron-deficient alkenes as well (Figure 2). Though activity and stereoselectivity differed on different substrates, each catalyst accepted most of the substrates tested. It is likely that activity on specific substrates can be optimized further, if desired, as has been shown in many other directed evolution studies.[26,27]

The small-molecule catalyzed enantioselective preparation of cyclopropyl esters from electron-deficient alkenes has previously been limited to making the *trans*-cyclopropanes,[28] whereas strategies to directly access 1-keto,2-ester or 1,2-diester *cis*-cyclopropanes (or their corresponding carboxylates) via enantioselective cyclopropanation are unknown. The biocatalysts, in contrast, enable access to the *cis*-1-keto,2-ester and *cis*-1,2-diester products in a

single, intermolecular step using an *E. coli*–based platform ((***1R***, ***2S***)- and (***1S***, ***2R***)-**3c**, (***1R***, ***2S***)-**3g**, Figure 2). Some of these products are precursors to valuable compounds: cyclopropyl esters of unbranched, aliphatic alkenes are used in fragrances, for example, including the essential odorants in frankincense.[29] Notably, the enzymes catalyze the reaction on 2-vinylpyridine (**2h**), which is a difficult substrate for many catalysts due to pyridine's propensity to coordinate to and inhibit metal centers. This cyclopropanation product is a precursor for an orphan GPR88 agonist.[30]

Enzymes are chemoselective and can generate desired products without additional steps to protect and deprotect other reactive functional groups on the same molecule. As shown in Figure 3, the enzymes described here, for example, can selectively cyclopropanate terminal alkenes in the presence of alcohol and carboxylic acid functional groups which often undergo competitive O–H insertion reactions with small-molecule carbene transfer catalysts like rhodium acetate dimer.[31] ApePgb AGW performed particularly well with unprotected 7-octen-1-ol (**2i**) and 7-octen-1-oic acid (**2j**), yielding products (***1R***, ***2S***)-**3i** and (***1R***, ***2S***)-**3j** at 77% and 64% isolated yield, respectively, in preparative-scale reactions. Some functional groups cannot be protected easily, and chemo- and regioselectivity is even more important in these cases. In the cases of 1,3-(*E*)-pentadiene (**2k**) and 1,3-(*Z*)-pentadiene (**2l**), all four engineered proteins cyclopropanate the terminal alkene with perfect regioselectivity, likely due to the steric constraints in each enzyme's active site that direct catalysis to the more accessible double bond. The diastereoselectivity varied for **3k** and **3l**, though the enantioselectivity for the major isomer remained high. As the electronic properties of **2k** and **2l** are similar, the difference in stereoselectivity likely reflects steric constraints of the enzyme active sites.

Citing the need for a greater reactivity of the metal center to cyclopropanate unactivated alkenes, Hartwig, Clark, and coworkers showed that heme proteins could bind an artificial iridium cofactor in place of iron heme and perform carbene transfer chemistry.[19] They showed that a protein's

active site can confer selectivity to noble-metal, small-molecule catalysts that can already catalyze the reaction.[19,20,32] Use of an artificial iridium cofactor (Ir(Me)PIX) required the lysis, purification, and *in vitro* metalation of the apoprotein with the Ir(Me)PIX, all of which add time and cost to catalyst preparation. Though it may be possible to perform these metalations *in vivo*,[33] the synthetic, noble-metal catalyst is far more expensive than the native heme cofactor, which is manufactured by the cell and loaded into the catalyst during protein expression *in vivo*. The use of iridium is also not ideal due to the negative impact mining and refining precious metals has on the environment.[34]

A noble-metal catalyst is not necessary, however, for these reactions. Two decades ago, Woo and coworkers showed that iron *meso*-tetrakis(pentafluorophenyl)porphyrin chloride (Fe(PFP)Cl) can catalyze the reaction of 2-ethyl-1-butene and EDA with 390 TTN; they reported the formation of cyclopropane products using 1-decene as well.[35] In fact, we observed that iron heme in aqueous buffer, with no protein, can catalyze the formation of **3a**, albeit with only 0.4 TTN. This basal activity is greatly enhanced and stereoselectivity is enforced by the protein environment, allowing the heme proteins described here to cyclopropanate a range of alkenes from electron-rich conjugated dienes to electron-deficient vinyl ketones and acrylates with high diastereo- and enantioselectivity. The primary factor in determining activity appears to be the binding of the alkene in a productive configuration: the heme's local protein environment can be molded to enhance activity and selectivity by optimizing the substrate binding modes. Different local heme environments can be accessed by screening natural and engineered protein diversity. Directed evolution then fine-tunes these features.

Metalloporphyrin catalysts have been used in synthetic chemistry for decades, but nature has used them for millions of years. Present in all forms of life on Earth, heme-binding proteins have diverse functions as well as promiscuous activities for which they were never selected, such as

the ability to form reactive carbene intermediates. We have taken advantage of this natural diversity to find catalysts for reactions not known to be catalyzed in biology, but that are synthetically useful and are driven by a synthetic carbene precursor (EDA).

While biocatalysts often possess very high selectivity, this selectivity can be synthetically limiting. A single enzyme may make only a single isomer, but access to other isomers may be equally important. Natural diversity can be leveraged effectively for this challenge. A combination of natural diversity and directed evolution let us realize the stereodivergent cyclopropanation of unactivated and electron-deficient alkenes in mild, aqueous conditions with a fully genetically encoded heme protein expressed in bacteria. This set of biocatalysts can serve as starting points for green, sustainable synthesis of valuable cyclopropanated products.

Supporting Information

The materials and experimental methods, detailed protein engineering strategies for each variant, and compound characterization are available in the Supporting Information.

Acknowledgments

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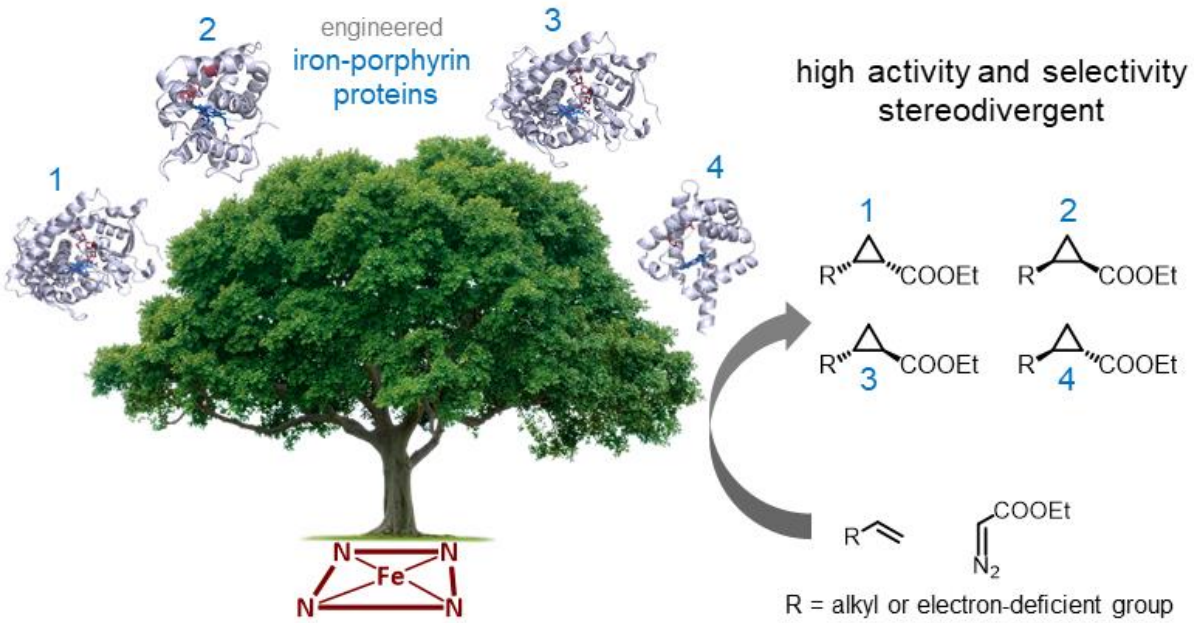


Table of contents graphic.

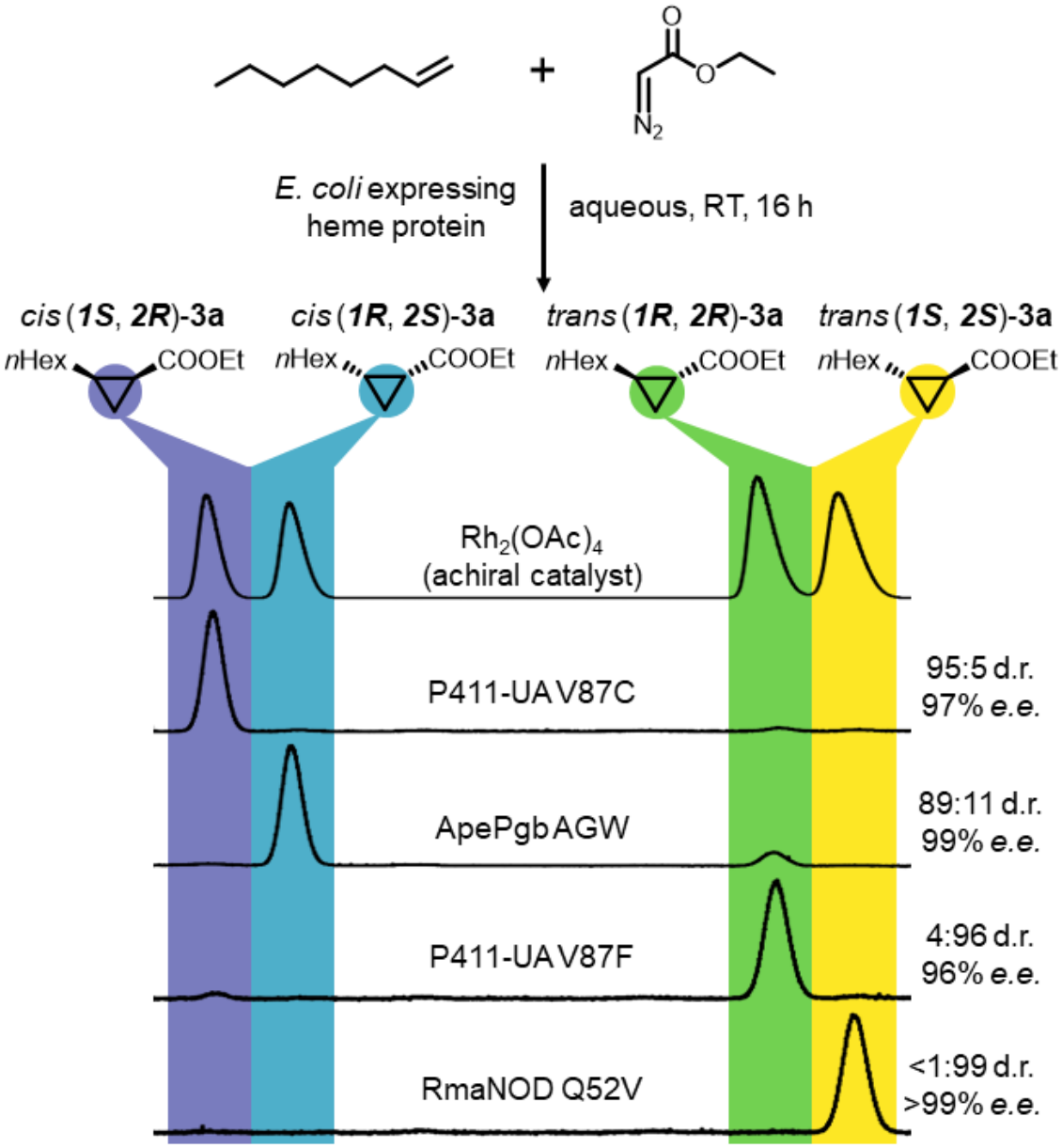
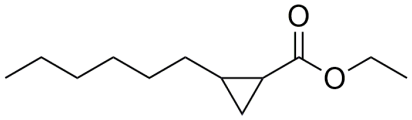
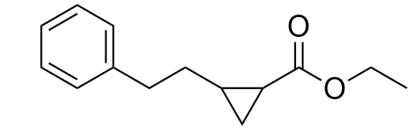
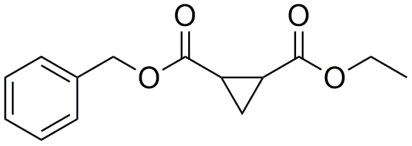
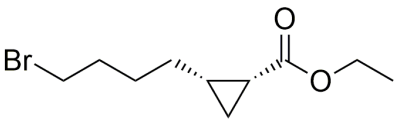


Figure 1. Stereoselective enzymatic cyclopropanation of the aliphatic alkene 1-octene **2a** and EDA **1** to obtain each of four stereoisomers of cyclopropane product **3a** with diastereoselectivies from 89:11 to <99:1 d.r. and enantioselectivies from 96% to >99% *e.e.*. Reaction conditions: whole *E. coli* cells in M9-N buffer, 25 mM glucose, 10 mM 1-octene **2a**, direct addition of 20 mM EDA **1** under anaerobic conditions, 5% ethanol cosolvent. Catalysts used: rhodium acetate dimer ($Rh_2(OAc)_4$) to form the racemic authentic standard, two variants of the engineered, serine-ligated cytochrome P450-BM3 (P411-UA V87C and P411-UA V87F), *Aeropyrum pernix* protoglobin W59A Y60G F145W (ApePgb AGW), and *Rhodothermus marinus* nitric oxide dioxygenase Q52V (RmaNOD Q52V). Protein sequences are available in the Supporting Information.

a

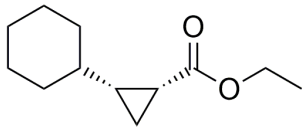
	P411-UA-V87C *(1S, 2R)*	ApePgb AGW *(1R, 2S)*	P411-UA-V87F *(1R, 2R)*	RmaNOD Q52V *(1S, 2S)*
3a 	270 TTN 95:5 d.r. 97% *e.e.*	490 TTN 89:11 d.r. 99% *e.e.*	310 TTN 4:96 d.r. 96% *e.e.*	100 TTN <1:99 d.r. >99% *e.e.*
3b 	1900 TTN 96:4 d.r. >99% *e.e.*	2400 TTN 84:16 d.r. 95% *e.e.*	1900 TTN <1:99 d.r. >99% *e.e.*	210 TTN 3:97 d.r. 98% *e.e.*
3c* 	2900 TTN 91:9 d.r. 96% *e.e.*	5400 TTN 71:29 d.r. 98% *e.e.*	2200 TTN 2:98 d.r. 96% *e.e.*	3700 TTN <1:99 d.r. >99% *e.e.*

b



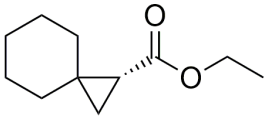
(***1R***, ***2S***)-**3d** 1100 TTN[1]

92:8 d.r. 99% *e.e.*



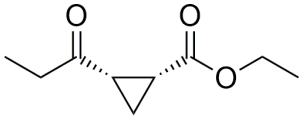
(***1R***, ***2R***)-**3e** 550 TTN[1]

76:24 d.r. 96% *e.e.*



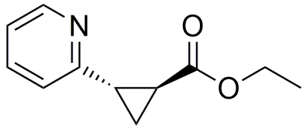
(***R***)-**3f** 610 TTN[1]

96% *e.e.*



(***1R***, ***2S***)-**3g** 610 TTN[1]

89:11 d.r. 99% *e.e.*



(***1S***, ***2S***)-**3h** 3100 TTN[2]

<1:99 d.r. 97% *e.e.*

Figure 2. Cyclopropanation substrate scope. a) Activity and selectivity of each protein variant against **3a**, **3b**, and **3c**. b) Activity and selectivity against a variety of alkenes. Activity and selectivity reported for single enzymes denoted by superscripts: 1: Ape AGW, 2: RmaNOD Q52V. Reaction conditions: whole *E. coli* cells (OD_{600} = 5 (ApePgb AGW, RmaNOD Q52V), OD_{600} = 20 (P411-UA V87C, P411-UA V87F) in M9-N buffer, 25 mM glucose, 10 mM 1-octene **2a**, direct addition of *2*0 mM EDA **1** under anaerobic conditions, 5% ethanol cosolvent. Chiral separation conditions reported in the Supporting Information. *: The benzyl ester of **3c** has IUPAC naming priority and therefore the chiral carbon numbering is reversed for these compounds.

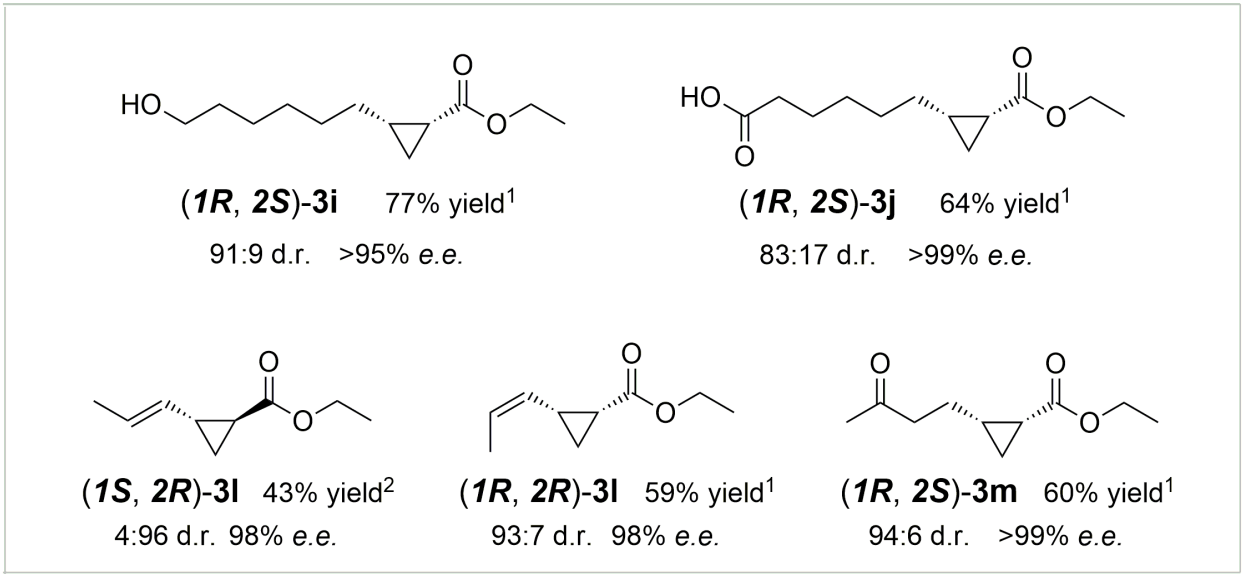


Figure 3. Preparative-scale syntheses of unactivated alkene cyclopropanation products. Preparative-scale reactions against substrates with free alcohol (7-octen-1-ol, **2i**), free carboxylic acid (7-octen-1-oic acid, **2j**)**,** the two diastereomers of 1,3-pentadiene **2k** and **2l,** and ketone (5-hexen-2-one, **2m**). Activity and selectivity reported for single enzymes denoted by superscripts:1: ApePgb AGW, 2: RmaNOD Q52V. Reaction and separation conditions are available in the Supporting Information.

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