Crotonases – Nature's Exceedingly Convertible Catalysts

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Abstract

The crotonases comprise a widely-distributed enzyme superfamily that has multiple roles in both primary and secondary metabolism. Many crotonases employ oxyanion hole-mediated stabilization of intermediates to catalyze the reaction of coenzyme A (CoA) thioester substrates (e.g., malonyl-CoA, α,β-unsaturated CoA esters) with both nucleophiles and, in the case of enolate intermediates, with varied electrophiles. Reactions of crotonases that proceed via a stabilized oxyanion intermediate include the hydrolysis of substrates including proteins, as well as hydration, isomerization, nucleophilic aromatic substitution, Claisen-type, and cofactor-independent oxidation reactions. The crotonases have a conserved fold formed from a central β-sheet core surrounded by α-helices, which typically oligomerizes to form a trimer, or dimer of trimers. The presence of a common structural platform and mechanisms involving intermediates with diverse reactivity implies that crotonases have considerable potential for biocatalysis and synthetic biology, as supported by pioneering protein engineering studies on them. In this Perspective article, we give an overview of crotonase diversity and structural biology, then illustrate the scope of crotonase catalysis and potential for biocatalysis.

Keywords

Crotonases, biocatalysis, proteases, oxyanion hole, coenzyme A, hydrolase, protease, enolate intermediate, protein engineering.

Introduction

Enzymes belonging to the widely distributed crotonase superfamily catalyze a very broad spectrum of reactions (Figure 1). The crotonases have multiple important and highly conserved metabolic roles in both eukaryotes and prokaryotes, as exemplified by the prototypical crotonases, enoyl-CoA hydratase (ECH) and enoyl-CoA isomerase (ECI), which are crucially involved in the β-oxidation pathway of fatty acid metabolism.[1] The term 'crotonase' has been used to refer specifically to ECH,[1] but it is used in this Perspective to refer to the entirety of the superfamily of enzymes bearing the crotonase-type fold. Crotonases are also involved in the biosynthesis of biomedicinally important secondary metabolites, including the carbapenem and glycopeptide antibiotics, and in microbial catabolic pathways associated with the degradation of amino acids, peptides, and pollutants, including chlorinated compounds (for previous reviews see Hamed *et al*.[2] and Holden *et al*.[3]).

Many of the reactions catalyzed by crotonases are analogous to well-established reactions proceeding via enol/enolate intermediates that are commonly used in the synthesis and functionalization of carbonyl compounds in synthetic organic chemistry. Such reactions include aldol and retro-aldol condensations, decarboxylations, hydrations and isomerizations of unsaturated carbonyl compounds, and amide hydrolysis (Figure 1). Some crotonase catalyzed reactions are beyond the routine scope of routine synthetic methodology, such as the efficient cofactor-free oxidation of a substrate as mediated by dioxygen[4] and the one step synthesis of functionalized heterocycles.[5] Coenzyme-A (CoA) thioester derivatives are the most common crotonase substrates, though many crotonase fold enzymes employ other substrate types, notably including proteins (Figure 1).[2,3]

The crotonase superfamily has >120,000 proposed members, according to the InterPro database (family ID IPR001753).[6] As indicated by sequence similarity network analyses, enzymes belonging to the crotonase superfamily are ubiquitous in all life forms and

62 manifest substantial sequence diversity (Figure 2, S1, Table S1). These analyses further
63 suggest that, as a group, the crotonase enzymes remain largely understudied (Figure S1), and
64 it is likely that the chemical diversity attributed to enzymes belonging to this superfamily will
65 continue to expand.

66 Crystallographic studies reveal that, despite often low overall sequence homology
67 (Figure 2), all crotonases manifest a conserved overall fold comprising repeated ββα
68 subunits, which form a scaffold of major and minor β-sheets in a roughly perpendicular
69 arrangement, surrounded by α-helices (Figure 3A, 3B).[2,3,7] By way of example, views from
70 crystal structures of the crotonase CarB reveal a major β-sheet comprised of six β-strands and
71 a minor β-sheet of three β-strands (Figure 3B).[8] Most crotonase enzymes tend to oligomerize
72 as trimers or dimers of trimers in solution (and in crystals) (Figure 3C), although some adopt
73 other oligomerization states; the *C*-terminal α-helical region is usually important in
74 oligomerization.[2]

75 Crystal structures of substrate or product complexes to crotonases reveal a conserved
76 horseshoe-like conformation for the bound CoA element, with the adenyl group oriented in
77 close proximity to the phosphopantetheinyl part and the thioester located near to/within the
78 active site (Figure 3D). The substrate binding pocket is typically made up of the minor β-
79 sheet and the neighbouring loops and α-helices. The active site of each crotonase monomer is
80 located near the interface of two adjacent monomers, and may consist in part of residues from
81 the *C*-terminal α-helices of a neighbouring monomer.[9]

82 As might be anticipated from their conserved fold and active sites, many reactions
83 catalyzed by crotonases have common mechanistic elements (Figure 1A). In particular, most
84 crotonase reactions involve activation of a carbonyl group by binding of the carbonyl oxygen
85 in an oxyanion hole, wherein hydrogen bonds with protein backbone amide groups polarize
86 and increase the electrophilicity of the substrate (often, but not always, a thioester) carbonyl

carbon. Binding in the oxyanion hole can not only enable direct nucleophilic addition onto the carbonyl group, but can also promote reactions involving an enolate intermediate, as exemplified by the different types of crotonase reactions summarized in Figure 1A. The key role of the oxyanion hole in crotonase catalysis is analogous to that of the oxyanion hole in the catalysis of the serine/cysteine proteases and other hydrolytic enzymes, such as the *N*-terminal nucleophilic hydrolases; indeed some crotonase superfamily members act as proteases, e.g., the bacterial caseinolytic and Tricorn proteases.[10]

Due to their ability to catalyze diverse reactions in a stereo- and regioselective manner using structurally related substrates and a common protein scaffold, the engineering of crotonase enzymes into robust biocatalysts holds considerable, as yet largely untapped, promise. Pioneering work has hinted at this potential, including applications for the production of biofuels;[11] the bioproduction of *n*-butanol has been engineered in bacteria via a metabolic pathway involving a crotonase.[12] Crotonase enzymes are also candidates for engineering studies aiming to produce (intermediates of) bioactive molecules, including pharmaceuticals and other high value chemicals, as well as chiral starting materials, as exemplified in substrate analogue studies with 6-oxocamphor hydrolase (6-OCH).[13–15] Of particular note from a commercial perspective has been the development of recombinant organisms which use bacterial enoyl-CoA hydratase/aldolase, a crotonase enzyme, coupled with a CoA-synthetase for conversion of ferulic acid to a 'natural' form of the expensive flavor vanillin (Figure 4).[16–18] More recently, a hydratase/lyase (VpVAN) catalyzing the conversion of ferulic acid itself into vanillin has been reported.[19] However, VpVAN shows higher sequence similarity to the cysteine proteases than the crotonases, and its role in vanillin synthesis has been subsequently questioned.[20]

In this Perspective, we illustrate the diverse scope of crotonase catalysis by describing the reactions catalyzed by and mechanisms proposed for six mechanistically diverse crotonases (Figure 1B). These enzymes represent distinct members of the crotonase structural superfamily, and have relatively low sequence similarity (Figure 2). We begin with a description of ECH and ECI, which catalyze relatively simple double bond hydration and isomerization reactions, but which have central roles in fatty acid β-oxidation; ECH and ECI were amongst the first crotonases to be characterized.[1] The ring-forming menaquinone biosynthetic enzyme MenB is discussed, with focus on its potential as an antibiotic target.[9] We then describe crotonases involved in the biosynthesis of the clinically important β-lactam and glycopeptide antibiotics. Recent substrate analysis and engineering work on CarB (carboxymethylproline synthase B), which catalyzes the committed step in carbapenem biosynthesis,[5] exemplifies the potential of crotonases for the stereoselective synthesis of heterocycles. DpgC (3,5-dihydroxyphenylacetyl-CoA 1,2-dioxygenase) is a chemically interesting cofactor-independent oxygenase that synthesizes precursors for glycopeptide antibiotic assembly.[21] 4-Chlorobenzoyl-CoA dehalogenase (4-CBD) is involved in the biodegradation of chlorinated aromatic compounds.[22] Finally, 6-oxocamphor hydrolase (6-OCH), which is involved in the bacterial degradation of camphor, is discussed with an emphasis on its ability to desymmetrize achiral substrates and provide enantioenriched products for synthetic chemistry.[14] We hope that this article will promote further studies on the biocatalytic potential of existing and new members of the crotonase superfamily.

ECH/ECI - Enoyl-CoA Hydratase/Enoyl-CoA Isomerase

The β-oxidation fatty acid pathway involves the catabolic transformation of long chain fatty acids into acetyl-CoA. ECH catalyzes the second step of the β-oxidation pathway, i.e., the *syn*-addition of a water molecule across the double bond of an α,β-unsaturated enoyl-

CoA thioester substrate (e.g., crotonyl- or methacrylyl-CoA, Figure 5A).[7,23] ECH displays activity towards unsaturated CoA thioesters with different chain lengths, although the turnover rate decreases for longer substrates.[24]

Crystallographic studies reveal that the ECH structure adopts a functional hexamer comprising two stacked trimers.[7] The CoA moiety of the substrate adopts the typical horseshoe conformation in a binding pocket formed from two adjacent monomers, while the active site catalytic residues are provided by a single monomer (Figure 5C). The active site of rat liver mitochondrial ECH contains an oxyanion hole formed from the backbone amides of Gly141 and Ala98, which serves to polarize the carbonyl group of the α,β-unsaturated enoyl-CoA substrate and stabilize the enolate intermediate. Two active site glutamate residues (Glu144 and Glu164) are proposed to act as a general base(s) for the conjugate addition of water onto the substrate enone, and to protonate the resultant enolate (Figure 5A, 5C). Introduction of two glutamate residues into the active site of 4-CBD (see below) in positions corresponding to these residues in ECH provided a 4-CBD variant with a hydratase activity not seen with wild-type 4-CBD,[25] highlighting the potential of rationally engineering crotonase enzymes.

Catalysis by ECI, which is closely related to ECH, enables fatty acids with double bonds not conjugated to the thioester/carboxylate carbonyl to enter the β-oxidation catabolic pathway (Figure 5B). Glu165 in ECI (numbering based on the rat liver mitochondrial enzyme), which is analogous to Glu164 in ECH, is essential for proton relay between C2 and C4 to form the corresponding 2*E*-enoyl-CoA.[26–28] As with ECH, two backbone amide protons form an oxyanion hole which activates the thioester carbonyl of the 3*E*- or 3*Z*-enoyl-CoA substrate. Crystallographic analyses of human ECI type 2 (or HsECI2) indicates that the *C*-terminal α-helix-10 is involved in substrate binding, and in combination with loop-2 and loop-4, in orienting the substrate for catalysis.[29] Studies on the binding of substrate analogues

to the yeast peroxisomal ECI imply extensive hydrogen bond networks involving the catalytic glutamate residue (Glu165) and the residues making up the oxyanion hole.[30]

Some enzymes (e.g., rat peroxisomal multifunctional enzyme, type 1) have both ECH and ECI activities; these enzymes employ an active site with two glutamate residues.[28,31] Through the use of an additional domain, some multifunctional crotonase enzymes can also catalyze a further step in fatty acid catabolism, i.e., the oxidation of the enoyl-CoA hydratase product.[28]

Substituting Lys242 with a non-basic residue in rat liver mitochondrial ECI resulted in a mutant protein with ECH activity.[32] Furthermore, rat mitochondrial ECH-1 (which has the two glutamate residues typical of ECH) has isomerase activity, albeit much lower than its hydratase activity.[33] While mutation of Glu144 to alanine in this enzyme diminished the isomerase activity by 10-fold, mutation of Glu164 to alanine decreased the isomerase activity 1000-fold; the hydratase activity was decreased 2000-fold for both mutants.[33] While the hydratase activity depends on both glutamate residues, the isomerase activity (as with dedicated ECI enzymes) relies mostly on a single glutamate. The catalysis of more than one reaction by a single active site, in combination with the observation that residue substitutions can be used to alter reaction pathways, highlights the potential of crotonase-fold enzymes as scaffolds amenable to engineering to elicit new activities.

MenB – 1,4-Dihydroxy-2-naphthoyl-CoA Synthase

Menaquinone has an essential role in the electron transport chain in many bacteria.[34] Enzymes involved in menaquinone biosynthesis are potential antibiotic targets, including the crotonase MenB (or 1,4-dihydroxy-2-naphthoyl-CoA synthase). MenB catalyzes the intramolecular Claisen condensation of *O*-succinylbenzoyl-CoA to form the naphthyl core of menaquinone (Figure 6A). The MenB mechanism is proposed to proceed via the concerted

185 protonation of the substrate carboxylate, thereby increasing its electrophilicity, and
186 deprotonation of the α-position of the CoA thioester (Figure 6A).[35] The resultant thioester
187 enolate is then proposed to undergo an intramolecular addition-elimination reaction with the
188 carboxylic acid of the intermediate; subsequent enolization of the two keto groups yields the
189 1,4-dihydroxy-2-naphthoyl-CoA product.

190 Crystallographic studies reveal that MenB exists as a dimer of trimers in the
191 crystalline state, as also observed in solution.[9,36,37] The MenB active site is primarily
192 composed of residues from a single monomer, with some residues contributed from the *C*-
193 terminal α-helices of an adjacent subunit.[37] Differences in the active site are observed in the
194 complex of MenB with product analogues, suggesting substantial conformational changes
195 during catalysis.[38,39] A co-crystal structure of *Escherichia coli* MenB with a substrate
196 analogue reveal details of MenB catalysis (Figure 6B),[35] such as the formation of an
197 oxyanion hole from the backbone amides of Gly86 and Gly133 which activates the substrate
198 to promote deprotonation at the α-position. The side chains of Tyr97 and Tyr258′ (from an
199 adjacent subunit) activate the substrate carboxylic acid by protonation to promote the
200 intramolecular addition-elimination reaction of the CoA enolate.[35]

201 Crystal structures of MenB from different bacterial species suggest mechanistic
202 variations (Figure 6B), including in the identity of the base responsible for generating the
203 thioester enolate.[35] Interestingly, the activity of *E. coli* MenB is dependent on bicarbonate, as
204 supported by crystallographic evidence which revealed a bicarbonate ion near the active site
205 (Figure 6C).[40,41] The dependence, or not, of catalysis on bicarbonate has been used to
206 categorize types of MenB enzymes; structural analyses of some type I MenB enzymes
207 manifest a bicarbonate ion bound next to an active site glycine residue, while type II enzymes
208 (e.g., from *Mycobacterium tuberculosis*)[36] contain an aspartate residue in place of the glycine
209 and do not show bicarbonate dependence (Figure 6C, 6D). Because the aspartate residue of

the type II enzymes is proposed to be the general base responsible for substrate thioenolate formation, the bicarbonate-dependence of the type I enzymes has led to the proposal that bicarbonate acts as the base[41] (however, crystallographic analyses of some type I enzymes do not show bound bicarbonate).[35] It has also been proposed that the substrate itself may act as a base during catalysis.[35] Based on a crystal structure of *E. coli* MenB with a bound substrate mimic, one oxygen of the substrate C7 carboxylate is likely < 3 Å away from the pro-2*S* proton.[35] Further, the corresponding C7 methyl ester is not a substrate, despite binding tightly to MenB,[35] consistent with involvement of the substrate C7 carboxylate in intramolecular proton transfer.

High-throughput screening for inhibitors of MenB from *M. tuberculosis* led to the identification of compounds with a 2-amino-4-oxo-4-phenylbutanoate scaffold.[42] However, such compounds were found to only inhibit MenB following a pre-incubation step, requiring a non-enzymatic elimination to form 4-oxo-4-phenylbut-2-enoate. Furthermore, addition of CoA to the reaction mixture greatly improved the extent of inhibition, resulting from the Michael addition of free CoA onto the α,β-unsaturated enone of the inhibitor (Figure 6E). Modeling studies suggest that these inhibitors may exploit structural features common to crotonases. In addition to the expected favourable binding of the inhibitor CoA moiety, the inhibitor carboxylate group is likely positioned in the oxyanion hole. Beyond MenB, such (precursors of) CoA-based inhibitors may represent a general approach to the inhibition of crotonase superfamily enzymes.

CarB - Carboxymethylproline Synthase

Crotonases play key roles in the biosynthesis of the medicinally important carbapenem antibacterials.[43] It has not yet been possible to develop fermentation routes to produce clinically useful carbapenems or precursors thereof; hence, unlike most other β-

lactam antibacterials, carbapenems are produced by total synthesis, with consequent cost of goods issues. There is thus interest in engineering carbapenem biosynthesis to improve production of existing clinically important carbapenems and to produce new β-lactam antibacterials. Unexpectedly, this work has heralded the potential of crotonase engineering for stereoselective control of enolate reactivity.

Three enzymes (CarA, B, C) are directly involved in the biosynthesis of the simplest carbapenem, (5*R*)-carbapenem-3-carboxylic acid (Figure 7A).[44] CarB, a crotonase superfamily enzyme from *Pectobacterium carotovorum*, catalyses the first step, i.e., the reaction of (2*S*)-pyrroline-5-carboxylic acid (P5C) and malonyl-CoA to give the β-amino acid (2*S*,5*S*)-carboxymethyl proline (*t*-CMP). *t*-CMP then undergoes CarA-catalyzed and ATP-dependent bicyclic β-lactam ring formation to give (2*S*,5*S*)-carbapenam-3-carboxylic acid. The 2-oxoglutarate dependent oxygenase CarC then catalyzes epimerization at C-5 and desaturation to give (5*R*)-carbapenem-3-carboxylic acid. ThnE, from *Streptomyces cattleya*, is proposed to play an analogous role to that of CarB in the biosynthesis of more complex carbapenems such as thienamycin. The (5*R*)-stereochemistry is required for carbapenem antibacterial activity; the reason for the apparently 'wasteful' epimerization at the beginning of this route is unknown.[2,45]

CarB has the conserved crotonase fold as revealed by crystallography, and is trimeric in solution.[8] CarB/ThnE catalysis is proposed to proceed via decarboxylation of malonyl-CoA to give an enolate (Figure 7B), which is stabilized by the conserved oxyanion hole. The enolate then likely reacts with the iminium form of P5C (in solution, P5C is in equilibrium with 5-hydroxyproline and glutamate semi-aldehyde) to form an enzyme-bound thioester intermediate, which undergoes hydrolysis, most likely via direct reaction with a glutamate (Glu131 in CarB) activated 'hydrolytic' water molecule.[46]

Studies on the substrate and product selectivities of wildtype and engineered variants of CarB and ThnE illustrate the potential of crotonases for stereocontrolled biocatalysis involving enolate intermediates (Figure 7C, D, E).[47–51] Although considerable work would be required to develop these unnatural CarB/ThnE reactions to commercial utility (likely using cell-based biosynthesis, in part due to the cost of making isolated malonyl-CoA derivatives), this work reveals the potential of crotonases for the highly stereocontrolled synthesis of functionalized heterocycles of pharmaceutical interest.

CarB has an apparently strict requirement for malonyl-CoA derivatives and the (*S*)-stereochemistry of pyrroline-5-carboxylate, but in other regards its selectivity is relaxed. Wild type CarB and active site variants can catalyze formation of 6- and 7-membered ring β-amino acid products from the appropriate aldehydes/imines (Figure 7C).[47] CarB/ThnE variants can produce products methylated at all the carbons of the product proline ring, including the sterically demanding quaternary C-2 and C-5 positions (Figure 7D).[49]

There is even more flexibility in terms of the malonyl-CoA derivatives accepted, and several straight and branched malonyl-CoA derivatives can be converted (Figure 7E).[48] The stereoselective catalysis of CarB variants can be coupled to the enzyme-catalyzed synthesis of malonyl-CoA derivatives by malonyl-CoA synthetase, or, in more limited cases, using crotonyl-CoA carboxylase reductase (Ccr) from crotonyl- or acryloyl-CoA.[50]

Di- or tri-alkylated CMP derivatives can also be synthesised using combinations of malonyl-CoA and P5C derivatives.[47,49,52] In many cases, the β-amino acid products of CarB catalysis have been converted into bicyclic β-lactams by CarA, which catalyzes the step after CarB in carbapenem biosynthesis (Figure 7A).[47,52] Such dual-enzyme catalysis has enabled the efficient production of multiple bicyclic β-lactams which would otherwise be challenging to efficiently synthesize via non-enzymatic methodology.[47]

The standout feature revealed by the substrate analogue and engineering work with CarB/ThnE is the potential for stereoselective control in crotonase catalysis. In the case of the conversion of the malonyl-CoA derivatives to give C-6 alkylated CMP derivatives, stereocontrol substantially depends on the selective generation and reaction of the appropriate trisubstituted (*E*)- or (*Z*)-enolate intermediate. Active site variants of CarB/ThnE, including at the oxyanion stabilizing site, have enabled production of C-6 methylated and ethylated derivatives of *t*-CMP with very high stereoselectivities for either the (6*S*)- or (6*R*)-products. In some cases, stereoselectivity can be enhanced by coupling (engineered) CarB/ThnE asymmetric catalysis with production of the C-2 alkylated malonyl-CoA derivatives using malonyl-CoA synthetase or Ccr.[48,50]

In the case of C-2 alkylated malonyl-CoA CarB substrates, the C-2 position undergoes epimerization under the assay incubation conditions. In cases where the rate of epimerization limits the stereoselectivity inherent in CarB catalysis, a malonyl-CoA epimerase can be added to improve stereoselectivity, including when using Ccr to prepare the malonyl-CoA derivative in a one pot three enzyme synthesis of C-5 ethylated *t*-CMP starting from crotonyl-CoA and CO_2.[51]

DpgC

Some of the most mechanistically interesting crotonase enzymes act as cofactor-independent oxygenases.[53] DpgC, a hexameric crotonase superfamily oxygenase produced by actinomycetes, is involved in the biosynthesis of 3,5-dihydroxyphenylgycine, a precursor to the glycopeptide antibiotics. DpgC catalyzes the dioxygen-dependent, but cofactor-independent, four-electron oxidation of 3,5-dihydroxyphenylacetyl-CoA (DPA-CoA) to give 3,5-dihydroxyphenylglyoxylate (DPGX) (Figure 8A).[4,21] DPGX is then converted to an

amino acid, as catalyzed by the transaminase HpgT, and incorporated into glycopeptide antibiotics (such as vancomycin and teicoplanin) by non-ribosomal peptide synthetases.

Crystallographic studies have examined the cofactor-independent nature of DpgC catalysis and identified a putative hydrophobic dioxygen-binding pocket near the substrate binding site (Figure 8B).[54–56] Mutations to the proposed dioxygen-binding pocket decreased the catalytic efficiency and raised the apparent K_M for oxygen.[57] Biophysical, modelling and mutagenesis studies indicate that dioxygen can follow different pathways to the active site, where it is concentrated in a position suitable for reaction with the substrate.[58]

While the DpgC reaction is unusual among crotonases, its proposed mechanism manifests features typical of the superfamily (Figure 8A).[54] Deprotonation of the α-position to the thioester carbonyl is proposed to be catalyzed by a water molecule, with the resultant enolate stabilized by an oxyanion hole formed by the backbone amides of Gly296 and Ile235. Electron transfer from the substrate enolate to the bound oxygen molecule is proposed to form a substrate radical cation and superoxide anion pair, which undergo reaction to give a peroxide. The distal oxygen of this peroxide may then react with the thioester carbonyl, again facilitated by the oxyanion hole (Figure 8A). The resultant dioxetanone intermediate can then undergo elimination to give the DPGX product.

4-CBD - 4-Chlorobenzoyl-CoA Dehalogenase

4-CBD catalyzes the hydrolytic dehalogenation of 4-chlorobenzoyl-CoA, forming 4-hydroxybenzoyl-CoA. Bacteria producing 4-CBD include *Pseudomonas* sp. CBS3 (or *Burkholderia* sp. CBS3),[22] *Arthrobacter* sp. 4-CB1,[59] Betaproteobacteria,[60] and *Arthrobacter* sp. TM-1.[61] 4-CBD is also able to hydrolyze other 4-substituted benzoyl-CoA substrates, including 4-fluoro-, 4-bromo-, 4-iodo-, and 4-nitrobenzoyl-CoA, suggesting scope for engineering work.[62,63] Crystallographic studies on 4-CBD from *Burkholderia* sp. CBS3 reveal

it oligomerizes to form a trimer with the participation of the *C*-terminal α-helices from one monomer in the active site of another, as observed with other crotonases, e.g., MenB (Figure 6B).[22,35]

The 4-CBD catalytic mechanism has been investigated experimentally and computationally (Figure 9A). The rate-determining step is proposed to be the nucleophilic addition of a carboxylate oxygen of Asp145 onto the C4 position of 4-chlorobenzoyl-CoA to form an enzyme-Meisenheimer complex (EMc) intermediate, as supported by QM/MM studies and Raman spectroscopy.[62,64,65] The backbone amide protons of Phe64 and Gly114 form an oxyanion hole (Figure 9B), which polarizes the substrate thioester carbonyl so increasing the electrophilicity of the substrate aromatic ring.[66,67] His90 is thought to increase the nucleophilicity of Asp145,[68,69] while Trp137 and Glu232 are proposed to orient Asp145 towards the substrate.[70,71] The EMc intermediate rearomatizes with loss of chloride, which is displaced into a pocket initially proposed to be formed by Phe64, Phe82, and Trp89,[69] although computational studies suggest the chloride receiving pocket may be formed from Ala86, His90, and Ile223′.[64] Rearomatization of the EMc results in an aryl-enzyme ester intermediate, which is then hydrolyzed (with His90 acting as a general base) to yield 4-hydroxybenzoyl-CoA.[69] Product release is proposed to proceed by a conformational change and ion shuffle, resulting in the extrusion of a proton and a chloride ion from the active site.

6-OCH - 6-Oxocamphor Hydrolase

6-OCH catalyzes the hydrolysis of the diketone 6-oxocamphor, giving a ~6:1 mixture of (2*R*,4*S*)- and (2*S*,4*S*)-α-campholinic acid (Figure 10A; note that a similar ratio of *cis*- and *trans*-isomers as a racemate was observed for the corresponding acid-catalyzed hydrolysis).[14] A crystal structure of 6-OCH from *Rhodococcus* sp. NCIMB 9784 has the typical hexameric

crotonase-type fold (Figure 10B), with the *C*-terminal α-helix of one monomer folding back over the core to cover the active site of the same monomer.[72]

Unusually for the crotonase superfamily, the 6-OCH substrate lacks a CoA thioester. Furthermore, a structure of the H122A variant of 6-OCH with bound product (2*S*,4*S*)-α-campholinic acid suggests that 6-OCH does not contain the typical crotonase oxyanion hole composed of backbone amide protons; instead the substrate enolate is likely stabilized by the side chains of Trp40 and His122.[73]

His145 is proposed to act as a general base in 6-OCH catalysis, catalyzing the addition of water to the pro-(*S*) face of symmetrical 6-oxocamphors (Figure 10A).[72] The resulting tetrahedral oxyanion can then undergo a retro-Claisen reaction, forming a carboxylic acid and enolate. While Trp40 and His122 likely stabilize the enolate intermediate, they may also limit the nucleophilic addition of water onto the ketone with which they interact.[73] The enolate may then be protonated on either face; protonation on the pro-(*R*) face is favored, resulting in a 6:1 mixture of (2*R*,4*S*)- and (2*S*,4*S*)-α-campholinic acid.

6-OCH can generate optically active products from achiral symmetrical substrates (Figure 10C), and is tolerant to a range of common organic solvents.[74] Furthermore, the activity and stereoselectivity of 6-OCH are preserved at temperatures up to 50 °C. As a consequence, 6-OCH has seen application for the asymmetric synthesis of substituted cyclopentanones and cyclohexanones from symmetrical bridged bicyclic 1,3-diketones.[13,14] In a one pot cascade process involving three enzymes, which nicely exemplifies how 'natural' enzymes can be combined for the efficient synthesis of unnatural asymmetric compounds from achiral precursors, the products of 6-OCH catalysis were sequentially modified by lipase catalyzed ester formation followed by transaminase catalyzed amine formation to give δ-amino esters.[75]

Various monocyclic ring systems have also been shown to be 6-OCH substrates. Simple 2,2-disubstituted-1,3-cyclohexadiones were converted by 6-OCH to give 1,5-keto acids, although only a racemic mixture of products was obtained from these substrates.[13,14] Fused 5,6 and 5,7 rings with non-enolisable 1,3-diketone systems also displayed reactivity with 6-OCH, producing enantioenriched products.[15]

Limitations to the substrates accepted by 6-OCH remain, with all substrates thus far identified containing a cyclic 1,3-diketone scaffold. In addition, substrates require a non-enolizable centre between the two ketones, thereby excluding many simpler 1,3-diketones.[15] However, the impact of active site residues on the substrate scope of 6-OCH has yet to be fully characterized and these enzymes are interesting targets for engineering to introduce new activities.

Conclusions and Future Perspectives

The examples in this Perspective illustrate the catalytic versatility of the crotonase superfamily in terms of both substrate and reaction selectivity. The available structural and mechanistic evidence suggests that the versatility of the crotonases arises substantially from their ability to efficiently form, stabilize, and control the reactivity of oxyanion intermediates, often in the form of enolates. The oxyanion is stabilized by conserved hydrogen bonds in the oxyanion hole, and general acid/base catalysis is commonly mediated by aspartate, glutamate, and histidine residues.[76] Although, oxyanion hole-type carbonyl activation is supported by other protein folds, it is possible that the crotonase fold is particularly good at supporting a diverse set of reactions/substrates – this hypothesis could be investigated by systematic designed studies employing both natural and unnatural folds. Many of the crotonase catalyzed reactions described here (Figure 1B) are analogous to reactions involving carbonyl compounds routinely used in organic synthesis. One obvious advantage of the enzymatic

transformations is stereocontrol. The stereoelectronic and mechanistic insights generated by studies on crotonases and related enzymes employing oxyanion intermediates[76] are providing inspiration for the development of non-protein based catalysts; pioneering work suggests there is considerable scope for the generation of powerful asymmetric catalysts.[77]

It is important to appreciate that the extent of crotonase catalysis extends well beyond the examples highlighted in this Perspective. As supported by bioinformatic analyses (Figure S1, Table S1), the full scope of crotonase catalysis has not yet been realized, and many members of this superfamily are understudied. Furthermore, several structurally 'atypical' crotonases are known that catalyze biologically important reactions. Interestingly, the majority of the atypical crotonases retain most of the characteristic elements of the conserved monomeric crotonase fold, but do not necessarily trimerize in the same manner as the more typical crotonases. Amongst the atypical members are the crotonase-fold serine proteases such as Clp, which is an antibacterial drug target,[78] and the biotin-dependent carboxylases/carboxyltransferases. A crystal structure of bacterial ClpP, the proteolytic domain of the caseinolytic protease Clp, reveals a crotonase-type fold distinct from the major families of serine proteases (Figure 11A).[79] In spite of its different fold, the key catalytic residues of ClpP required for proteolysis are positioned similarly as in other classes of serine proteases (e.g., subtilisin), in an apparent example of convergent evolution (Figure 11B). Such convergence is supported by subsequently solved structures of further serine proteases with a crotonase-type fold (e.g., photosystem II D1 C-terminal processing protease, tricorn protease).[80,81] Another biologically interesting atypical crotonase is acetyl-CoA carboxylase (ACC), which is involved in the regulation of fatty acid metabolism.[82] This enzyme catalyzes carboxylation of acetyl-CoA to give malonyl-CoA, which is a substrate for other crotonases. The enolate of acetyl-CoA, stabilized by an oxyanion hole, is positioned by ACC to react

429 with a covalently bound carboxybiotin molecule, which is generated through the action of a
430 biotin carboxylase domain.[83]

431 The conservation of key catalytic features in both typical and atypical crotonases,
432 coupled to the range of reactions catalyzed by the crotonases, suggests that there is
433 considerable scope for engineering them, both in terms of tweaking the selectivity of the
434 existing reactions they catalyze and to develop new reactions. In contrast to the oxyanion
435 hole, CoA binding, and general acid/base machinery, there appears to be much more variation
436 in the specific substrate binding pockets of crotonases. The results of the limited protein
437 engineering and synthetic biology work carried out on the crotonases, such as with 6-OCH,[75]
438 the carboxymethylproline synthases,[50] and vanillin production,[84] suggest that they may be
439 amenable to the development of biocatalysts of industrial utility.

440 Although not a limitation for all crotonases (e.g., 6-OCH),[13] in the case of the CoA-
441 employing crotonases, most short term industrial applications of crotonase catalysis are likely
442 to be in cells due to the current high cost of CoA thioesters. The pantetheine group of the
443 CoA present in typical crotonase substrates binds in a conserved and relatively rigid manner
444 in a tunnel leading to the enzyme active site (Figure 3D), acting as a handle to locate the
445 reactive elements of the intermediate at the active site. One productive line of future
446 investigation could be to engineer crotonases that do not require CoA thioesters, but which
447 catalyze the diverse reaction types presently catalyzed by crotonases using either truncated
448 CoA thioesters, or preferably, simple acids/esters/amides. Given the range of electrophiles
449 naturally accepted for reaction with enolates by crotonases, including gases such as dioxygen
450 and carbon dioxide, and the proven utility of reactions of non-enzyme derived enolates with
451 electrophiles, engineering crotonase-bound enolate intermediates to react with unnatural
452 electrophiles is of particular interest.

To date, protein engineering on crotonases has been guided by structural knowledge gained from crystallography (>85 crystal structures deposited by mid 2017 in the PDB; Table S1), which has provided information on active sites that has informed on stereoelectronic details of catalysis[76] and been used to alter catalytic properties of crotonases.[32,50] The oliogomeric nature of most crotonases and the conformational changes that likely occur during catalysis (probably underestimated by crystallographic studies) means that knowledge of solution structures of crotonases, in particular in complex with substrates, will be informative with respect to future engineering efforts. Such knowledge may take some time to emerge, and given that the interactions between monomers are, at least sometimes, involved in catalysis, it may be non-trivial to apply towards the design of new catalysts. Hence, semi-rational approaches to altering crotonase catalysed reactions, employing directed evolution type methods are of interest,[85] as is the potential design of consensus, possibly monomeric, crotonases for use as templates on which to engineer tailored active sites.

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Supporting Information. SSN analyses of the crotonase superfamily, summary of Swiss-Prot reviewed crotonase enzymes associated with enzymatic activity and crystallographic studies.

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638 **Figure Legends**

639 **Figure 1. Reactions catalyzed by crotonase superfamily enzymes (crotonases).** (A)
640 Crotonases catalyze a diverse range of reactions, most commonly with substrates activated as
641 coenzyme A (CoA) thioesters (highlighted in purple). These reactions tend to involve the
642 stabilization of an enolate (or tetrahedral alkoxide) intermediate via an oxyanion hole
643 (orange). (B) Scheme showing the reactions catalyzed by crotonase enzymes highlighted in
644 this Perspective.

645 **Figure 2. Representative sequence similarity network[86] for the crotonase superfamily,**
646 **colored by the domains of life, and displaying the distribution of the enzymes discussed**
647 **in the Perspective (indicated with large red nodes).** Each node represents a set of protein
648 sequences that are >65% identical (total number of nodes = 16916). Edges linking nodes are
649 drawn if the identity between a pair of nodes is better than a Blast E value of $1x10^{-40}$. The
650 total number of proteins with a crotonase fold is estimated to be ~122,924 according to the
651 InterPro database (Family ID IPR001753).[6] The figure was generated using the EFI web
652 tool[87] (minimum alignment score = 50) and Cytoscape 3.5.1 software.[88] The network reveals
653 the relative size of clusters of nodes (of likely functionally related enzymes); note the
654 ECH/ECI cluster is the largest.

655 **Figure 3. Typical structural features of crotonase enzymes.** (A) Topology of a CarB
656 monomer, displaying the repeated ββα motif. Residues involved in the highly, but not
657 universally, conserved oxyanion hole are indicated with asterisks. β-Strands are indicated in
658 yellow, while α-helices are in blue, or green for the *C*-terminal α-helix. (B) View from a
659 crystal structure of a CarB monomer from *Pectobacterium carotovorum*, with bound acetyl-
660 CoA (represented as yellow sticks; PDB 2A81). (C) The trimeric oligomerization state of
661 CarB (PDB 2A7K); note the role of the *C*-terminal α-helices α6, α7, and α10 in
662 oligomerisation. (D) The horseshoe-like conformation of acetyl-CoA bound to CarB (PDB

663 2A81); inset shows the chemical structure of a generic CoA thioester. Note that electron
664 density was not observed for the full CoA substrate in this structure, as indicated with the
665 black line. This unresolved part of the substrate extends through a tunnel towards the active
666 site.

667 **Figure 4. Involvement of a crotonase in vanillin production.** Reaction scheme showing the
668 formation of vanillin from ferulic acid in *Streptomyces* sp. strain V-1. Scheme adapted from
669 Yang *et al*.[17] The intermediate shown in square brackets was not observed.

670 **Figure 5. ECH/ECI mechanism and structural features.** (A) Proposed outline mechanism
671 of ECH catalysis (numbering according to the rat liver mitochondrial enzyme). (B) Proposed
672 outline mechanism of ECI catalysis (numbering according to the rat liver mitochondrial
673 enzyme). (C) View from rat liver mitochondrial ECH active site showing key residues
674 interacting with substrate analogue acetoacetyl-CoA (PDB 1DUB).[7] Dotted lines represent
675 hydrogen bonds between the substrate thioester carbonyl oxygen and the backbone amide
676 protons of Ala98 and Gly141. Glu144 and Glu164 are indicated in the same colors as in panel
677 A.

678 **Figure 6. MenB mechanism and structural features.** (A) Proposed mechanism of MenB
679 catalysis. (B) View from the *Escherichia coli* MenB active site with bound substrate mimic
680 *O*-succinylbenzoyl-aminoCoA (OSB-NCoA), using dotted lines to represent hydrogen bonds,
681 the two oxyanion holes formed from Gly86 and Gly133, and from Tyr97 and Tyr258′ (PDB
682 3T88).[35] Note that Tyr258' belongs to an adjacent monomer of EcMenB. (C) View from the
683 *E. coli* MenB active site showing a bound bicarbonate ion (PDB 4ELS).[41] In some cases a
684 bicarbonate ion may provide general acid base catalysis.[41] (D) View from the *Mycobacterium*
685 *tuberculosis* MenB active site showing bound product analogue 1-hydroxy-2-naphthoyl-CoA
686 (1-HNA-CoA) and putative basic residue Asp185 (PDB 4QIJ).[39] The hydrogen bonds

687 between the product thioester carbonyl oxygen and the backbone amide protons of Gly105
688 and Gly161 are represented as dotted lines. The putative general base Asp185 is shown. (E)
689 Scheme showing the non-enzymatic generation of CoA-based inhibitor adducts from 2-
690 amino-4-oxo-4-phenylbutanoates.[42]

691 **Figure 7. Role of carboxymethylproline synthases in carbapenem biosynthesis.** (A)
692 Biosynthesis of the simplest carbapenem as catalyzed by the crotonase CarB, the synthetase
693 CarA, and the oxygenase CarC. (B) Proposed outline mechanism of CarB catalysis. (C)
694 Capacity of CarB and variants to catalyse the formation of different ring sizes (5- to 7-
695 membered *N*-heterocycles). (D, E) Biocatalytic production of unnatural carbapenams by
696 engineered CarB/CarA sequential catalysis, employing alkylated substrate analogues
697 (including those generated via tandem catalysis by MatB/Ccr in panel E).

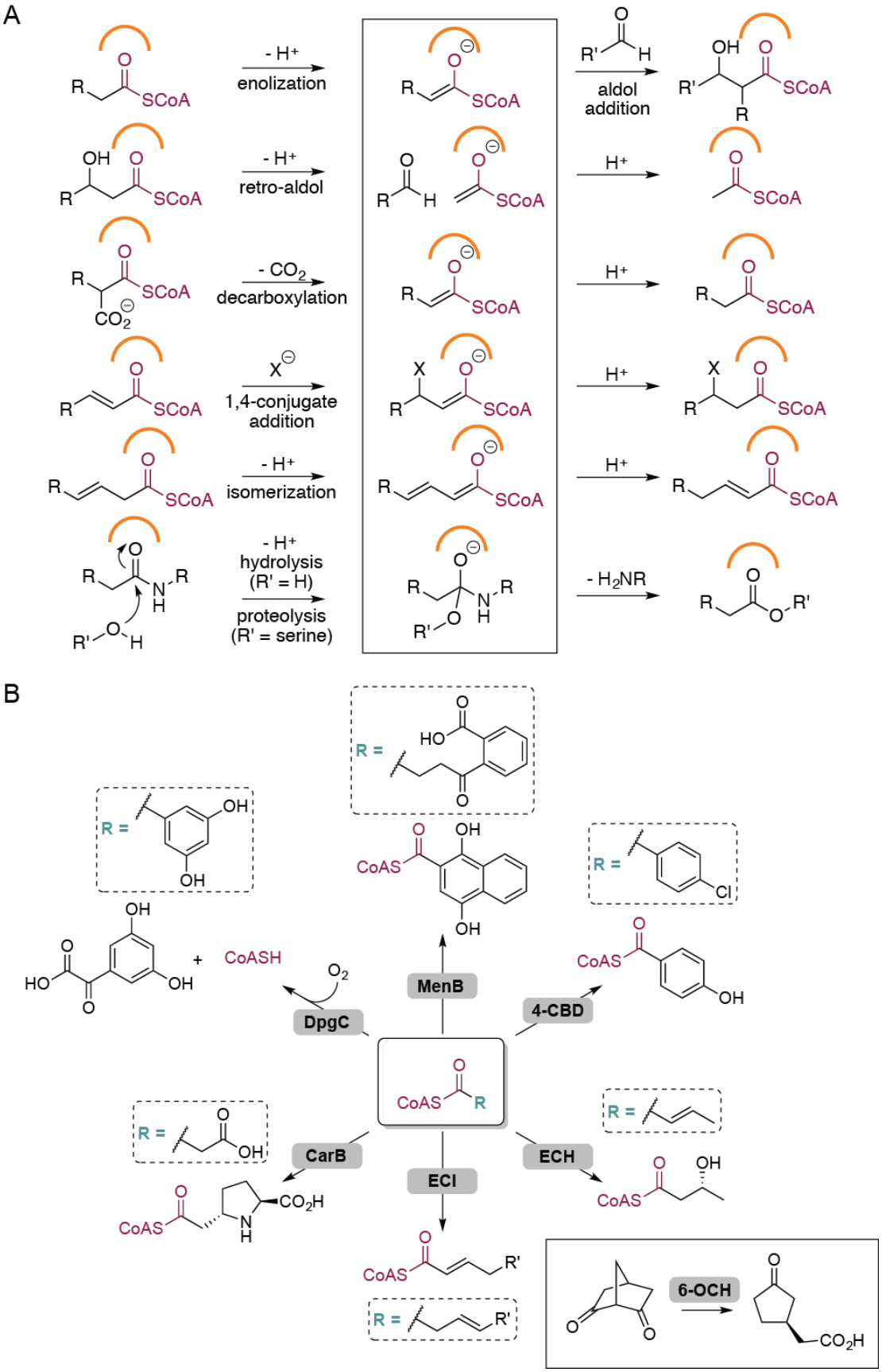
698 **Figure 8. Catalytic and structural features of DpgC.** (A) Proposed mechanism of DpgC
699 catalysis. (B) View of a crystal structure of DpgC from *Streptomyces toyocaensis* with bound
700 substrate analogue DPA-NH-CoA, showing dioxygen bound in a hydrophobic pocket (PDB
701 2NP9).[54] Note that the residues thought to make up the hydrophobic pocket are shown as dark
702 blue sticks. The hydrogen bonds between the substrate analogue carbonyl oxygen and the
703 backbone amide protons of Ile235 and Gly296 are represented by dotted lines.

704 **Figure 9. Mechanistic and structural features of 4-CBD.** (A) Proposed outline mechanism
705 of 4-CBD catalysis. (B) View from crystal structure of the *Burkholderia* sp. CBS3 4-CBD
706 active site showing a bound molecule of 4-chlorobenzoylacetyl-CoA (4-CBA-CoA) (PDB
707 1NZY).[22] Hydrogen bonds between the substrate thioester oxygen and the backbone amide
708 protons of Phe64 and Gly114 are represented as dotted lines. The nucleophilic Asp145
709 residue involved in formation of the Meisenheimer complex is shown as sticks.

Figure 10. Mechanistic, structural and biocatalytic features of 6-OCH. (A) Proposed outline mechanism of 6-OCH catalysis. (B) View from a crystal structure of the *Rhodococcus* sp. NCIMB 9784 6-OCH H122A variant with bound product α-campholinic acid (PDB 1SZO).[73] Note that the oxyanion hole is proposed to be formed by the side chains of Trp40 and His122 (mutated to alanine in this structure). The hydrogen bond between the substrate/product carbonyl and the indole side-chain of Trp40 is represented by a dotted line. The proposed general base, His145, is shown in a stick representation. (C) Substrate scope of 6-OCH, showing conversion of monocyclic and bicyclic non-enolizable 1,3-diketones.

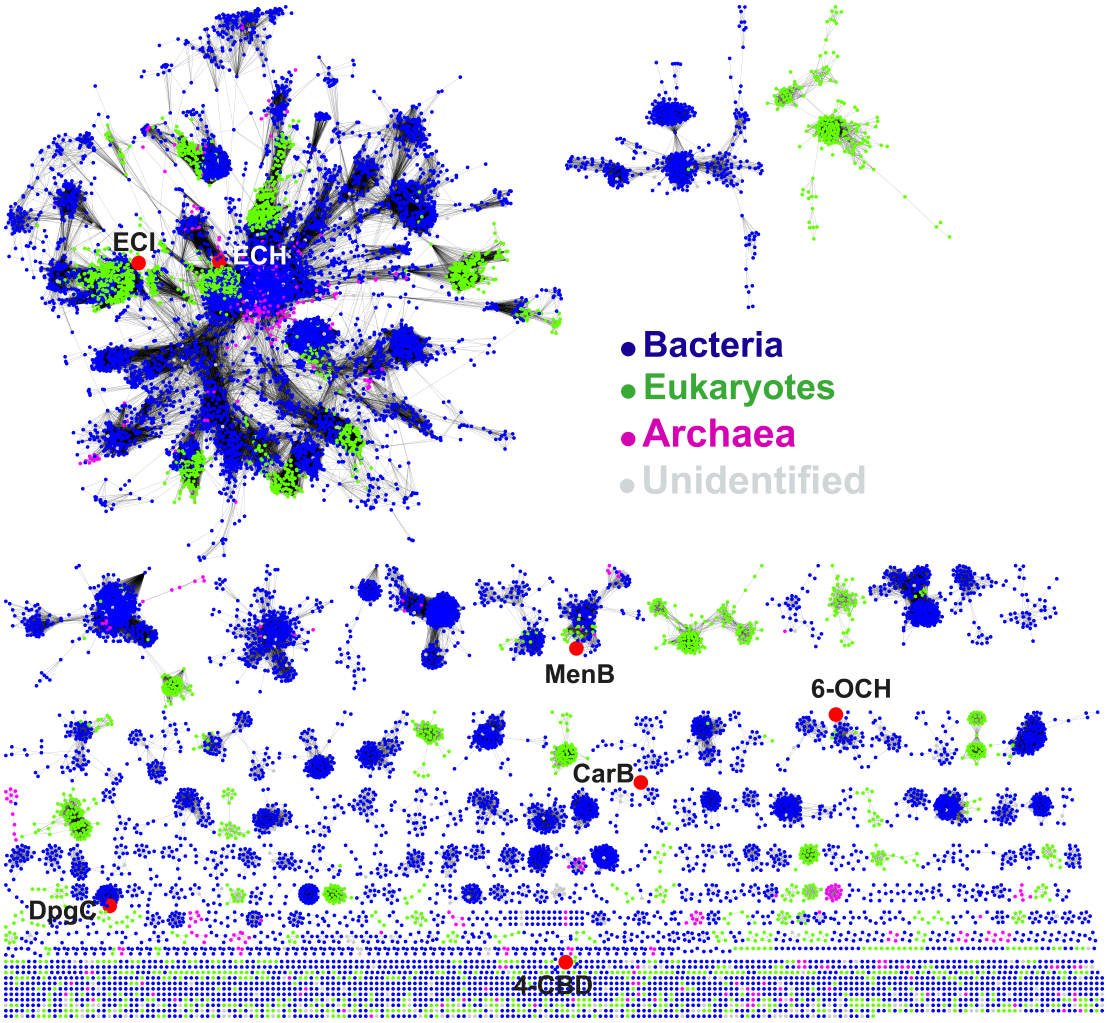
Figure 11. Comparison of serine protease active sites. (A) View from a crystal structure of the *Escherichia coli* crotonase-fold serine protease, ClpP (PDB 1TYF).[79] Ser97 acts as a nucleophile, as assisted by the general base His122, while the backbone amide protons of Gly68 and Met98 form an oxyanion hole. (B) View from a crystal structure of the serine protease subtilisin from *Bacillus amyloliquefaciens* (PDB 1ST2).[89] Ser221 acts as a nucleophile, while His64 acts as a general base; the oxyanion hole is composed of the backbone amide proton of Ser221 and the side-chain of Asn155.

727 **Figure 1.**



728

729 **Figure 2.**



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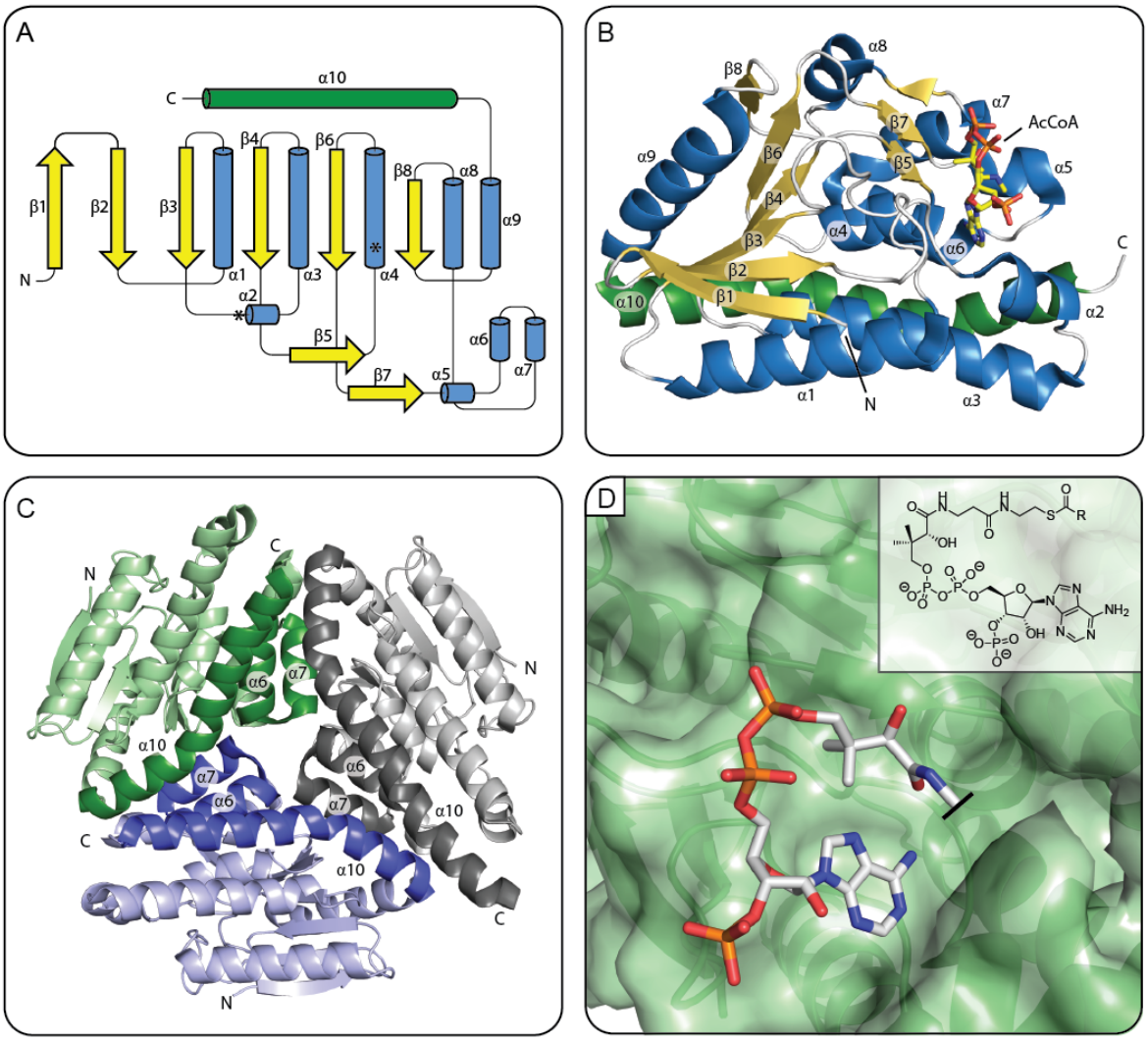
731

732 **Figure 3.**



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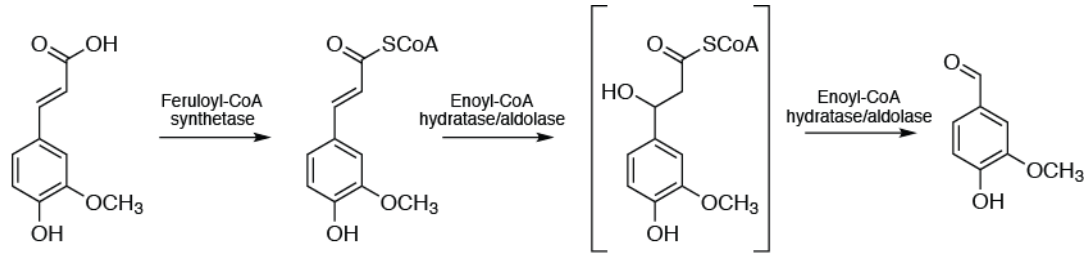
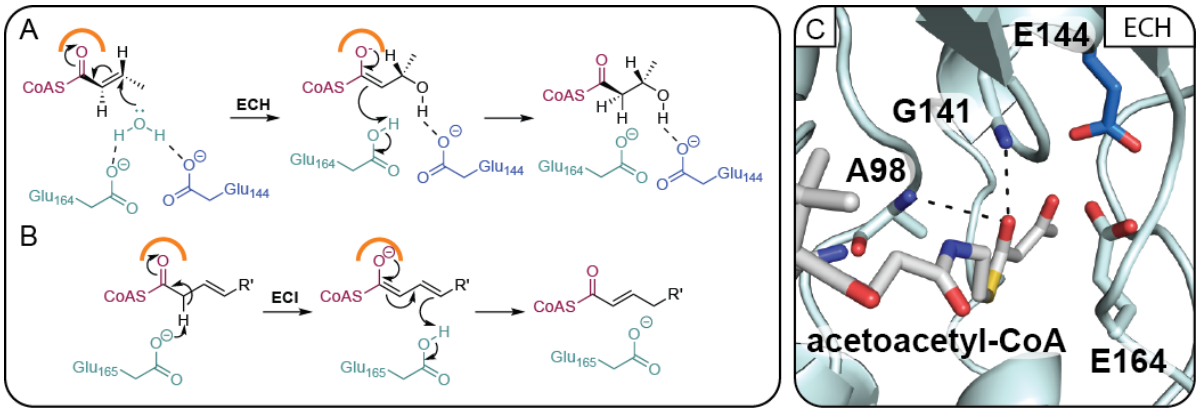
734

Figure 4.


Feruloyl-CoA synthetase
Enoyl-CoA hydratase/aldolase
Enoyl-CoA hydratase/aldolase
Ferulic acid
Feruloyl-CoA
Vanillin

738 **Figure 5.**



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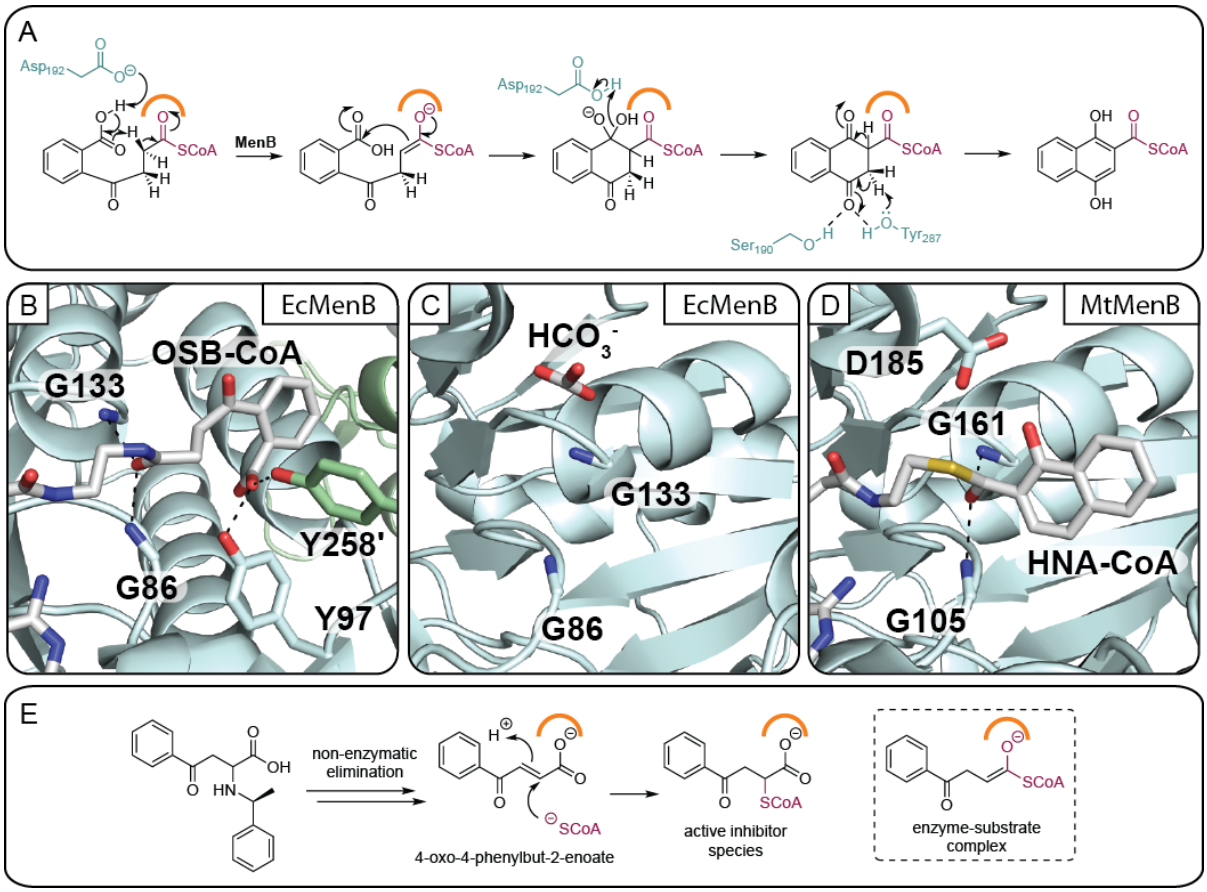
740

741 **Figure 6.**



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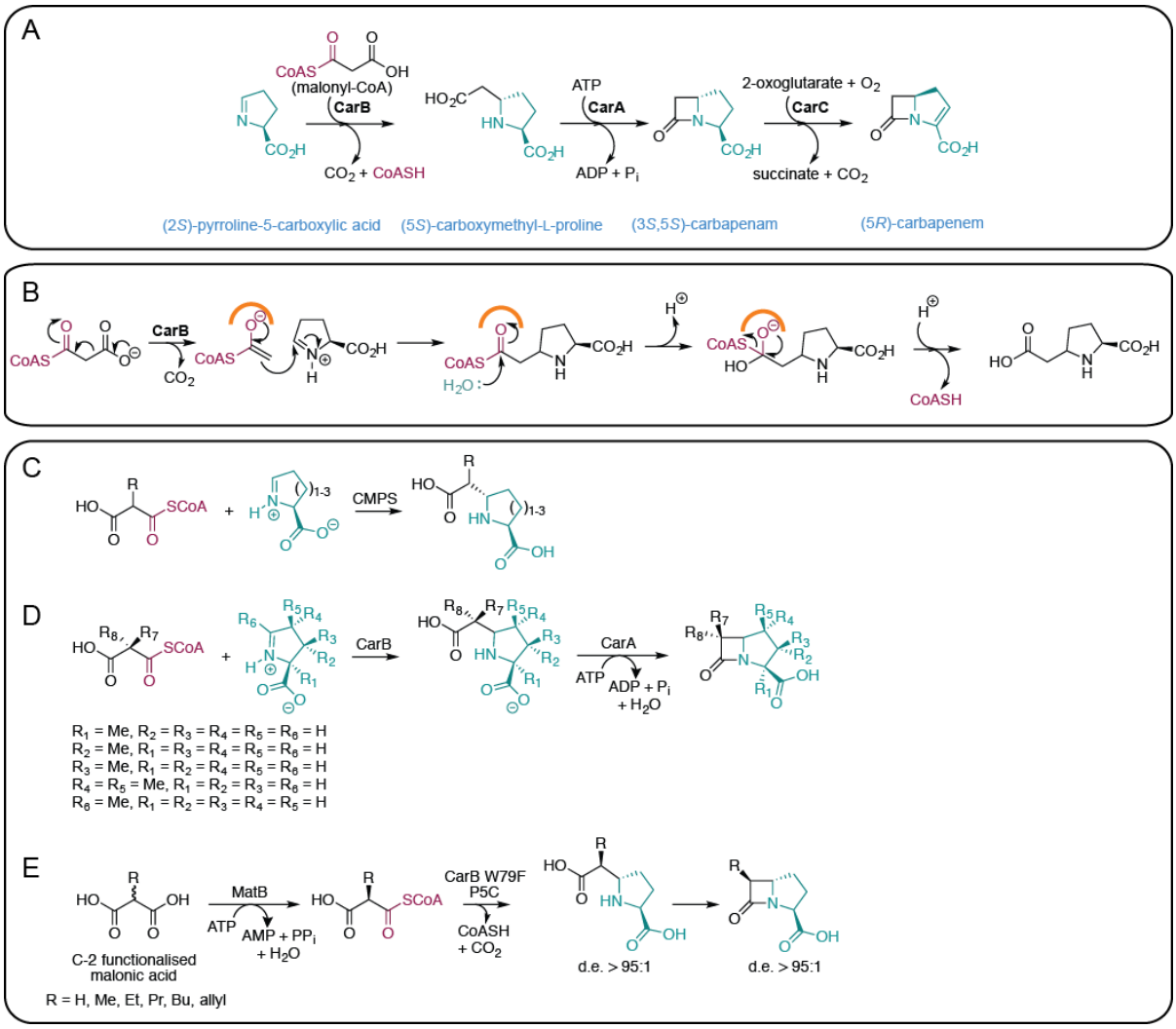
743

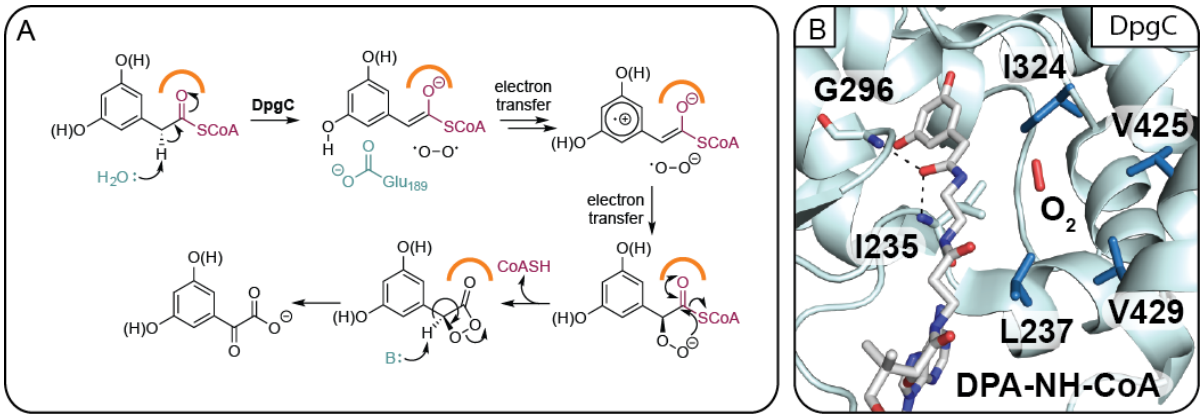
744 **Figure 7.**



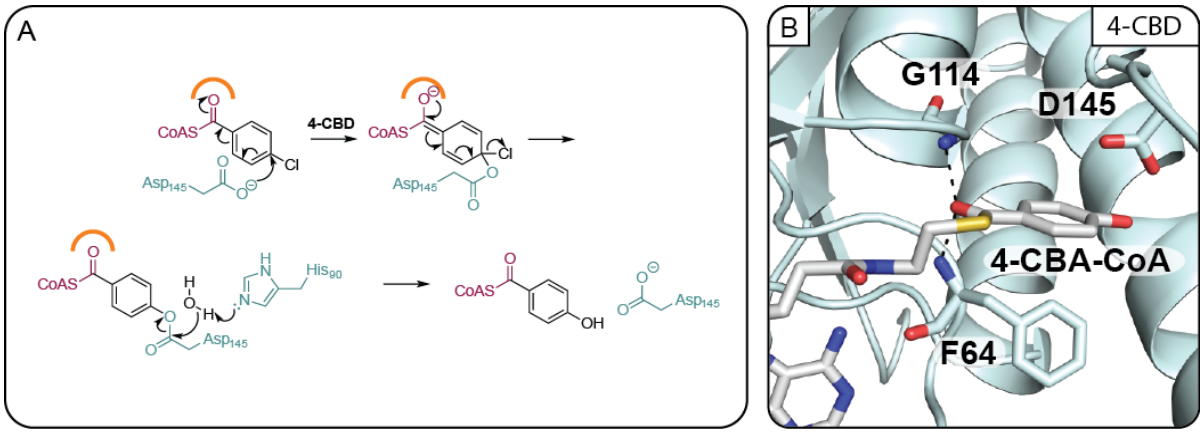
745

746

Figure 8.


A
O(H)
(H)O
SCoA
H
H2O :
DpgC
O
H
Glu189
electron transfer
electron transfer
CoASH
B:
B
DpgC
G296
I324
V425
O2
I235
L237
V429
DPA-NH-CoA

750 **Figure 9.**



751

752

Figure 10.

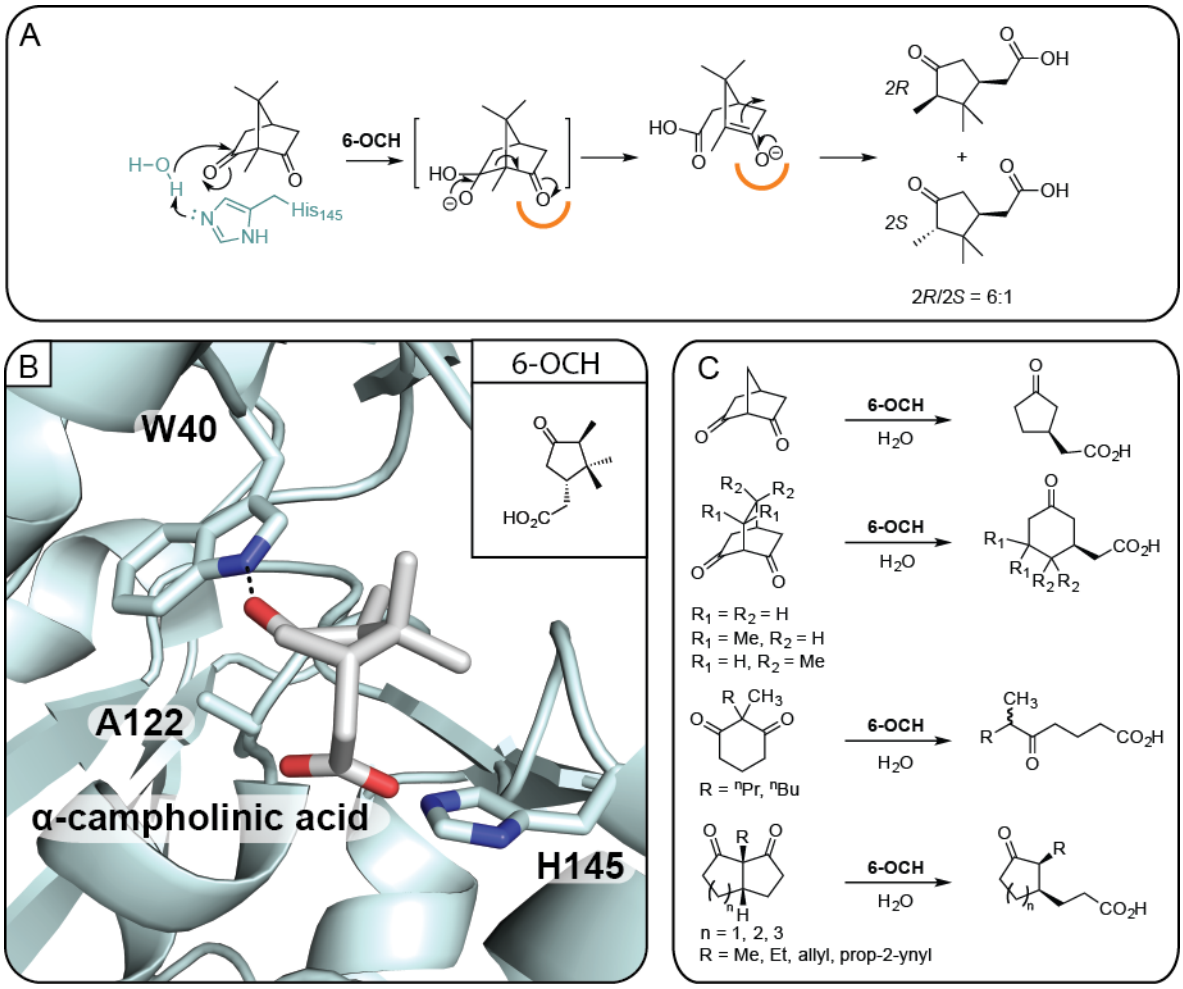

A
6-OCH
His145
2R
2S
OH
2R/2S = 6:1
B
6-OCH
W40
A122
α-campholinic acid
H145
C
6-OCH
H2O
CO2H
R1 = R2 = H
R1 = Me, R2 = H
R1 = H, R2 = Me
R = nPr, nBu
n = 1, 2, 3
R = Me, Et, allyl, prop-2-ynyl

Figure 11.