Halohydrin dehalogenases in organic synthesis

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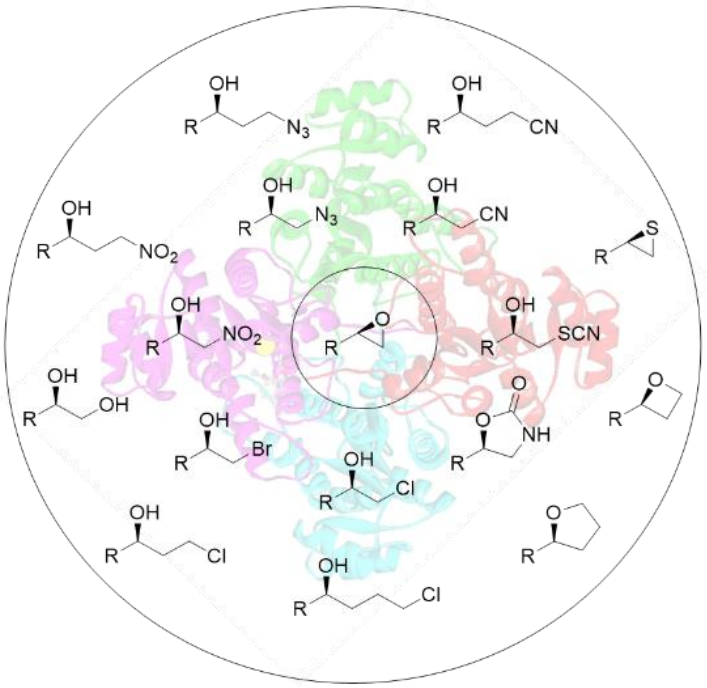
Abstract:

Halohydrin dehalogenases (HHDHs) constitute a remarkable class of biocatalysts that have been successfully applied in the asymmetric synthesis and transformation of epoxides. Their catalytic promiscuity and ability to mediate enantioselective ring-opening reactions have significant implications for the preparation of chiral intermediates used in pharmaceuticals, agrochemicals, and fine chemicals. To date, many HHDHs have been identified, characterized, engineered, and employed in the synthesis of epoxides, β-substituted alcohols, oxazolidinones, thiiranes, and, more recently, oxetanes and tetrahydrofurans. This review provides a general overview on the biocatalytic applications of HHDHs in organic synthesis.

Graphical abstract:



1. Introduction

Enzymes have emerged as powerful catalysts in organic synthesis, offering a sustainable and highly selective alternative to traditional chemical methods [1][2] As nature's catalysts, enzymes facilitate a wide range of chemical transformations under mild reaction conditions,

typically operating at ambient temperature, atmospheric pressure, and near-neutral pH. These characteristics significantly reduce energy consumption and minimize the formation of unwanted by-products. One of the most significant advantages of enzymatic catalysis is its exceptional selectivity. Enzymes exhibit excellent chemo-, regio-, and stereoselectivity, enabling the efficient synthesis of complex molecules with high purity and often eliminating the need for extensive protection and deprotection strategies.[3-4] This is particularly valuable in the pharmaceutical, agrochemical, and fine chemical industries, where the production of enantiomerically pure compounds is of critical importance.[5-7]

Halohydrin dehalogenases (HHDHs), previously known as haloalcohol dehalogenases, halohydrin epoxidases or halohydrin hydrogen-halide lyases, are enzymes that belong to the class of lyases (EC 4.5.1.-) and catalyze the formation and conversion of epoxides.[8] They were initially discovered as components of bacterial metabolic pathways for degradation of halogenated compounds, catalyzing the cyclization of vicinal haloalcohols to epoxides.[9] Structurally, HHDHs are homotetramers composed of two dimers, with one active site located in each monomer, and based on sequence and structural similarities, belong to a superfamily of short-chain dehydrogenase/reductase (SDR) proteins.[10] However, unlike other SDR enzymes, HHDHs do not bind cofactors. Instead of the nicotinamide cofactor binding site found in SDR enzymes, a nucleophile binding pocket is present at a corresponding position.[11]

The active site contains a highly conserved catalytic triad consisting of Ser–Tyr–Arg. Mechanistically, the serine residue forms a hydrogen bond with the oxygen atom of the substrate hydroxyl group, thereby positioning the substrate near the tyrosine residue. Tyrosine acts as a catalytic base and deprotonates the hydroxyl group, enabling nucleophilic attack by the resulting alkoxide ion on the vicinal carbon atom. This leads to epoxide ring closure accompanied by the departure of a halide ion, which is stabilized through binding interactions within the anion-binding pocket. The role of arginine is to lower the pK_a of the tyrosine hydroxyl group through hydrogen-bond formation. (Fig. 1A) Analogous to the ring-closing mechanism, the epoxide substrate is positioned within the active site through hydrogen-bond formation between the epoxide oxygen atom and the hydroxyl group of serine, facilitating nucleophilic attack by a nucleophile bound in the anion-binding pocket. The resulting alkoxide ion subsequently abstracts a proton from the tyrosine hydroxyl group, which is stabilized by hydrogen-bonding interactions with arginine (Fig. 1B).[12]

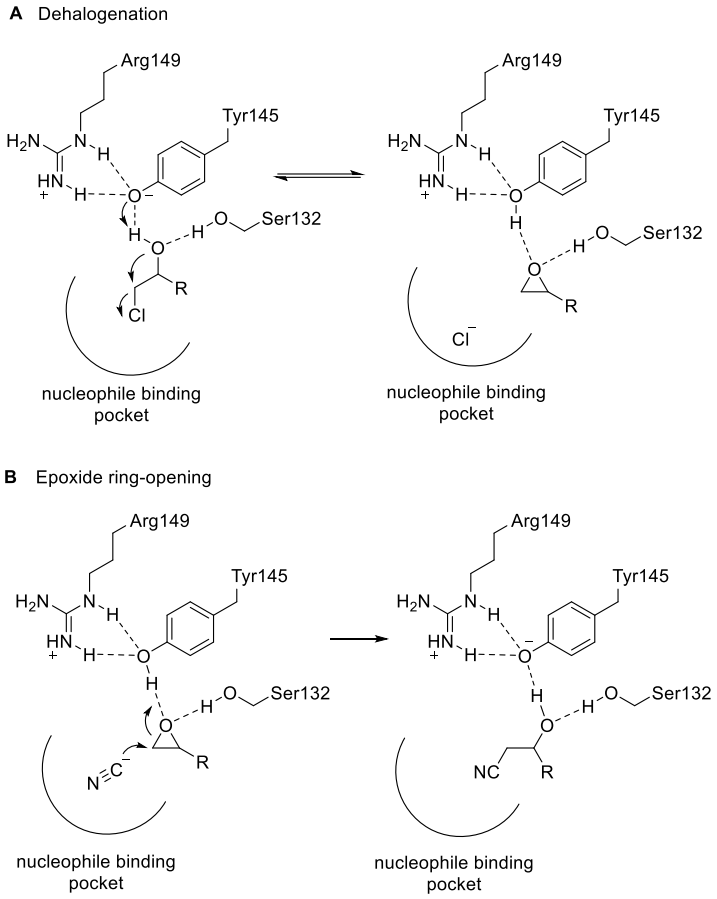


Fig. 1 Catalytic mechanism of halohydrin dehalogenases in dehalogenation (A); and epoxide ring-opening (B); residue numbering according to HheC [13]

While knowledge of HHDHs was previously limited to several characterized enzyme sequences that were classified into three phylogenetic subtypes (HheA, HheB and HheC), more than sixty potential HHDH sequences have recently been identified in public genomic databases using data mining.[14] Today, the classification of HHDHs is expanded by four new subtypes (HheD, HheE, HheF and HheG) in accordance with similarities in genetic sequences and bioactivity.[15]

From the synthetic point of view, HHDHs catalyze the reversible dehalogenation of vicinal haloalcohols, yielding the corresponding epoxides (Fig. 2A). Beyond their native reactivity, HHDHs display notable catalytic promiscuity, enabling the nucleophilic ring-opening of epoxides with a range of small anionic nucleophiles, including azide, cyanide, nitrite, cyanate, and thiocyanate (Fig. 2B).[16] This versatility enables the highly regio- and stereoselective formation of C–N, C–C, C–O, and C–S bonds, providing access to diverse β-substituted alcohols and enantioenriched epoxides of considerable synthetic value. Recent advances in the protein engineering of HHDHs have expanded their catalytic repertoire to include novel reactions such as oxetane formation and oxetane ring-opening, tetrahydrofuran formation and dehalogenative hydroxylation of ε-haloalcohols to form ε-diols (Fig. 2C).[17-19] To date, the HHDH-catalyzed ring opening of tetrahydrofurans has not been achieved experimentally, reflecting the considerable challenge associated with this transformation.

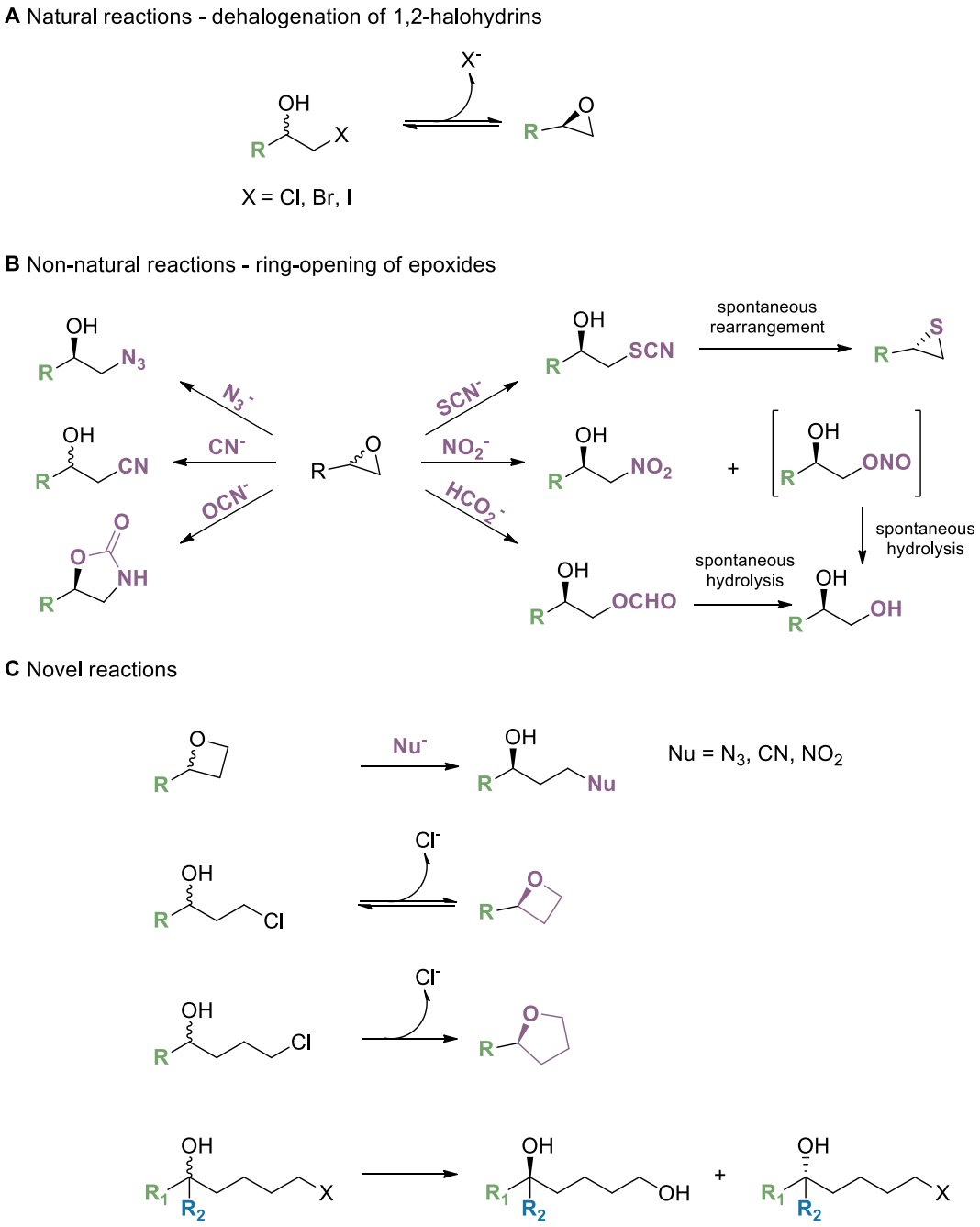


Fig. 2 Organic reactions catalyzed by HHDHs

Despite their significant potential in asymmetric synthesis, HHDHs also exhibit several limitations for the use in organic synthesis.[20] Their natural substrate scope is relatively narrow, with reduced activity toward bulky or highly functionalized hydrophobic compounds. HHDHs typically operate in aqueous media, which leads to mass-transfer limitations at higher substrate loadings due to poor solubility of many organic substrates. More importantly for process applications, increasing substrate concentrations can result in decreased catalytic performance due to substrate and/or product inhibition.[21-22] Furthermore, many HHDHs show limited tolerance toward organic co-solvents, which are often required to improve substrate solubility. Such solvents can induce partial unfolding, active-site perturbation, or

reduced catalytic efficiency by acting as inhibitors.[23] Although HHDHs accept a set of anionic nucleophiles, this range remains limited compared to conventional chemical synthesis, and both activity and stereoselectivity can vary significantly depending on the enzyme variant and substrate.

Comprehensive information on the discovery, structural and mechanistic characteristics, as well as the biocatalytic applications of HHDHs, can be found in previous review articles and book chapters.[1][8][13][20][24]

Although HHDH-catalyzed transformations have been studied extensively, most investigations have remained limited to microscale or screening-scale experiments performed at low substrate concentrations. Until recently, reports describing their implementation on synthetically relevant scales were relatively rare. Although the number of such studies has gradually increased in recent years, examples involving product isolation and subsequent incorporation into multistep synthetic sequences remain limited. Moreover, advances in enzyme discovery,[14] protein engineering,[25-33] and reaction engineering,[21-22][34] have significantly expanded the substrate scope and operational stability of HHDHs, enabling their application to increasingly complex and industrially relevant transformations. This is particularly noteworthy given that HHDH-catalyzed processes provide access to enantiomerically pure compounds bearing tetrasubstituted stereogenic carbon centers — highly valuable yet very challenging structural motifs in modern synthetic chemistry.[35] This review focuses on the synthetic applications of HHDH catalyzed transformations, and only studies reporting preparative procedures and isolated products are included.

2. Epoxide ring-opening reactions

2.1. Synthesis of hydroxynitriles

Following pioneering discoveries by Castro *et al.* [9] and Nakamura *et al*. [36] that halohydrin dehalogenases (HHDHs) catalyze the conversion of haloalcohols to epoxides and can utilize cyanide ion as a nucleophile, Nakamura *et al.* subsequently employed the HheB enzyme from *Corynebacterium sp.* strain N-1074 (referred to as H-lyase B in their study) for the synthesis of (*R*)-γ-chloro-β-hydroxybutyronitrile from epichlorohydrin or 1,3-dichloro-2-propanol (Fig. 3).[37] This work has laid the foundation for the application of HHDH-catalyzed ring-opening reactions in biocatalysis.

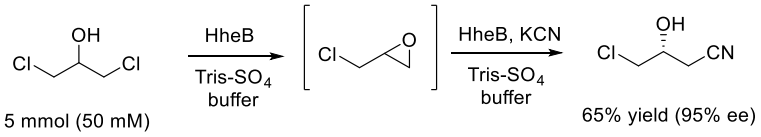


Fig. 3 HheB-catalyzed synthesis of (*R*)-γ-chloro-β-hydroxybutyronitrile

Majerić Elenkov *et al.* investigated HHDH catalyzed ring-opening of epoxides with cyanide ion on a several structurally different aliphatic epoxides using HheA, HheB, and HheC enzymes.[38] Activity, enantioselectivity and enantiopreference were found to depend on the type of enzyme and the substrate structure. HheC was found to be the most selective among the

tested enzymes and resolution of 2,2-disubstituted epoxides proceeds with very high enantioselectivity. Application of the method in the synthesis of enantiopure tertiary alcohols that are difficult to prepare using other methods, was shown on two structurally different compounds (Fig. 4).[39]

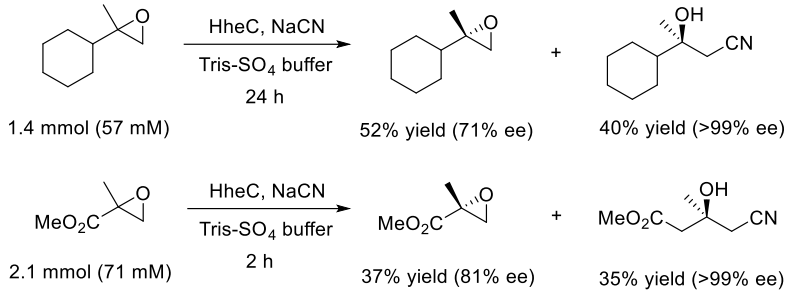


Fig. 4 HheC-catalyzed synthesis of tertiary alcohols

A variant HheC-W249P-catalyzed nucleophilic ring-opening of a series of fluorinated styrene oxide derivatives with cyanide ions with excellent enantioselectivity (*E*-values up to >200). However, due to lower enzymatic activity in cyanolysis reaction, very high catalyst loadings and prolonged reaction time were required to obtain β-hydroxy nitrile in 30% yield and 98% ee (Fig. 5).[40]

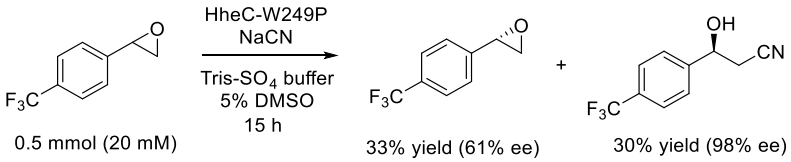


Fig. 5 HheC-W249P-catalyzed cyanolysis of fluorinated styrene oxide derivatives

In their study, Guan *et al.* demonstrated that engineered halohydrin dehalogenases can catalyze the asymmetric cyanation of epoxides by employing cyanohydrins as cyanide donors instead of sodium cyanide. After enzyme screening and protein engineering, the optimized biocatalysts efficiently converted aryl (Fig. 6), alkyl (Fig. 7), and spiro epoxides (Fig. 8) into the corresponding β-hydroxy nitriles, using the *in situ* release of cyanide ion from mandelonitrile, reaching enantiomeric excesses of up to >99% ee, while the isolated yields were as high as 47%. [41]

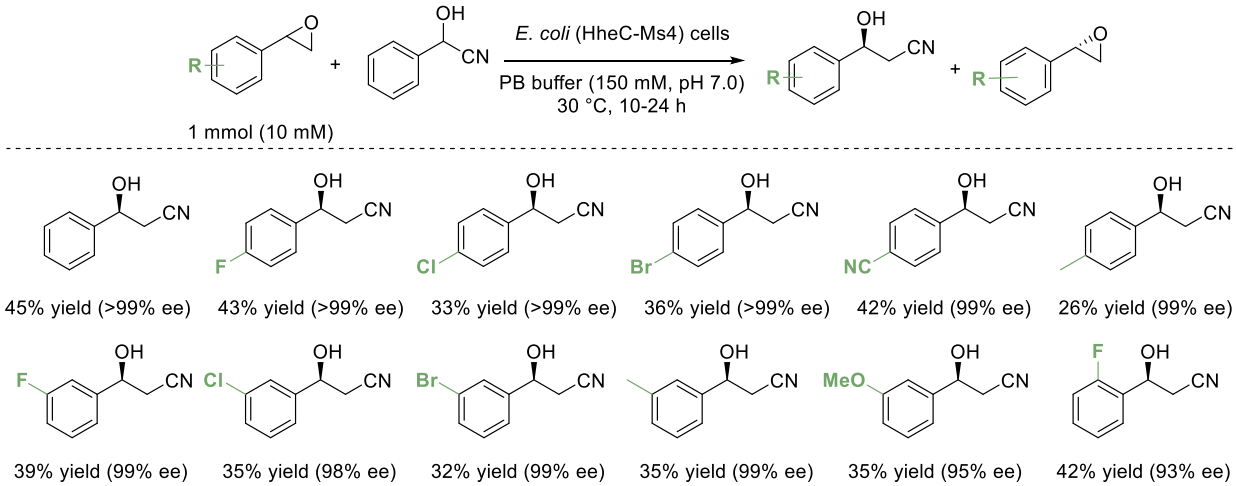


Fig. 6 HheC-Ms4-catalyzed cyanation of aryl epoxides

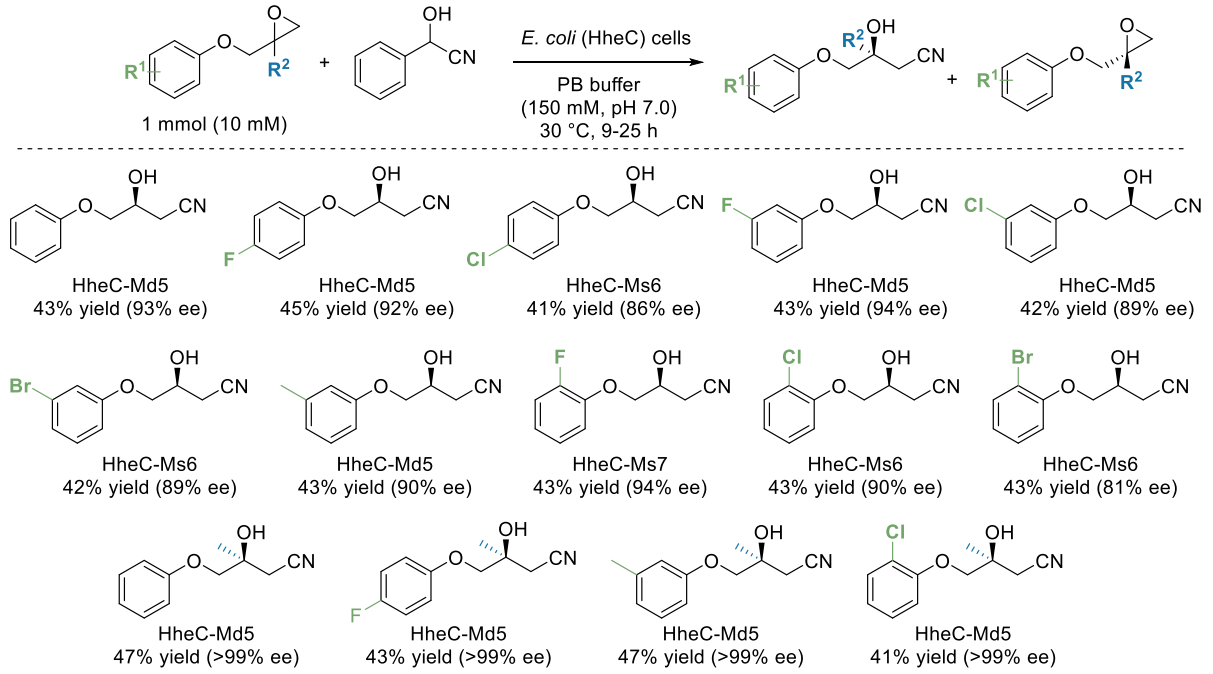


Fig. 7 Cyanation of alkyl epoxides catalyzed by HheC variants

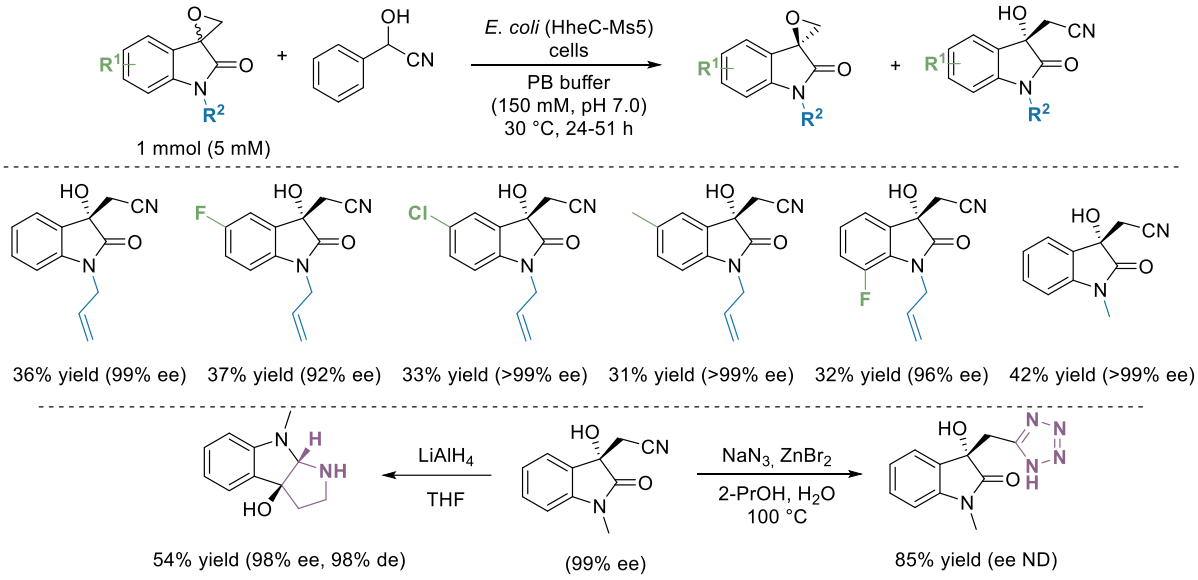


Fig. 8 HheC-Ms5-catalyzed cyanation of spiroepoxides

2.2. Synthesis of azido alcohols

One of the most extensively studied ring-opening reactions catalyzed by HHDHs involves the use of azide as the nucleophile, typically in the form of sodium azide. Spelberg *et al.* were the first to demonstrate that azide can serve as a nucleophile in HHDH-catalyzed reactions, reporting highly enantioselective and β-regioselective azidolysis of (substituted) styrene oxides using the HHDH from *Agrobacterium radiobacter* AD (HheC) (Fig. 9).[42] To avoid an uncatalyzed chemical side reaction, sodium azide was added slowly over 24 h keeping the azide concentration low.

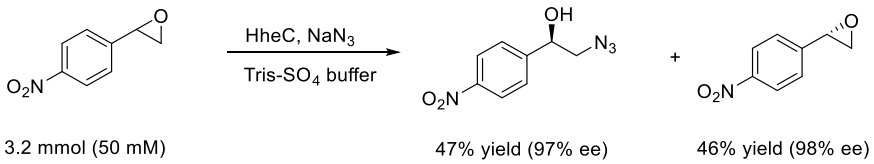


Fig. 9 HheC-catalyzed azidolysis of *p*-nitrostyrene oxide

Molinaro *et al.* described a method for the HHDH resolution of 2-alkyl-2-aryl disubstituted epoxides using commercially available Codex HHDH enzymes and sodium azide (Fig. 10).[43] This method allowed regio- and enantioselective synthesis of novel (*R*)-1-azido-2-arylpropan-2-ols in 19–45% yield and 88–99% ee depending on the epoxide structure. Furthermore, obtained azido alcohols were used for the preparation of enantiomerically enriched amino alcohols and aziridines.

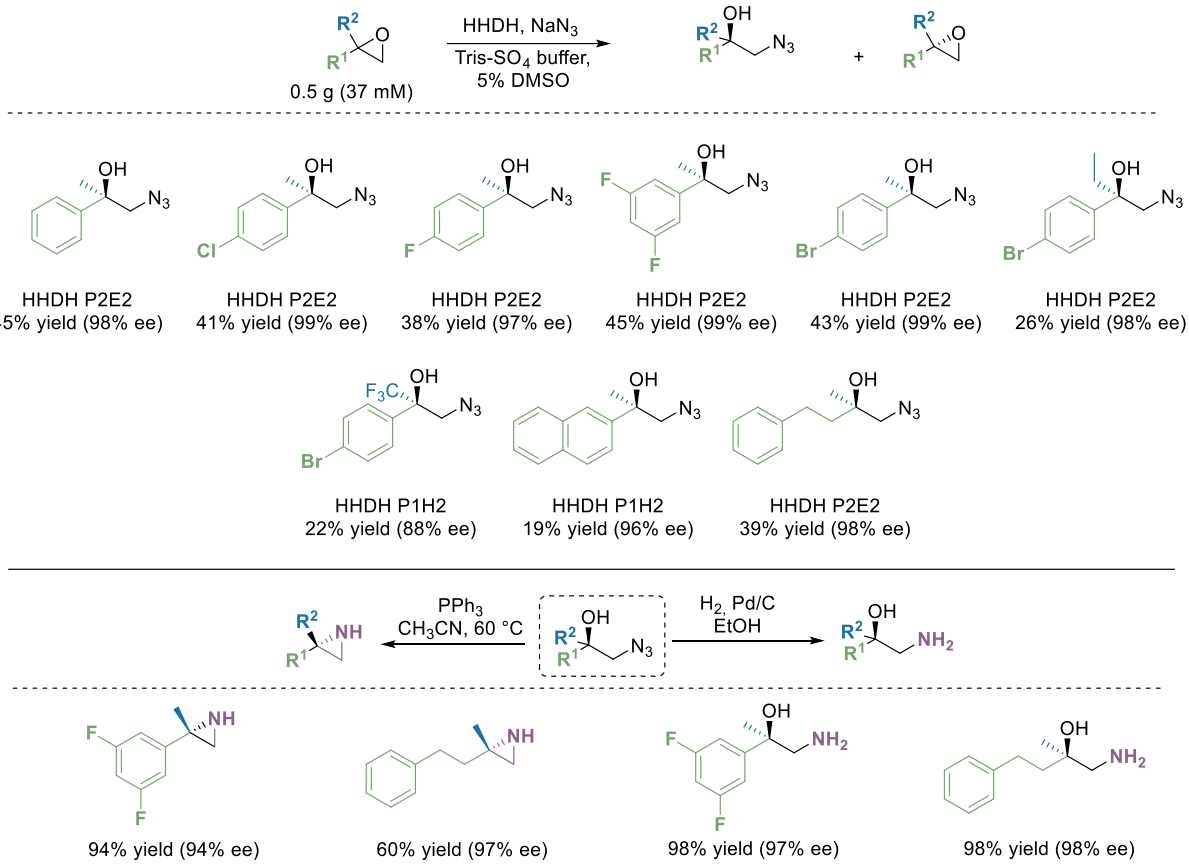


Fig. 10 HHDH-catalyzed azidolysis of 2-alkyl-2-aryl disubstituted epoxides

Majerić Elenkov *et al.* expanded the substrate scope of HHDHs to spiroepoxides. Highly regioselective azidolysis of spiroepoxides containing 5-, 6-, and 7-membered cycloalkane rings was reported using the enzyme from *Arthrobacter sp.* AD2 (HheA), (Fig. 11).[44] Corresponding β-azido alcohols were obtained in almost complete regioselectivity (96–100%) and in 75–96% yield.

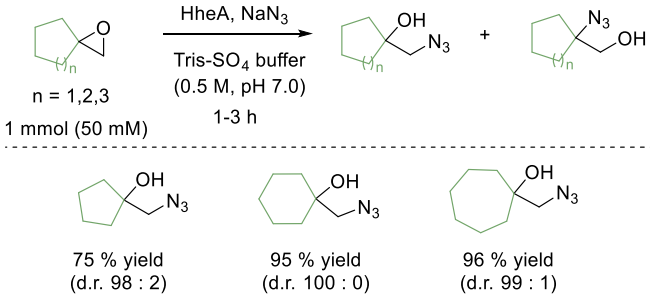


Fig. 11 Azidolysis of spiroepoxides using HheA

Calderini *et al.* investigated a panel of 22 HHDHs (representatives of six phylogenetic subtypes A through G) in the conversion of aliphatic and aromatic vicinally di-substituted *trans*-epoxides using azide as nucleophile and demonstrated that the substrate scope of HHDHs, in particular of HheG enzyme, is not limited to terminal epoxides.[45] Selected regioselective reactions were

performed on preparative scale (200 mg of epoxide) by means of whole-cell biocatalysis with HheE and HheE5, respectively (Fig. 12).

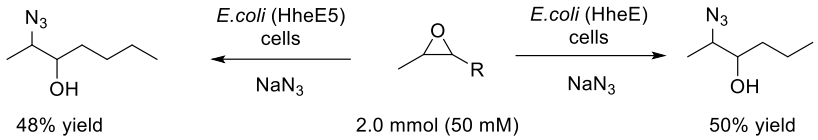


Fig. 12 Azidolysis of non-terminal epoxides using HheE and HheE5

An *et al.* showed that HheG (from *Ilumatobacter coccineus sp*.) exhibits excellent α-regioselectivity in the azidolysis of styrene oxide derivatives (most of HHDHs are β-regioselective), producing the corresponding racemic 2-azido-2-aryl-1-ols with the yields up to 96% (Fig. 13).[46]

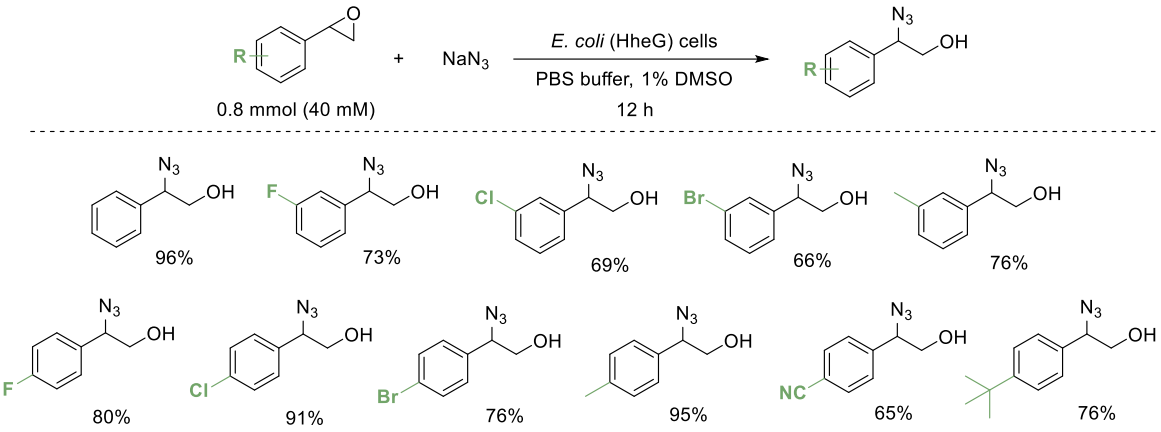


Fig. 13 HheG-catalyzed azidolysis of styrene oxide derivatives

Zhang *et al.* developed an efficient HheC-catalyzed kinetic resolution via azidolysis for the synthesis of a range of chiral spiroepoxyoxindoles and 3-(azidomethyl)-3-hydroxyindoles in good yields (up to 48% isolated yield) and high optical purity (up to >99% ee).[47] For the resolution of sterically demanding substrates (e.g., those bearing substituents at the 4-position of the indole moiety or an allyl group on the nitrogen atom), engineered HheC variants 2-H6 (N176A/T134I) and 2-E3 (N176A/T134S) were employed (Fig. 14). The synthetic value of the method was demonstrated by a gram-scale kinetic resolution performed at a substrate concentration of 30 mM, affording the epoxide in 39% yield and >99% ee, and the azido alcohol in 47% yield and 97% ee. Both products were further converted into several valuable synthetic building blocks (Fig. 15).

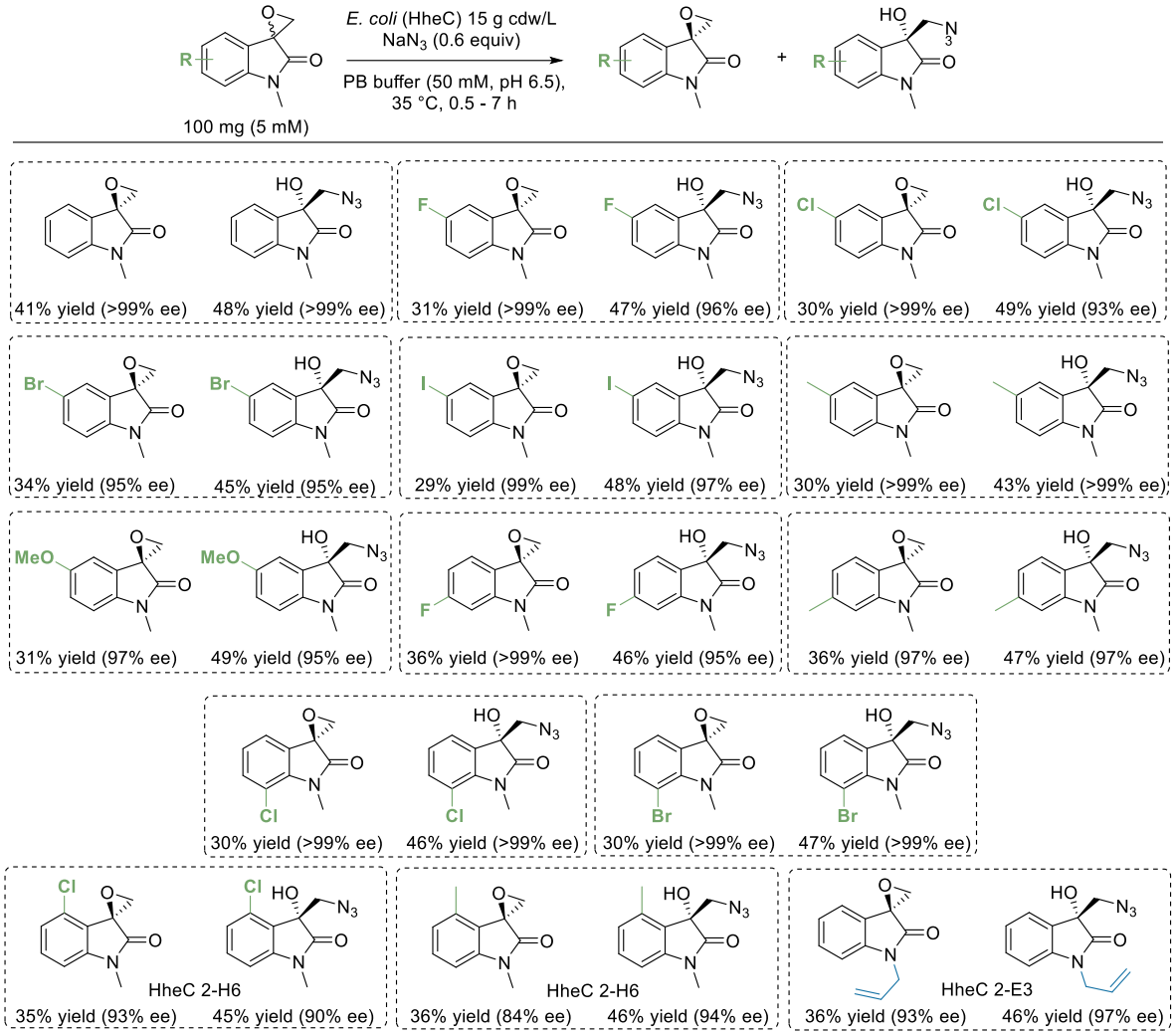


Fig. 14 HheC-catalyzed azidolysis of spiroepoxyoxindoles

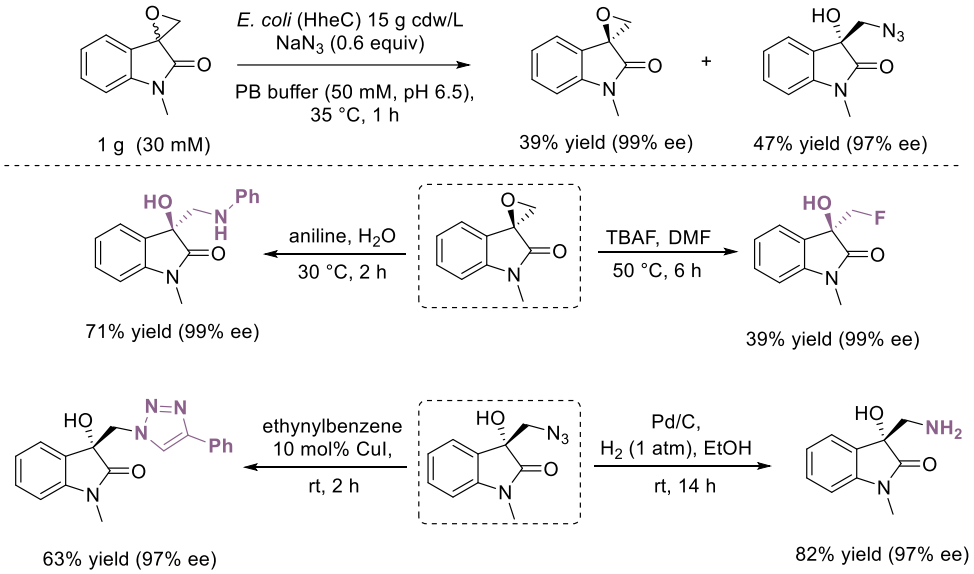


Fig. 15 HheC-catalyzed gram-scale azidolysis of spiroepoxyindole and further synthetic transformations of obtained products

Kolman *et al*. expanded the substrate scope of HHDH enzymes to propargylic epoxides (Fig. 16).[48] Azide-mediated kinetic resolution of these epoxides was investigated using the stereocomplementary HHDHs: HheC and HheA2-N178A, which exhibit opposite enantioselectivities. Sequential application of two enzymatic reactions to the p-tolyl propargylic epoxide enabled the stereoselective synthesis of both secondary azido alcohol enantiomers and (*R*)-primary azido alcohol, each with >99% ee, accompanied by complete conversion of the starting epoxide. The resulting azido alcohols were subsequently employed in click reactions with terminal alkynes, affording triazolyl propargylic alcohols in >99% ee and 90–98% yield. For the primary azido alcohol, the click reaction was followed by an intramolecular cyclization, providing a dihydrofuranyl triazole motif present in biologically active compounds.

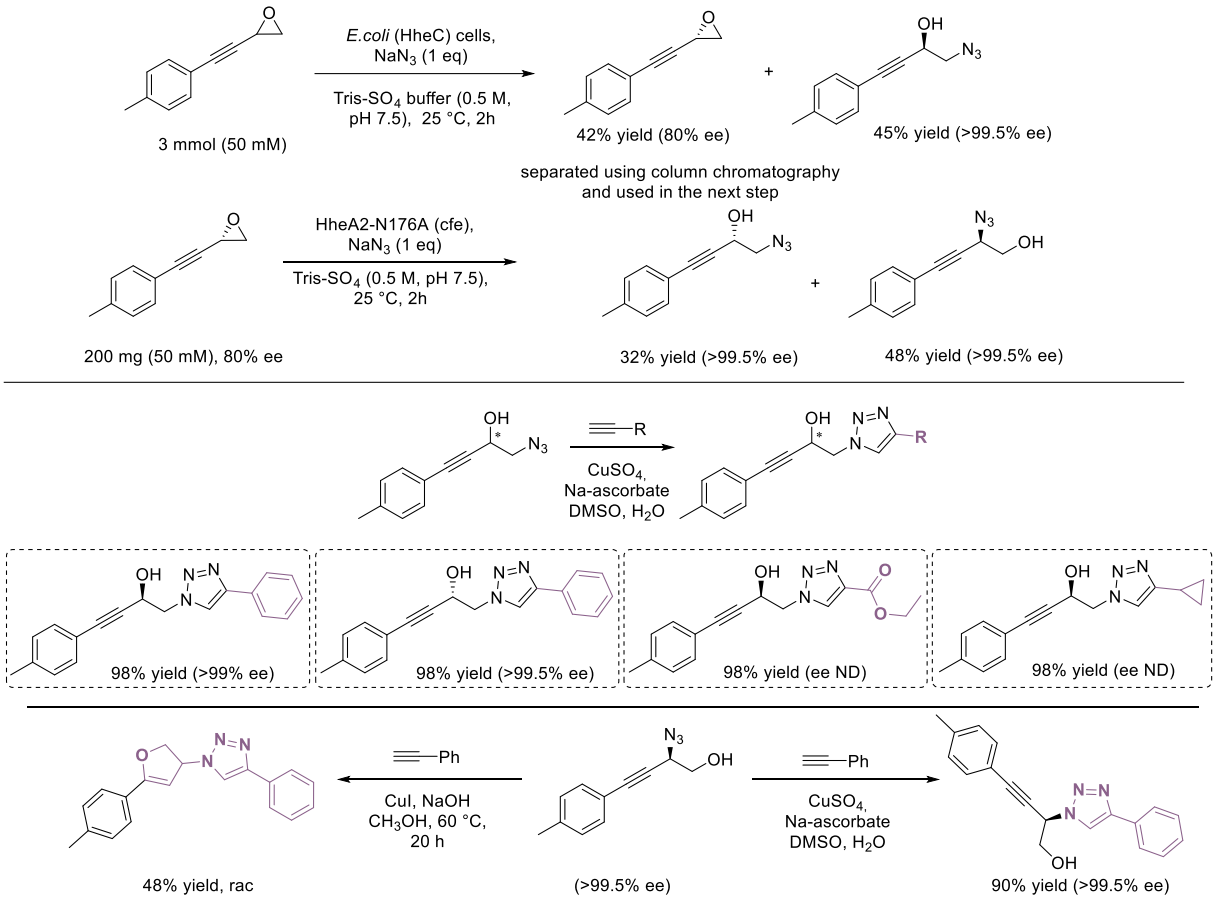


Fig. 16 HheC and HheA2-N176A-catalyzed sequential azidolysis of *p*-tolyl propargylic epoxide

Based on previous results using several available HheC variants,[40],[49] Kolman and Milčić *et al.* further investigated the HheA2-N178A variant, displaying high (*S*)-enantioselectivity, although with compromised regioselectivity (preferentially attacking at the Cβ-position) for the synthesis of a wide range of fluoroaromatic azido alcohols (Fig. 17). Biotransformations using 100 mM substrate concentration were carried out in a two-phase system, and the products were isolated in 43–>99% ee.[50]

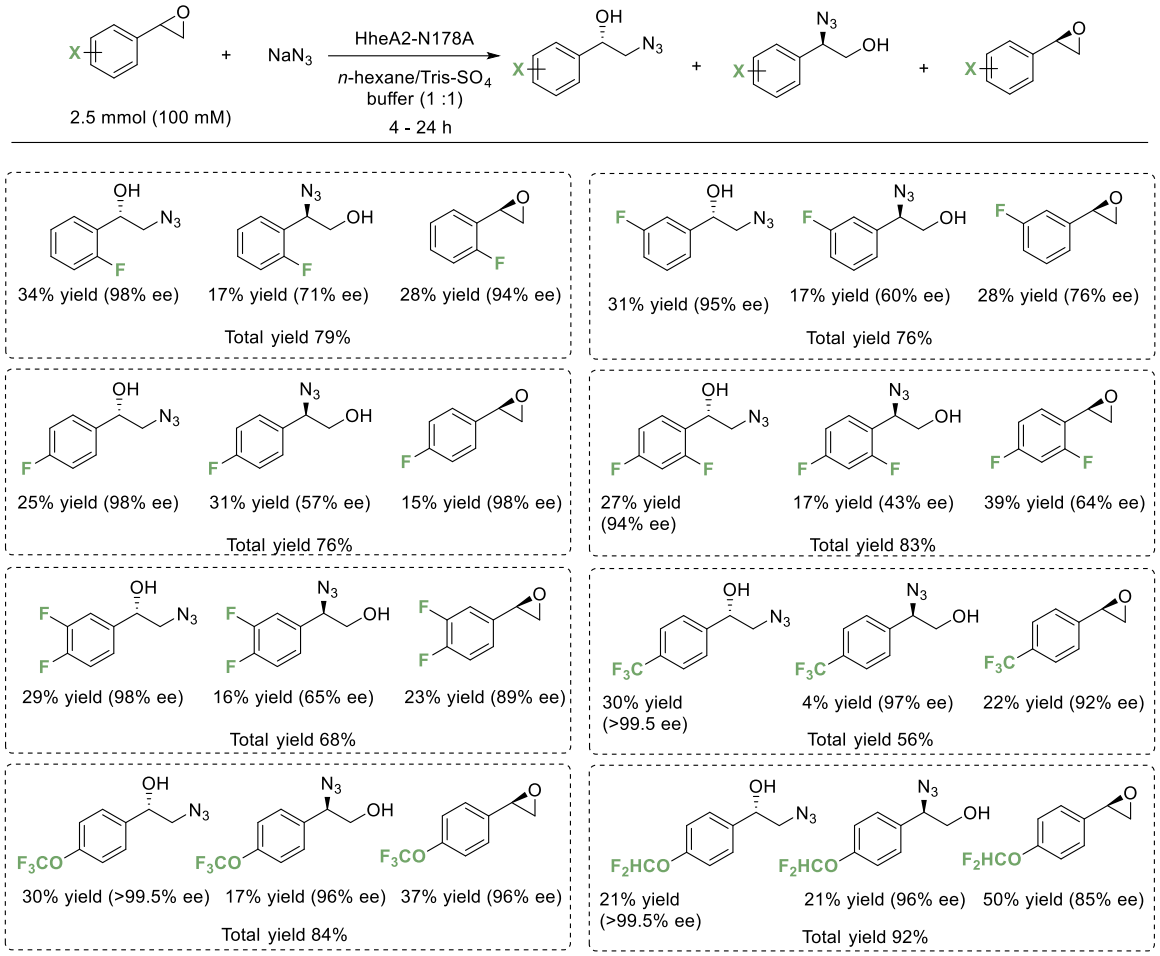


Fig. 17 HheA2-N178A-catalyzed synthesis of fluoroaromatic azido alcohols

2.3. Synthesis of 2-oxazolidinones

Hasnaoui-Dijoux *et al.* were first to report that, in addition to halides, azide, cyanide and nitrite, HHDHs can also accept cyanate, thiocyanate, and formate ions as nucleophiles in ring-opening reactions of epoxides [16] Following that research, Majerić Elenkov *et al.* explored HheC-catalyzed ring-opening of terminal epoxides using cyanate ion as a nucleophile in the synthesis of 2-oxazolidinones (Fig. 18A).[51] 5-Substituted 2-oxazolidinones were formed in high yields and with high enantiomeric excess (69–98% ee), (Fig. 18B).

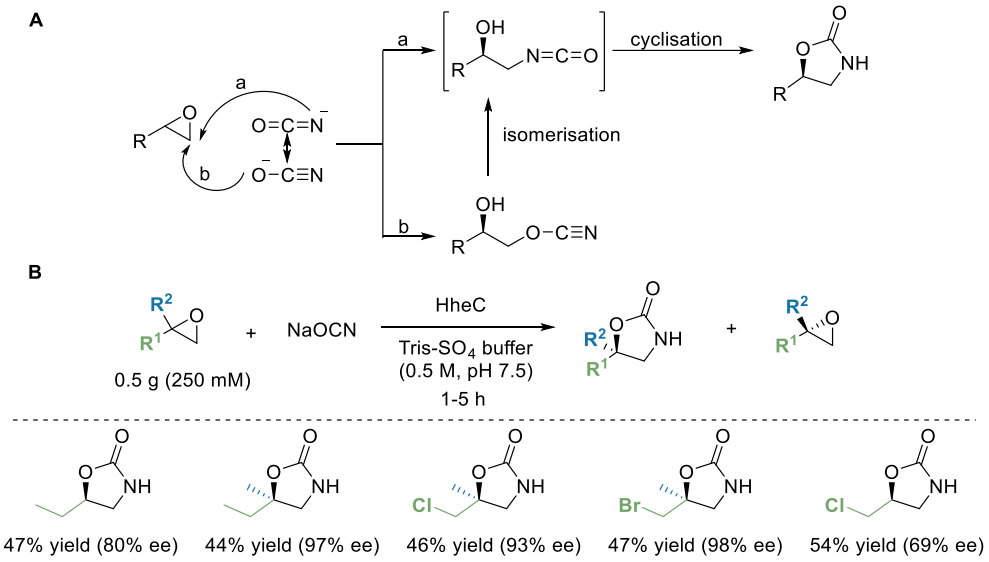


Fig. 18 A Mechanism of 2-oxazolidinone formation; B HheC-catalyzed synthesis of 5-substituted 2-oxazolidinones

Wang *et al.* explored the use of HHDHs for the synthesis of the oxazolidinone drug metaxalone and several related analogues through the ring-opening of epoxides with cyanate [52]. Protein engineering of HHDHamb from *Acidimicrobiia bacterium* enabled gram-scale preparation of both enantiomerically enriched and racemic metaxalone. The chiral product was obtained in 44% isolated yield with 98% ee, whereas the racemic form was produced in 81% yield. The same strategy was applied to the synthesis of several metaxalone analogues, providing optically active derivatives in 28–40% yield (90–99% ee) and racemic analogues in 77–92% yield (Fig. 19).

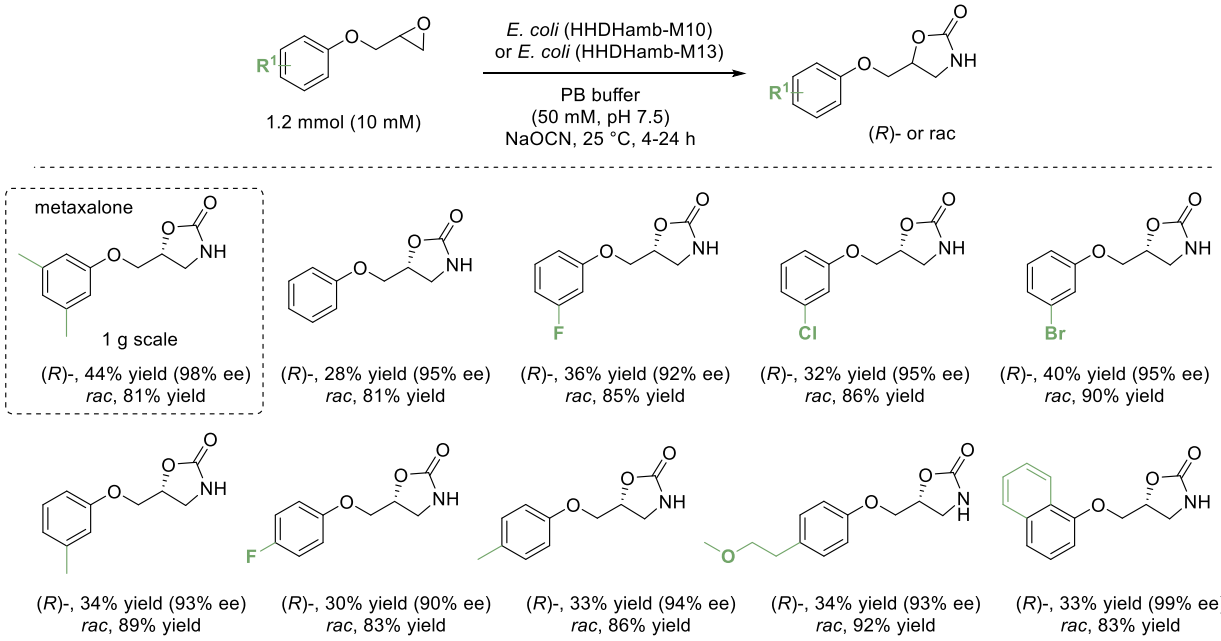


Fig. 19 Synthesis of metaxalone and analogues using HHHDamb variants

Zhou *et al.* evaluated a panel of ten HHDHs to identify biocatalysts suitable for the synthesis of chiral 4-aryl-2-oxazolidinones.[53] Among the enzymes examined, *Ab*HheG displayed an uncommon preference for α-regioselective ring opening of racemic styrene oxide with cyanate, although its enantioselectivity remained low (E < 3). To overcome this limitation, the authors engineered the substrate-binding pocket and obtained the *Ab*HheG variant Y15M/N182S, which exhibited improved regio- and stereoselectivity. This engineered enzyme enabled the preparation of (*R*)-4-aryl-2-oxazolidinones in 18–52% yield with enantiomeric excesses ranging from 87 to 99% ee (Fig. 20). In contrast, the wild-type enzyme converted (*R*)-styrene oxides into the corresponding (*S*)-configured products, providing access to both enantiomeric series. Crystallographic analysis combined with molecular docking suggested that the enhanced selectivity of the engineered variant originated from an altered substrate-binding mode, in which substrate reorientation within the active site favored the desired catalytic pathway.

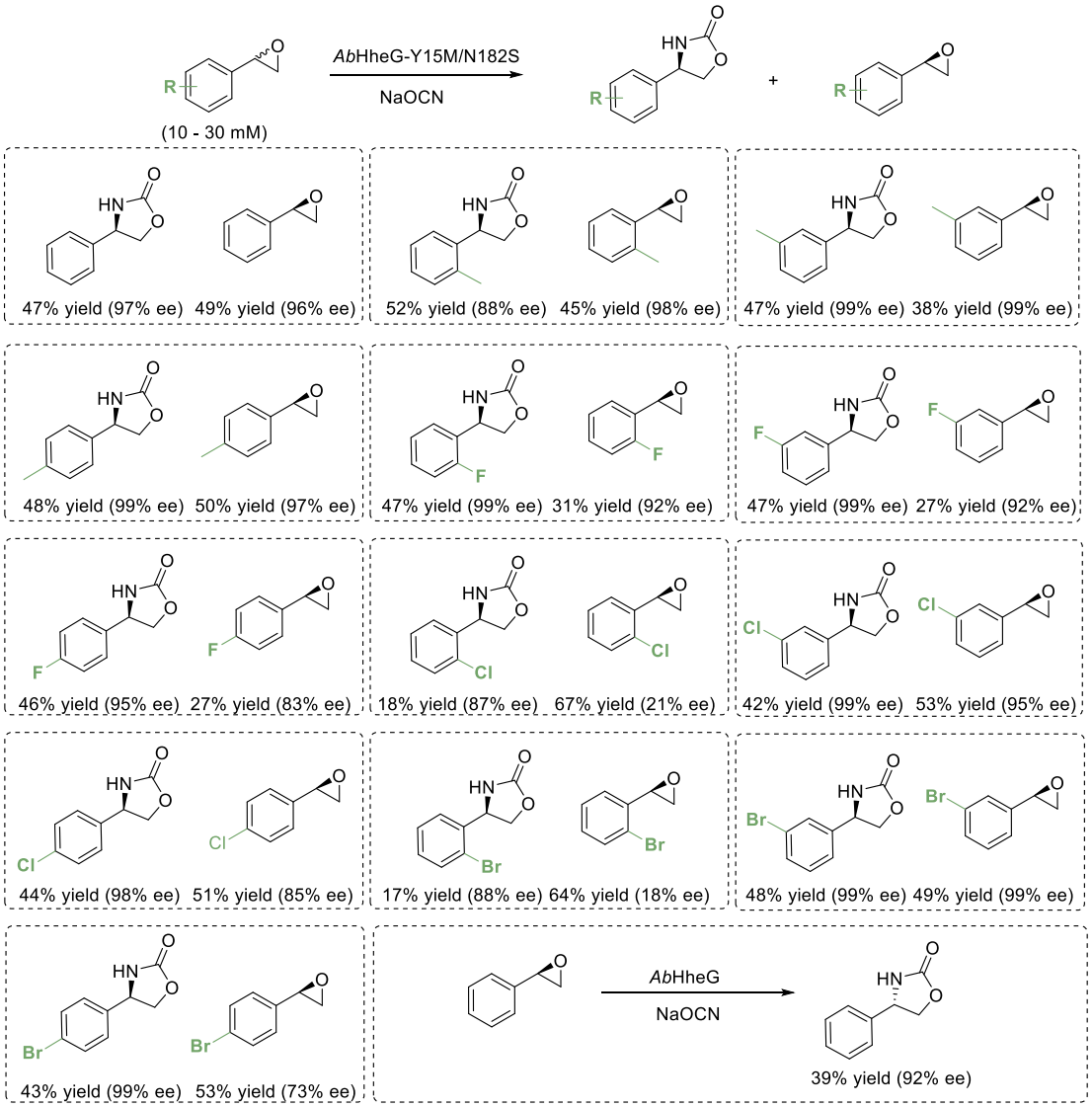


Fig. 20 Synthesis of (*R*)-4-aryl-2-oxazolidinones using *Ab*HheG-Y15M/N182S

To further study the use of HHDHs in real industrial applications, Yang *et al.* screened a diverse panel of 48 WT HHDHs for their ability to convert aliphatic cyclohexene oxide to *trans*-

hexahydro-2(3H)-benzoxazolone.[54] Using HheG from *Ilumatobacter coccineus*, in form of a whole cell biocatalyst, reaction was scaled up from analytical to pilot-scale manufacturing. pH control was achieved using hydrolysis of a co-solvent ethyl acetate, and the product was obtained in 87% yield and 53% ee (Fig. 21).

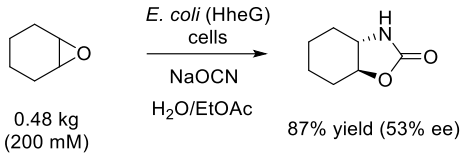


Fig. 21 Pilot-scale synthesis of *trans*-hexahydro-2(3H)-benzoxazolone

Ma *et al*. developed a synthesis of chiral and racemic spirooxazolidinones based on the HheG catalyzed ring expansion reaction of spiroepoxides with NaOCN.[55] By performing screening and protein engineering of HheG enzyme and using newly designed HheG-M6 (I104F/N196S/F200W) and HheG-M7 (I104F/N196G) variants, both chiral and racemic spirooxazolidinones were synthesized in 24−47% yields (90−98% ee) and 69−98% yields, respectively (Fig. 22). Furthermore, a chemoenzymatic approach was developed to circumvent the intrinsic 50% theoretical yield limitation associated with kinetic resolution. Racemic 1-methylspiro[indoline-3,2'-oxiran]-2-one was converted on a gram-scale using HheG-M6 and NaOCN to obtain (*S*)-epoxide (45% yield, 81% ee) and (*R*)-oxazolidinone (42% yield, 95% ee). (*S*)-Epoxide was racemized using a $BiCl_3$ catalyzed ring-opening to obtain a chlorohydrin that was transformed again to (*R*)-oxazolidinone through sequential enantioselective dehalogenation and ring expansion reactions using HheG-M6 variant. The oxazolidinone product was obtained in 59% yield and used in several representative reactions. The biocatalytic method was also applied to the efficient synthesis of the drug fenspiride (Fig. 23).

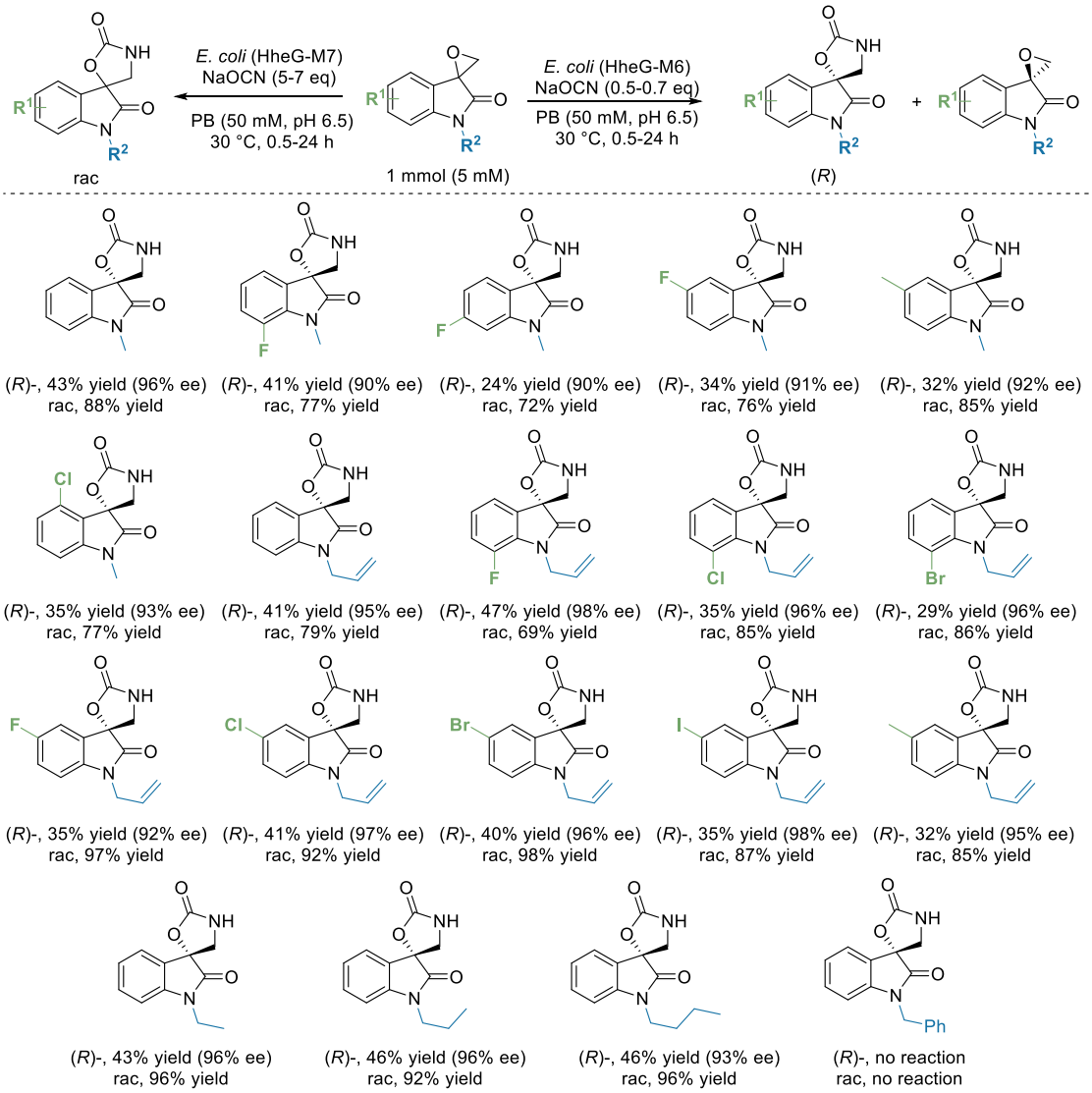


Fig. 22 Synthesis of chiral and racemic spirooxazolidinones using HheG-M6 and M7 variants

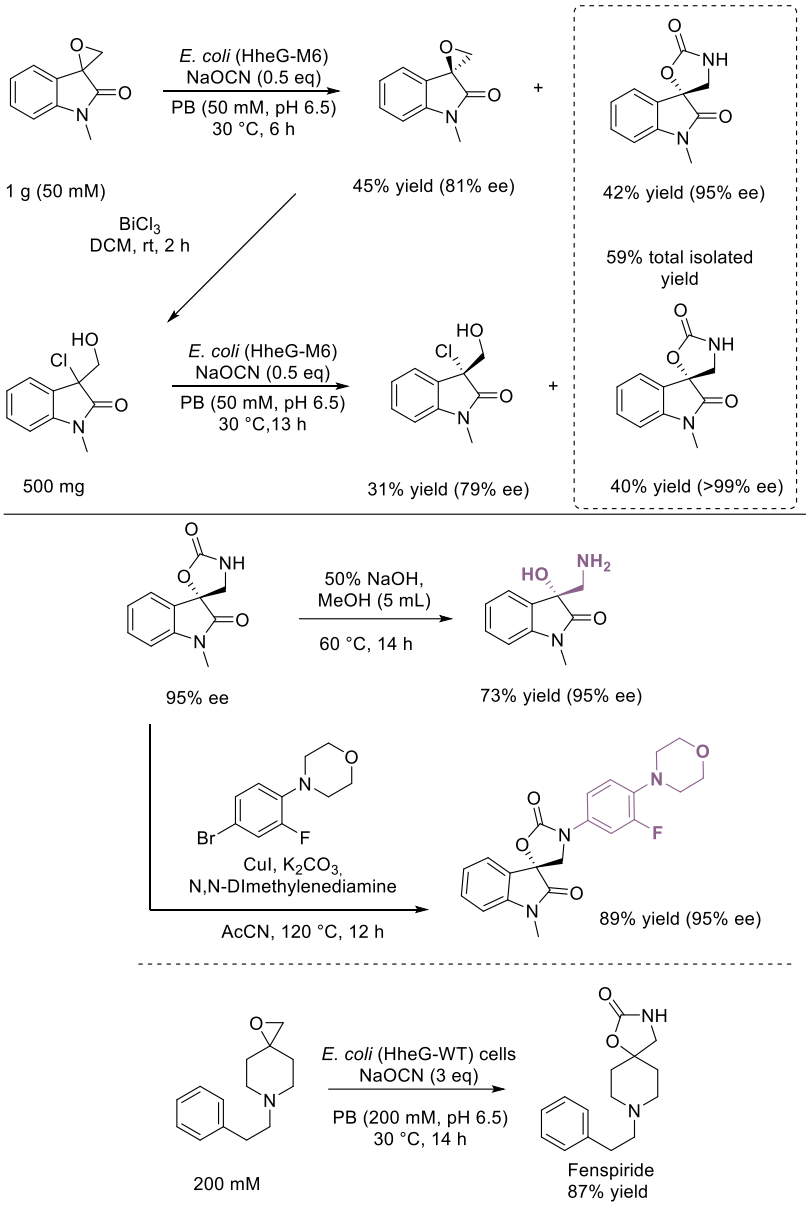


Fig. 23 Gram-scale resolution of 1-methylspiro[indoline-3,2'-oxiran]-2-one and synthetic transformations of obtained products including the synthesis of fenspiride

Wan *et al.* reported the use of HHDH from *Ilumatobacter coccineus* (HheG), which exhibited an unusual and high α-regioselectivity in the synthesis of 4-aryloxazolidinones from styrene oxides, while with very low enantioselectivity.[56] Building on this research, and on a resolved structure of wild-type HheG determined by Schallmey's group,[57] Zhou, X.-Y. *et al.* have tailored HheG enzyme using protein engineering (HheG-M1 and HheG-M2 variants) to enhance its *R*-enantioselectivity for the synthesis of chiral 4-aryloxazolidinones via α-regioselective ring-opening of aryl epoxides with cyanate.[58] Additionally, they achieved the inversion of its regioselectivity and enantioselectivity, resulting in a variant HheG-M8 (T106V/V162W/T195A/N196G/Y197W/F203L/A206M) capable of yielding chiral 5-aryloxazolidinones via β-regioselective and *S*-enantioselective ring-opening of aryl epoxides with cyanate. The gram-scale biotransformation was done on styrene oxide using HheG-M1

(I104H) variant, yielding chiral (*S*)-4-phenyloxazolidin-2-one (39% yield, 95% ee). The authors also showed that chiral 4-aryloxazolidinones and 5-aryloxazolidinones can be synthesized simultaneously in a one-step, one-pot manner, utilizing the two regio- and enantio-complementary mutants HheG-M1 and HheG-M8. The reaction was demonstrated on 2-phenyloxirane furnishing (*S*)-4-phenyloxazolidin-2-one (49% yield and 90% ee), and (*S*)-5-phenyloxazolidin-2-one (45% yield and 98% ee), that can easily be separated by column chromatography (Fig 24).

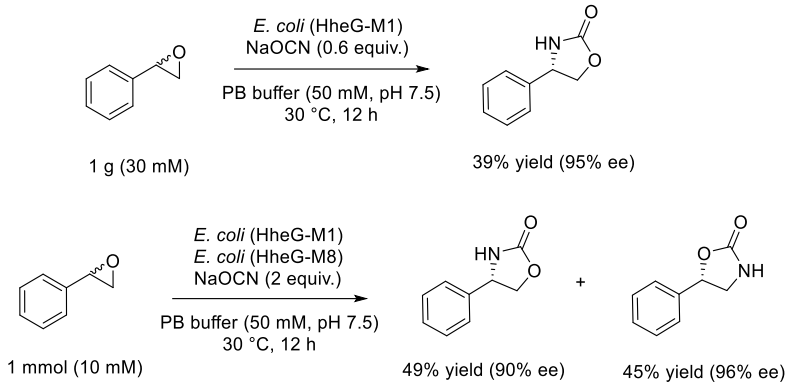


Fig. 24 Gram-scale synthesis of (*S*)-4-phenyloxazolidin-2-one; and one-step, one-pot, two enzyme synthesis of (*S*)-4-phenyloxazolidin-2-one and (*S*)-5-phenyloxazolidin-2-one

2.4. Synthesis of thiocyanatohydrins and thiiranes

Even though thiocyanate ion (SCN^-) has been reported as an acceptable nucleophile to HHDH by Hasnaoui-Dijoux *et al.*[16] so far there are only several papers that investigate this approach on a synthetically relevant scale. There are two possible products of epoxide ring-opening reaction with thiocyanate, depending on the pH of the reaction mixture: thiocyanatohydrins in neutral and mildly acidic, and thiiranes in basic reaction conditions conditions as a result of intramolecular rearrangement of thiocyanatohydrins (Fig. 25).[59]

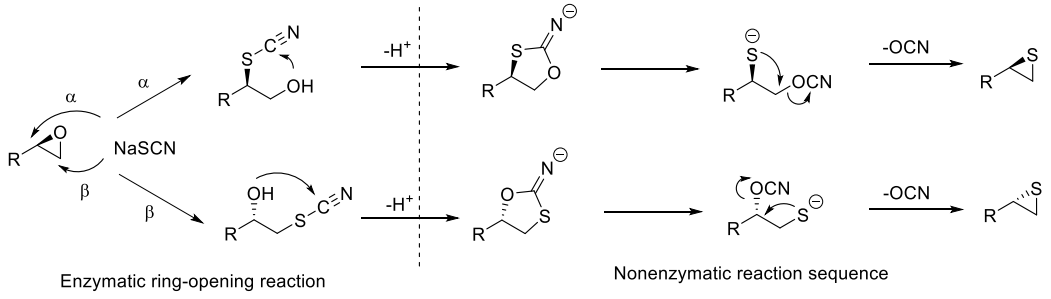


Fig. 25 Possible products of epoxide ring-opening with thiocyanate[59]

Ma *et al.* identified and further engineered a novel HHDHapb from *Alphaproteobacteria bacterium* strain, HHDHapb-M4 (I137C/Y181F) and HHDHapb-M7 (A82T/I137Q/F140H/A173P). These engineered enzymes catalyzed the enantioselective thionation of diverse aryl- and alkyl-substituted epoxides, providing the corresponding thiiranes in up to 43% isolated yield and 98% ee. The synthetic potential of the approach was further

validated on a 1.5 g scale using a racemic epoxide, while two subsequent transformations of the chiral thiiranes demonstrated the utility of the products (Fig. 26).[59]

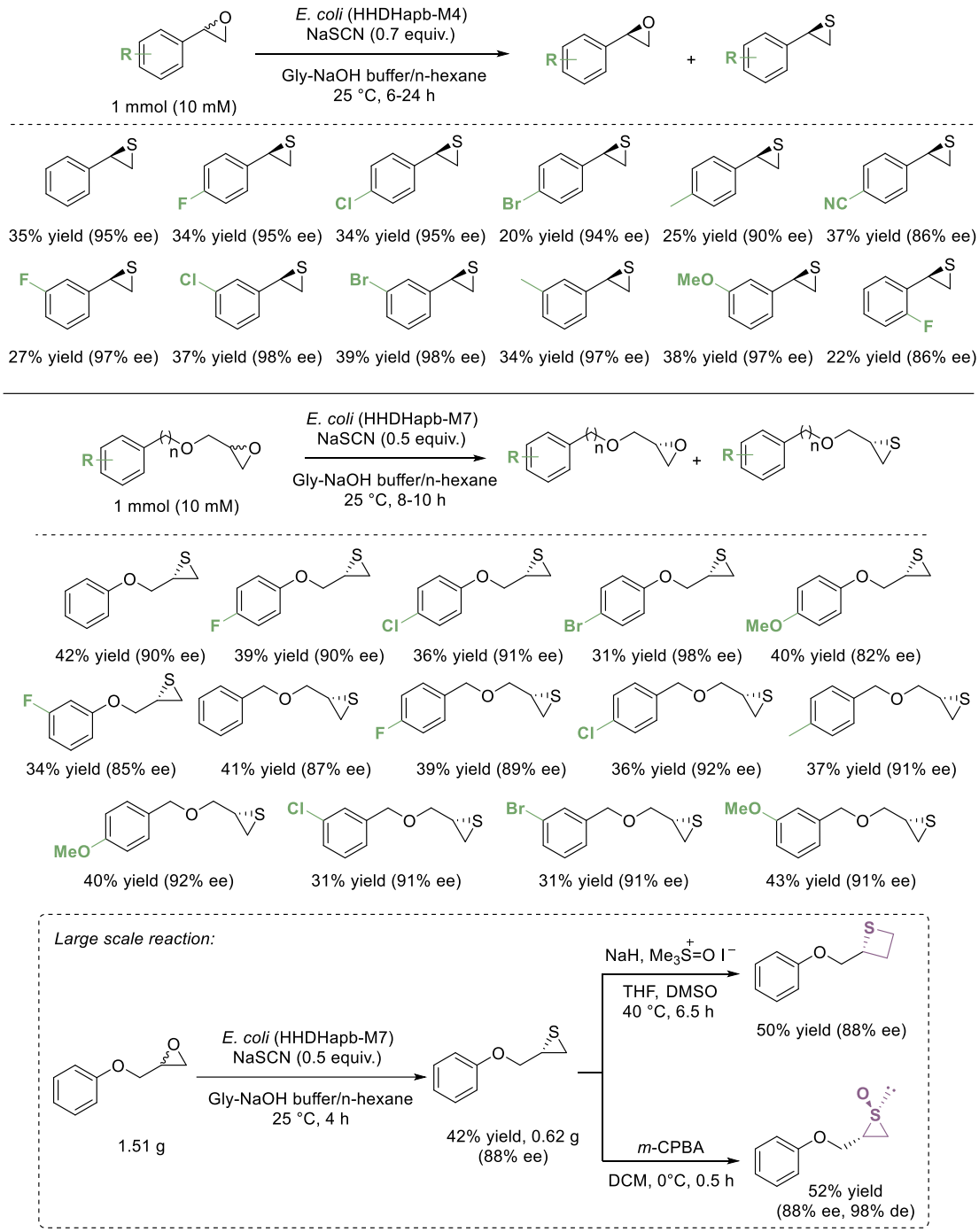


Fig. 26 Biocatalytic thionation of aryl- and alkyl-substituted epoxides

Solarczek *et al*. have explored the substrate scope of the two G-types HHDHs, HheG2 and a newly identified HheG3 with a variety of sterically demanding cyclic epoxides and a range of anionic nucleophiles such as azide, cyanide, cyanate, thiocyanate, formate, and nitrite.[60] Reactions proceeded with 9–70% yield and in 5–90% ee. Reactions with sodium thiocyanate

using HheG3 enzyme are shown in Fig. 27. Product thiocyanates were obtained as inseparable mixtures with isothiocyanates (resulting from rearrangement of thiocyanates).

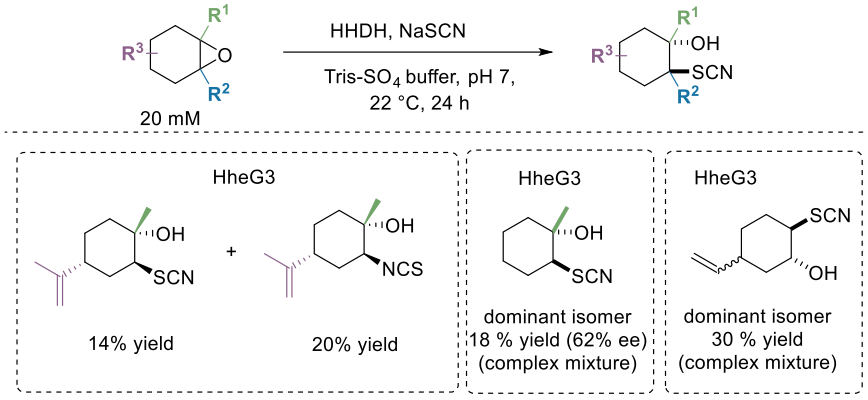


Fig. 27 Reactions with sodium thiocyanate using HheG3 enzyme

Building on earlier findings by Majerić Elenkov *et al.* that group B HHDHs are highly enantioselective toward sterically demanding 2,2-disubstituted epoxides with sodium azide,[61] Mehić *et al.* further demonstrated that HheB (*Corynebacterium sp.*) and HheB2 (*Mycobacterium sp.*) efficiently catalyze enantioselective epoxide ring-opening across diverse substrates and nucleophiles.[62] Reactions were performed on four sterically demanding substrates to obtain products with quaternary carbon stereocenter in high ee (94–99% ee) including thiocyanates in 23–36% yield and 80–98% yield (Fig. 28).

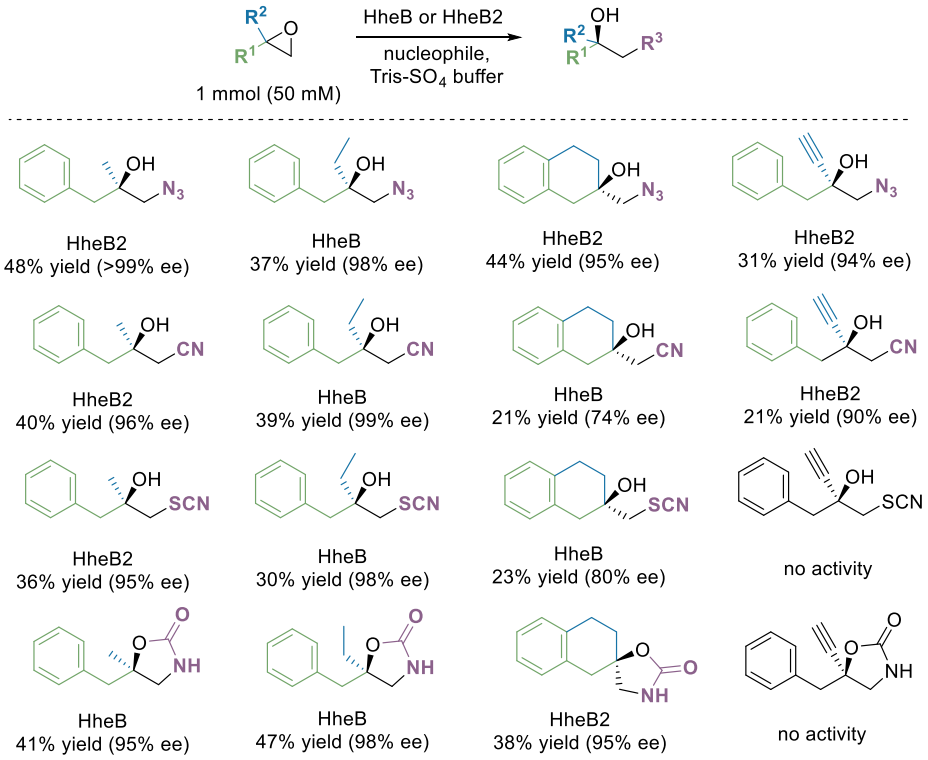


Fig. 28 HheB and HheB2-catalyzed epoxide ring-opening using different nucleophiles including NaSCN

2.5. Synthesis of nitroalcohols and diols

Epoxide ring-opening (ERO) reaction with nitrite ion can proceed via O- or N-attack on α- or β- carbon atom of the epoxide ring, forming four different isomers: two diastereomeric nitrite esters that spontaneously hydrolyse to diols; and two diastereomeric 1,2-nitroalcohols (Fig. 29)

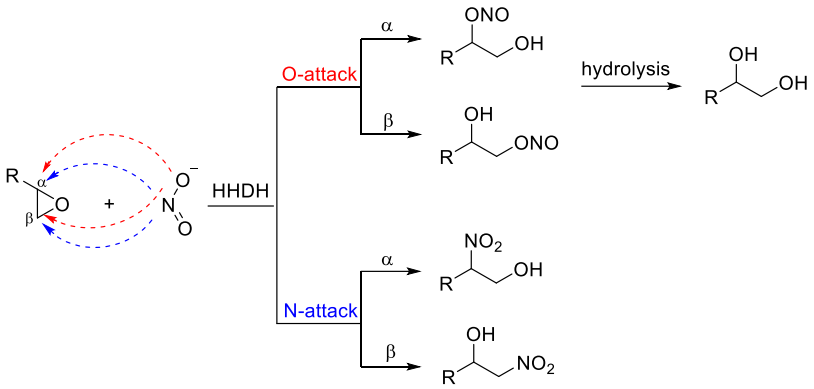


Fig. 29 Possible reaction pathways for ERO with nitrite [63]

Hasnaoui *et al.* were the first to investigate the use of the HheC from *Agrobacterium radiobacter* AD1 in the nitrite-mediated epoxide ring-opening.[64] The enzyme promoted the enantioselective β-regioselective conversion of *p*-nitrostyrene oxide in the presence of sodium nitrite, producing a hydroxynitrite intermediate via nucleophilic attack of nitrite oxygen. Because this intermediate is unstable under the reaction conditions, it undergoes spontaneous hydrolysis to the corresponding diol with *ee* >91%. Consequently, the HheC/nitrite system effectively performs a highly enantioselective epoxide hydrolysis. This catalytic property was subsequently exploited by Fuchs *et al.* in the enantiocomplementary chemoenzymatic synthesis of both (*R*)- and (*S*)-chromanemethanol, an important precursor for vitamin E derivatives (Fig. 30).[65]

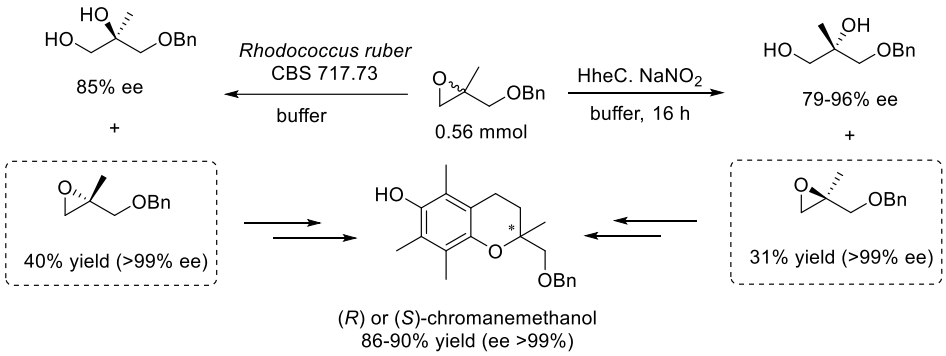


Fig. 30 Chemoenzymatic synthesis of (*R*) and (*S*)-chromanemethanol

According to the motif-based database mining methodology proposed by Schallmey's group, [14], Wang H. *et al*. discovered and used HHDHamb enzyme from *Acidimicrobiia bacterium,* that can work at lower pH and low temperatures, for the nitration of epoxides using $NaNO_2$. The reactions are chemo-, regio-, and enantioselective, and delivering enantiopure primary nitroalcohols in up to 41% isolated yield and >99 % ee.[63] HHDHamb was successfully

combined with styrene monooxygenase (SMO) in a two-enzyme biocatalytic cascade for one-pot synthesis of (*R*)-2-nitro-2-phenylethan-1-ol from the styrene. The obtained nitro alcohol was further transformed into amino alcohol and oxazolidinone (Fig. 31).

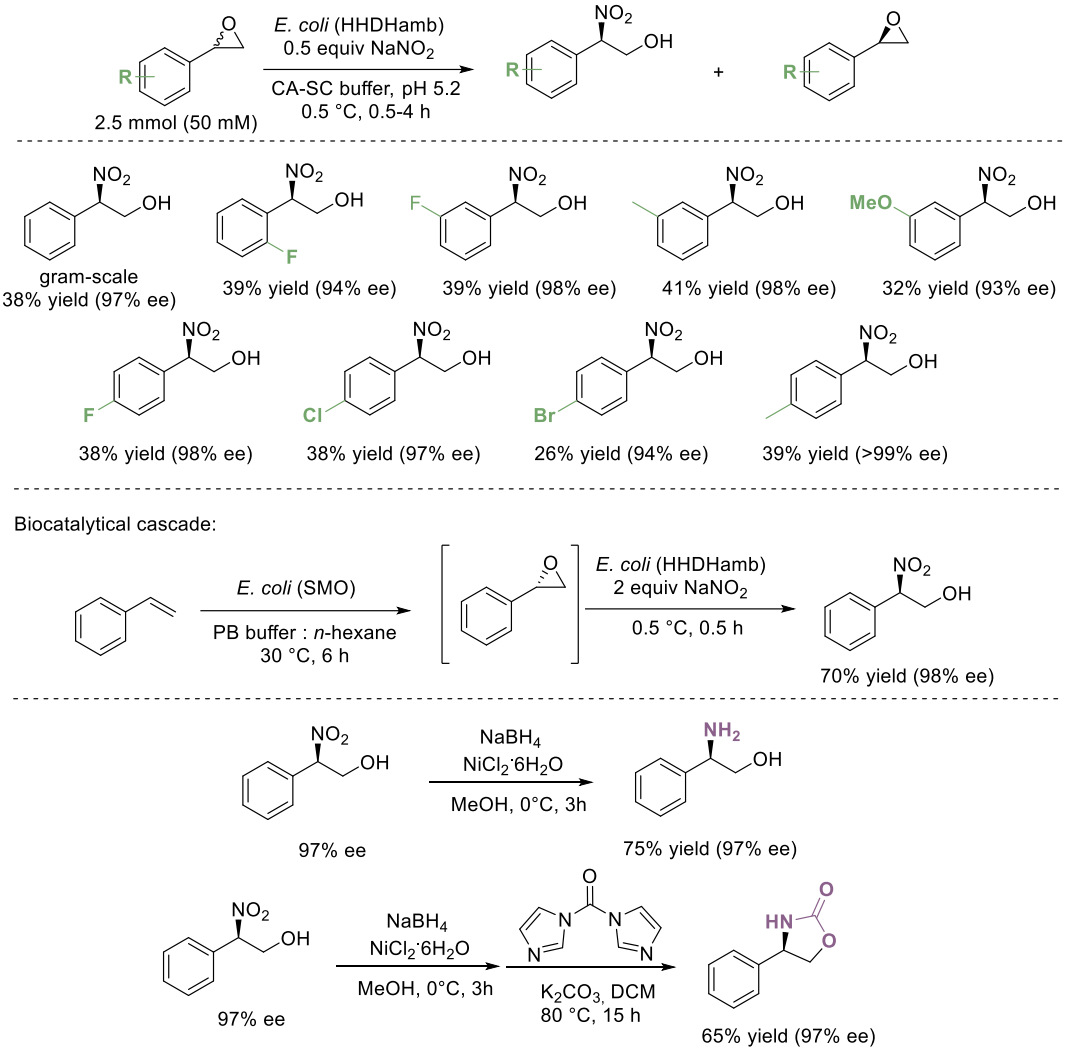


Fig. 31 HHDHamb-catalyzed nitration of epoxides

The same group of authors further engineered HHDHamb for nitration of various phenyl glycidyl ethers to the corresponding chiral β-nitroalcohols.[66] The chiral (*R*)- and (*S*)-1-nitro-3-phenoxypropan-2-ols were synthesized by the several enantiocomplementary HHDHamb variants, which showed good catalytic efficiency (up to 43% isolated yields) and high optical purity (up to >99% ee). The nitration of phenyl glycidyl ether was also performed at high substrate concentrations of up to 1000 mM. The applicability of the method was further demonstrated by the synthesis of both enantiomers of β-adrenergic blocker metoprolol from a corresponding β-nitroalcohol (Fig. 32).

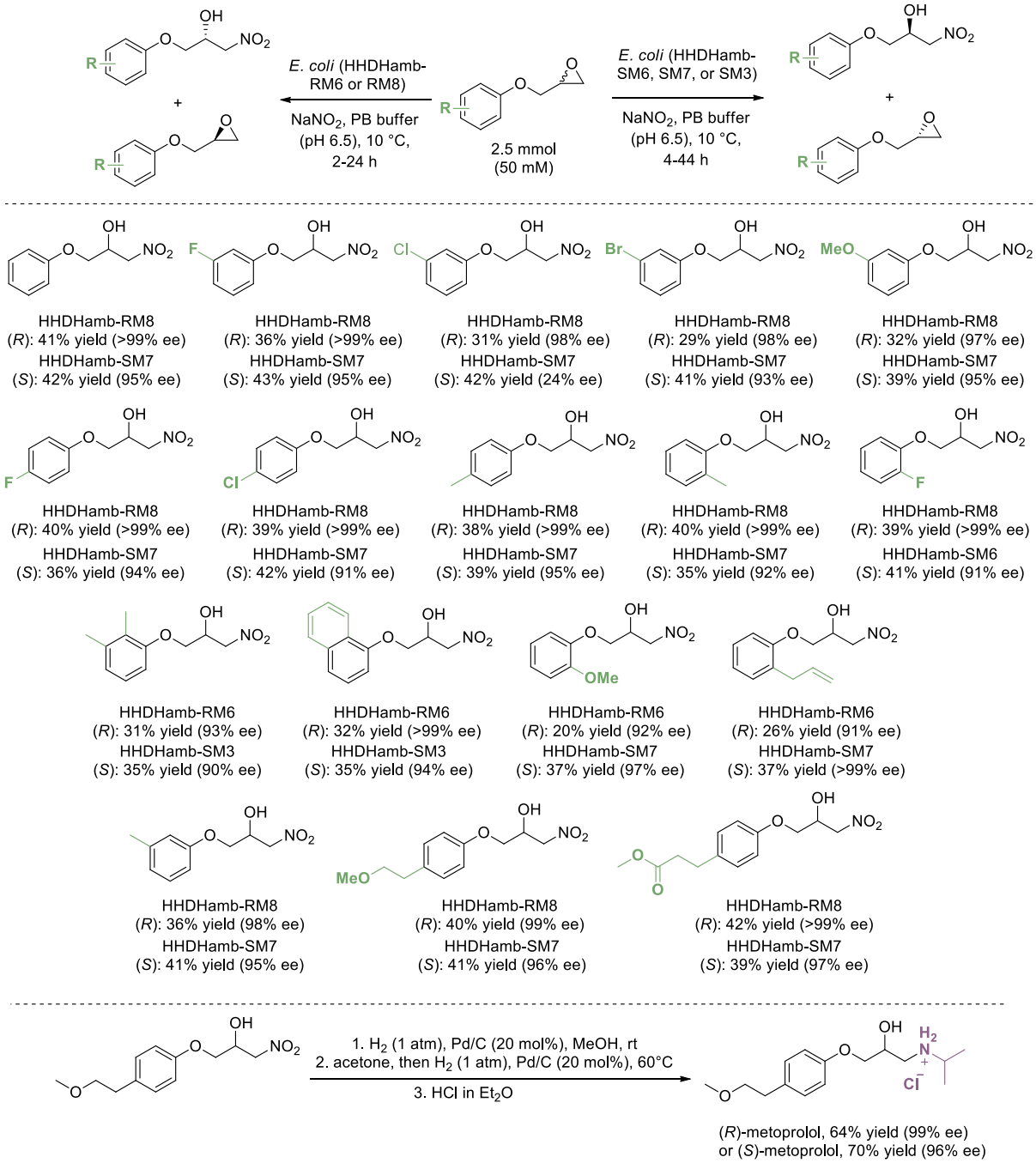


Fig. 32 HHDHamb-catalyzed nitration of various phenyl glycidyl ethers

Miao *et al.* engineered the HheC-M6 mutant (F12Y/P84T/F86W) for preparative-scale synthesis of enantiopure vicinal diols (up to 89% yield and 90% ee) from a broad range of racemic epoxides (Fig. 33) or β-haloalcohols (Fig. 34) via nitrite-mediated enantioconvergent hydrolysis.[67] Reactions involving β-haloalcohols are an example of a single-enzyme cascade reaction and are discussed in detail *vide infra*. Enantioconvergent catalysis enables the transformation of racemic substrates into a single enantiomeric product, thereby circumventing the inherent 50% theoretical yield limitation of kinetic resolution.[68] Structural and mechanistic insights revealed that the HheC-M6 mutant accommodates both enantiomers of the

substrate but steers them toward a single diol product through differential binding and transition-state stabilization.

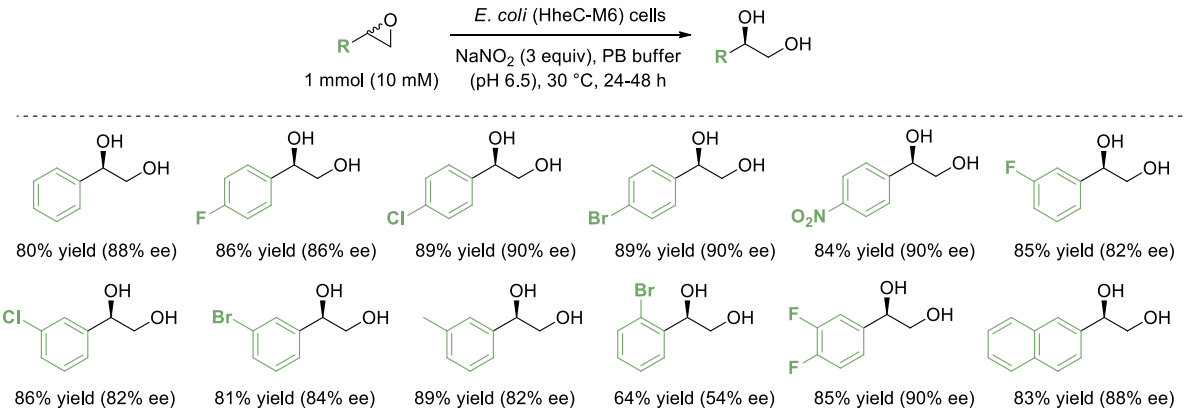


Fig. 33 Nitrite-mediated enantioconvergent hydrolysis of racemic epoxides

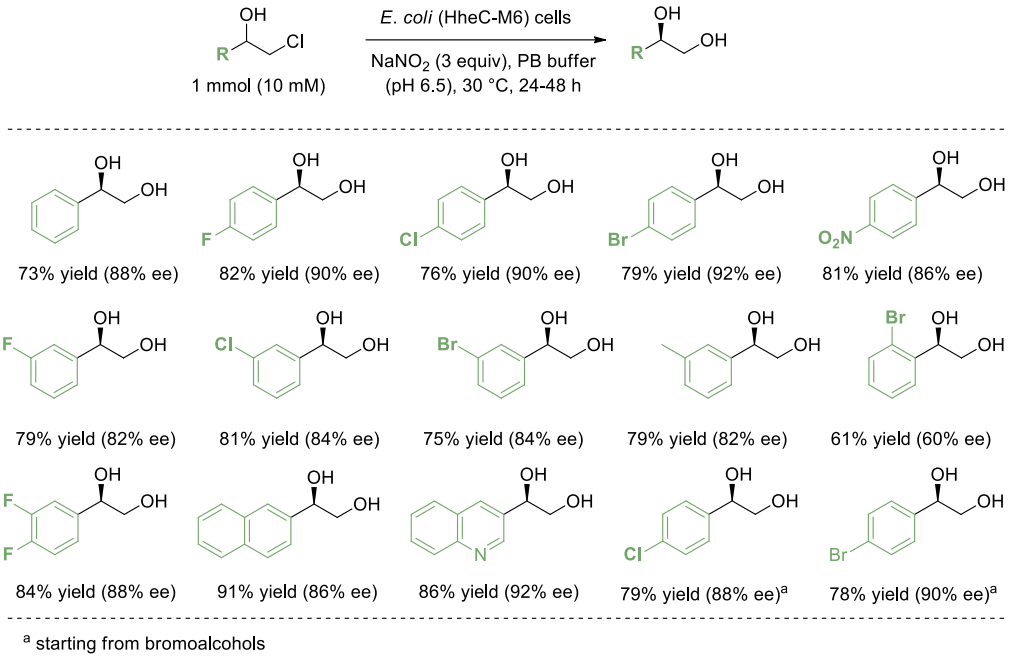


Fig. 34 Nitrite-mediated enantioconvergent synthesis of vicinal diols from of racemic β-haloalcohols

3. Dehalogenation of 1,2-haloalcohols

Although the natural function of HHDHs is the dehalogenation of vicinal haloalcohols to form epoxides, this reaction is rarely employed as a standalone transformation. The main reason is its reversible nature, which limits the effectiveness of the reaction unless the resulting epoxide is further converted through nucleophilic ring-opening, thereby shifting the reaction equilibrium toward product formation.

Haak *et al.* used HheC for enzymatic kinetic resolution of chloroalcohols, to obtain chiral 3-alkenyl and heteroaryl chloroalcohols in up to 47% yield and up to >99% ee (Fig. 35).[69] It is

presumed that the spontaneous hydrolysis of the formed epoxide pulls the equilibrium to the right side, thus ensuring excellent ee's of the remaining chloroalcohols. The applicability of this methodology was also shown on multigram scale, where toluene was employed as a co-solvent in a 1:1 ratio with Tris–SO_4 buffer.

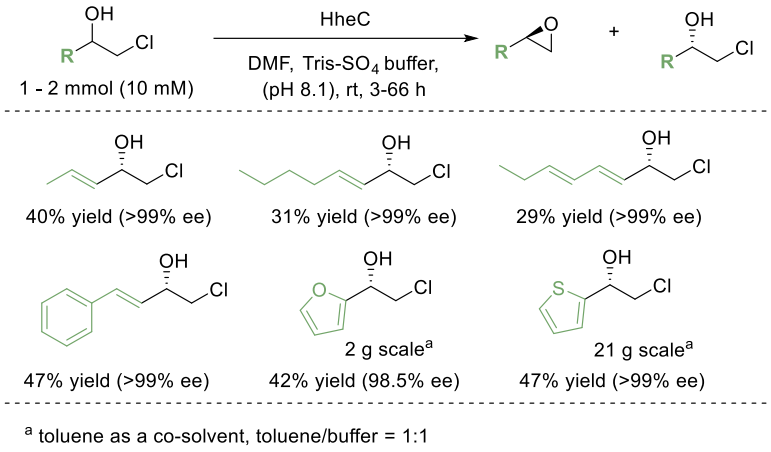


Fig. 35 HheC-catalyzed kinetic resolution of chloroalcohols

4. Cascade reactions

HHDHs can catalyse cascade reactions involving sequential ring-closure and ring-opening, as well as multienzyme cascades in combination with compatible biocatalysts, such as alcohol dehydrogenases or epoxide hydrolases. Combination with traditional chemical catalysts is also feasible. These cascades may be performed either simultaneously in a one-pot process or sequentially in separate reaction vessels. Compared with conventional multistep synthesis, cascade reactions eliminate the need for intermediate isolation and purification, improve overall process efficiency, and enable the rapid construction of molecular complexity in a single operation.

4.1. Cascade reactions using single enzyme

Dehalogenation of vicinal chloroalcohols in the presence of a nucleophile results in two consecutive reactions catalysed by a single HHDH. This unique property of HHDH enables one-step synthesis of β-substituted alcohols and oxazolidinones from chloroalcohols. The value of this approach is manifold. It eliminates the drawback of reversibility of dehalogenation reaction and incomplete conversion to epoxide, by shifting the equilibrium to the product side. Due to two sequential kinetic resolutions the product ee is higher compared to a single step. This strategy was first demonstrated by Nakamura *et. al.* on the synthesis of (*R*)-γ-chloro-β-hydroxybutyronitrile from 1,3-dichloro-2-propanol, as previously mentioned in section 2.1.[37]

Majerić Elenkov *et. al.* investigated a sequential kinetic resolution of methyl 4-chloro-3-hydroxybutanoate methylester catalyzed by HheC-W249F variant in the synthesis of (*S*)-methyl 4-cyano-3-hydroxybutanoate methyl ester in 40% yield (97% ee), and (*S*)-methyl 4-chloro-3-hydroxybutanoate methylester with 41% yield (95% ee) (Fig. 36). The reaction was carried out in aqueous solution under mild conditions and provided access to a useful statin side-chain building block.[70]

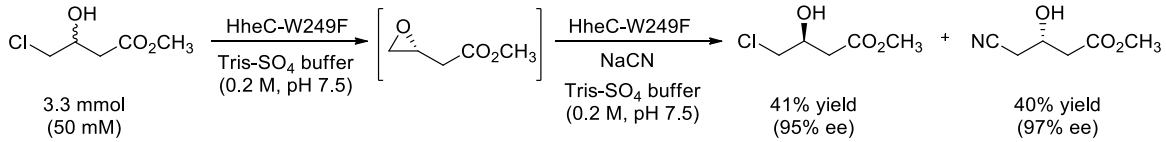


Fig. 36 Sequential kinetic resolution of methyl 4-chloro-3-hydroxybutanoate methylester

Wan *et al.* used the HheC-W249F variant for the synthesis of a key precursor of atorvastatin: (*S*)-4-chloro-3-hydroxybutyronitrile in one step, starting from prochiral 1,3-dichloro-2-propanol. Product was obtained in 86% yield with 97.5% ee (Fig. 37).[71]

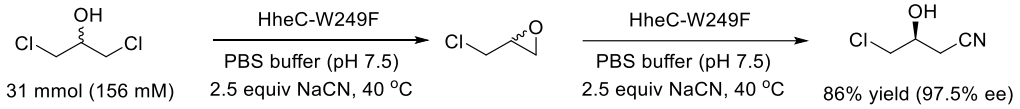


Fig. 37 Desymmetrization of 1,3-dichloro-2-propanol

Xu and Huang *et. al.* described a biocatalytic method for the stereodivergent synthesis of chiral epoxides and oxazolidinones via HHDH-catalyzed desymmetrization reactions.[72] Stereocomplementary HHDHs enabled the desymmetrization of 2-substituted-1,3-dichloro-2-propanols, providing epoxides in yields of up to 95% and with enantiomeric excesses of up to 99% via a single-step dehalogenation reaction (Fig. 38). The same approach also enabled the synthesis of chiral oxazolidinones in up to 88% yield and >99% ee via a tandem dehalogenation–cyanate-mediated epoxide ring-opening process (Fig. 39). The practicality of this biocatalytic desymmetrization strategy was further demonstrated by gram-scale reactions and subsequent derivatization of the products.

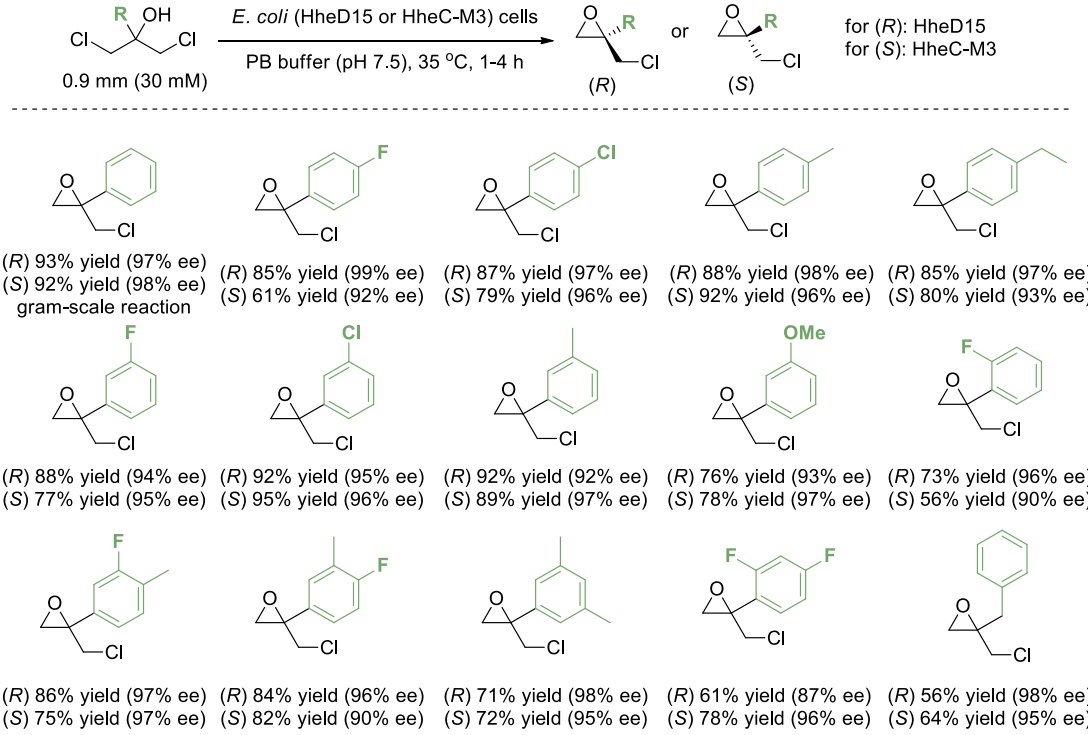


Fig. 38 Desymmetrization of 2-substituted-1,3-dichloro-2-propanols

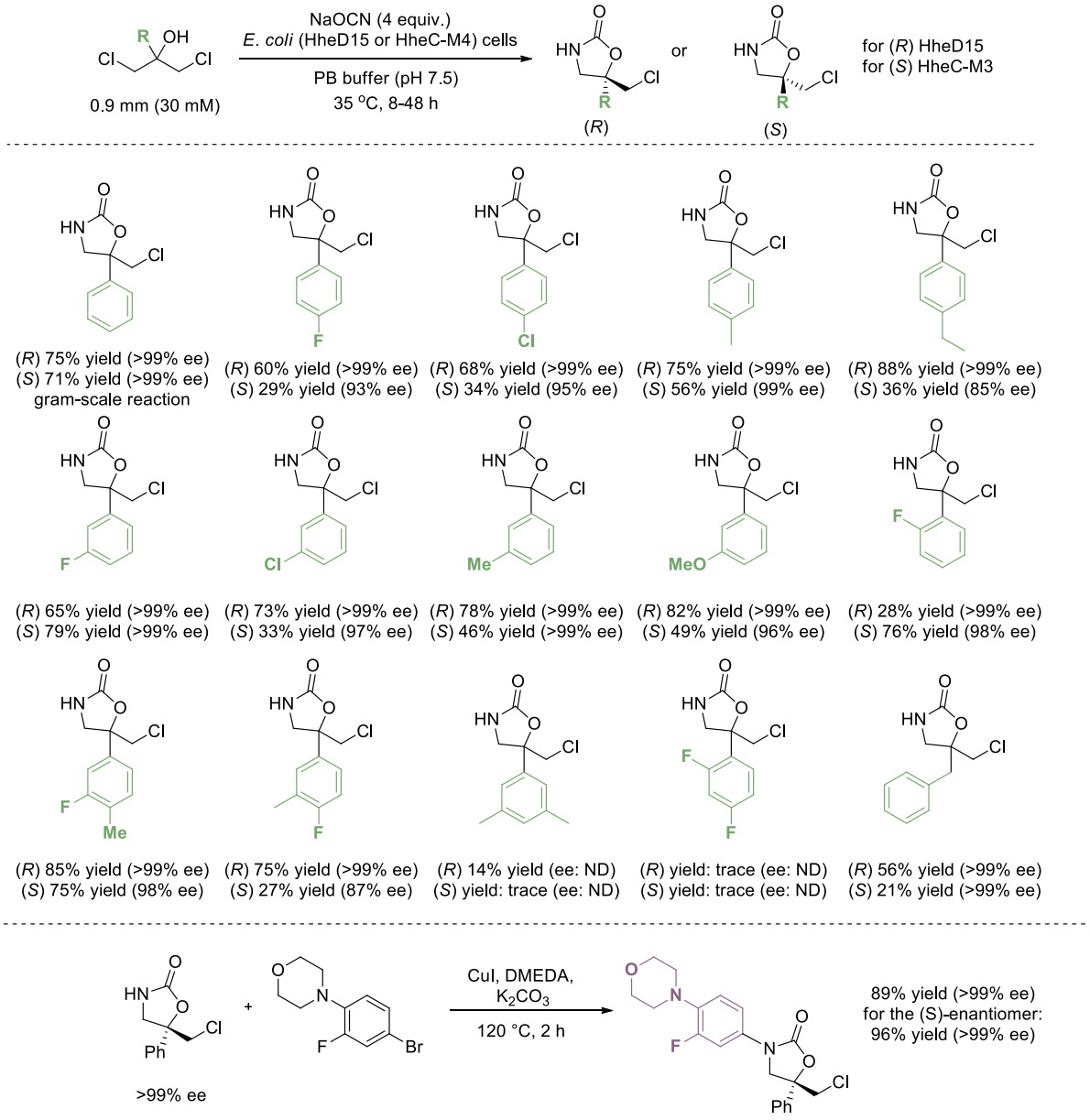


Fig. 39 Dehalogenation of 2-substituted-1,3-dichloro-2-propanols followed by cyanate-mediated epoxide ring-opening

4.2. Cascade reactions using multiple enzymes

Compared to single-enzyme systems, multi-enzyme catalysis enables complex reaction cascades and eliminates the requirement for isolation and purification of intermediates.

Ma *et al.* reported a biocatalytic route to an important intermediate used in the industrial production of atorvastatin, combining ketoreductase- and halohydrin dehalogenase-catalyzed transformations in a two-step, three-enzyme cascade.[73] In the first step, ethyl 4-chloroacetoacetate was stereoselectively reduced by a ketoreductase (KRED), while NADPH regeneration was achieved using glucose dehydrogenase (GDH) and glucose, affording ethyl (*S*)-4-chloro-3-hydroxybutyrate in 96% isolated yield and >99.5% ee. The second step employed an engineered HHDH to replace the chlorine substituent with a cyano group using in

situ-generated HCN, yielding ethyl (*R*)-4-cyano-3-hydroxybutanoate in 93% isolated yield and >99.5% ee (Fig. 40).

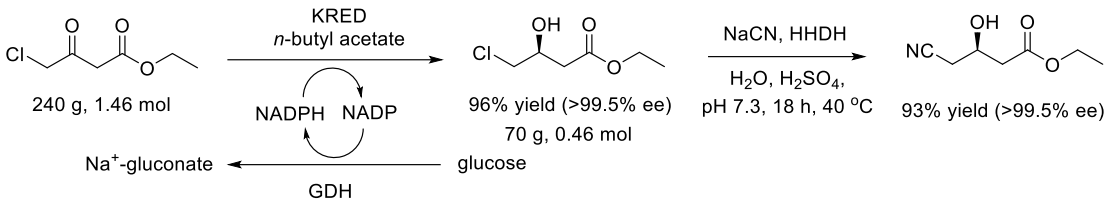


Fig. 40 Three-enzyme process for the synthesis of (*R*)-4-cyano-3-hydroxybutanoate

Seisser *et al.* and subsequently, Schrittwieser *et al.* developed an enzymatic cascade for the preparation of enantiomerically pure β-azido alcohols and β-hydroxynitriles starting from prochiral α-chloroketones. [74-75] In this strategy, the stereogenic center was established during the initial alcohol dehydrogenase (ADH)-catalyzed reduction, affording optically pure chloroalcohol intermediates. These intermediates were then converted *in situ* into epoxides, which underwent HheC- or HheB-catalyzed nucleophilic ring opening with azide or cyanide to furnish the desired products in 38–61% yield (Fig. 41). Because the HHDHs used in the second stage are essentially non-enantioselective, the overall stereochemical outcome was determined by the choice of ADH in the first step, allowing straightforward access to either product enantiomer with excellent optical purity

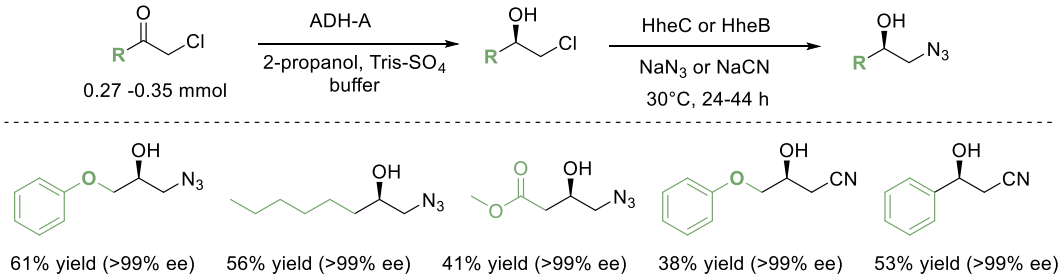


Fig. 41 Two-enzyme cascade reaction for the synthesis of β-azido alcohols and β-hydroxynitriles

Chen *et al.* designed a whole-cell biocatalytic system for the synthesis of enantiomerically pure β-hydroxynitriles from prochiral α-haloketones by co-expressing alcohol dehydrogenases, cofactor-regenerating enzymes, and a HHDH in *Escherichia coli*.[76] Two recombinant strains, Eco RBH and Eco SFH, were constructed to incorporate alcohol dehydrogenases with complementary stereoselectivities. As a result, the cascade enabled the selective preparation of either (*R*)- or (*S*)-β-hydroxynitriles with high enantiomeric purity, depending on the engineered strain employed (Fig. 42).

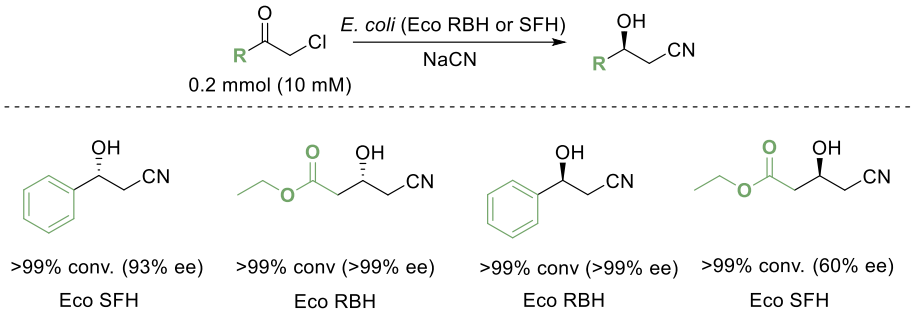


Fig. 42 Cascade reaction using *E. coli* cells with two co-expressed enzymes (Eco RBH or Eco SFH)

Jin *et al.* developed a two-step biocatalytic process for the synthesis of (*R*)-epichlorohydrin from 1,3-dichloro-2-propanol using immobilized recombinant *Escherichia coli* cells expressing a HHDH and an epoxide hydrolase.[77] The cascade was carried out in a specially designed two-reactor system, where cyclohexane served as a co-solvent during the initial HHDH-catalyzed dehalogenation to suppress the reverse reaction and minimize non-enzymatic hydrolysis of the epoxide intermediate. The resulting reaction mixture was subsequently subjected to an epoxide hydrolase-catalyzed kinetic resolution, affording (*R*)-epichlorohydrin in 26% yield with 99% ee (Fig. 43). Building on this concept, Xue *et al.* integrated both enzymatic activities into whole-cell catalysts co-expressing the HheC P175S/W249P variant and an epoxide hydrolase.[78] This cascade enabled the efficient production of (*S*)-epichlorohydrin, in up to 91% yield and 99% ee.

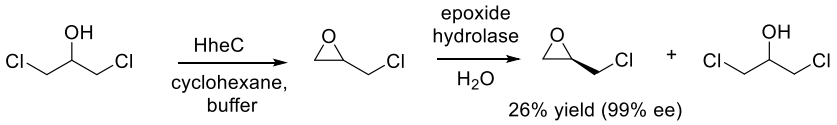


Fig 43. Synthesis of (*R*)-epichlorohydrin

Yao *et al.* developed a bienzymatic cascade for the synthesis of ethyl (*R*)-3-hydroxyglutarate (EHG) from ethyl (*S*)-4-chloro-3-hydroxybutyrate (ECHB) using recombinant *Escherichia coli* strains expressing either separate or co-expressed HHDH and nitrilase enzymes.[79] In this system, the HHDH variant from *Agrobacterium radiobacter* AD1 catalyzed the formation of the nitrile intermediate, which was subsequently hydrolyzed by the *Arabidopsis thaliana* nitrilase. Because elevated concentrations of both substrate and cyanide negatively affected nitrilase activity, substrate and NaCN were supplied by fed-batch addition to maintain efficient catalyst performance. Under the optimized reaction conditions, the cascade converted 1.2 M substrate to ethyl (*R*)-3-hydroxyglutarate in 87% yield.

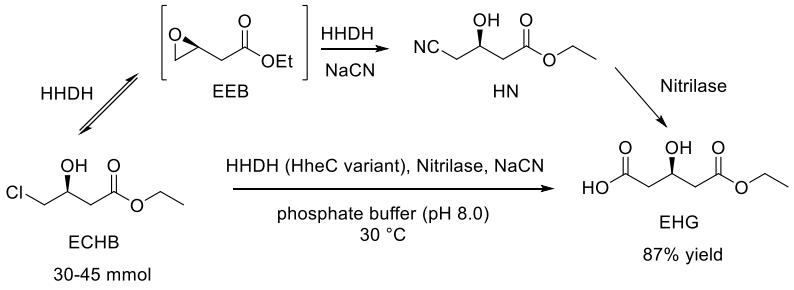


Fig 44. Synthesis of ethyl (*R*)-3-hydroxyglutarate (EHG)

Bogale *et al.* developed an integrated biocatalytic process that combined the haloalkane dehalogenase DhaA31 with the HheC to convert the environmental pollutant 1,2,3-trichloropropane (TCP) into enantiomerically pure (*S*)-2,3-dichloro-1-propanol (2,3-DCP).[80] During process optimization, the authors identified product inhibition arising from a cyclic reaction between epichlorohydrin (ECH) and 1,3-dichloro-2-propanol (1,3-DCP), which compromised the efficiency of the HheC-catalyzed kinetic resolution. This limitation was overcome by introducing an HZD-9 resin for *in situ* removal of the inhibitory product. Under the optimized conditions, complete conversion of TCP was achieved, affording (*S*)-2,3-DCP in 40% yield with an enantiomeric excess exceeding 99% (Fig. 45). The resin-assisted process was further demonstrated in a 0.5 L batch reactor and retained 64% of its initial activity after six consecutive cycles, highlighting its potential for the simultaneous remediation of chlorinated waste streams and production of valuable chiral intermediates.

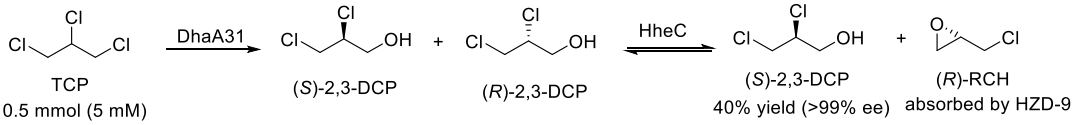


Fig. 45 Synthesis of 2,3-dichloro-1-propanol (2,3-DCP) from pollutant 1,2,3-trichloropropane (TCP)

Following the identification of the highly (*S*)-enantioselective HheA10 from *Tsukamurella* sp. 1534,[81] Cui *et al.* incorporated this enzyme into a one-pot biocatalytic cascade for the asymmetric synthesis of β-haloalcohols from halohydrocarbons.[82] The process combined $P450_{PL2}$-4-catalyzed hydroxylation with the stereoselective dehalogenation activity of HheA10, enabling the preparation of optically active β-haloalcohols with excellent enantiomeric purities (98–99% ee) (Fig. 46). This study illustrates how enzymes with complementary catalytic functions can be integrated into cascade systems to achieve efficient asymmetric synthesis from simple starting materials.

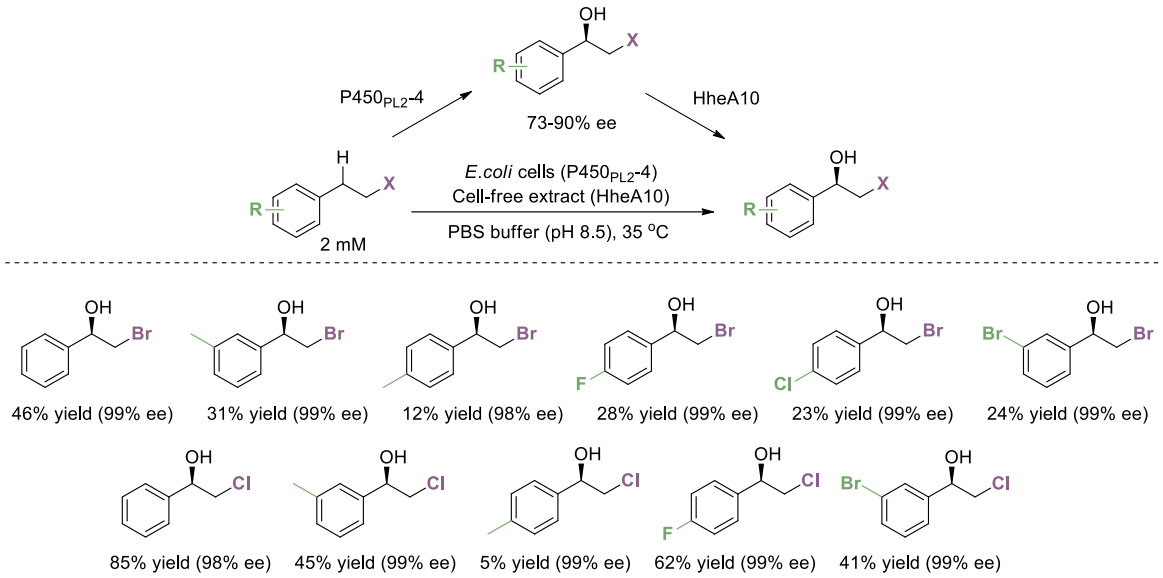


Fig. 46 Cascade reaction for the synthesis of β-haloalcohols from halohydrocarbons

Zhou *et al.* reported an enzymatic cascade that combines styrene monooxygenase (SMO) with the HheG to convert styrene derivatives into enantiomerically enriched oxazolidinones.[83] In this one-pot process, SMO first generated the corresponding epoxide intermediate, which subsequently underwent HheG-catalyzed ring opening with cyanate to furnish the target oxazolidinones. The cascade provided the desired products with excellent regio- and stereoselectivity, affording yields of up to 70% and enantiomeric excesses exceeding 99% (Fig. 47).

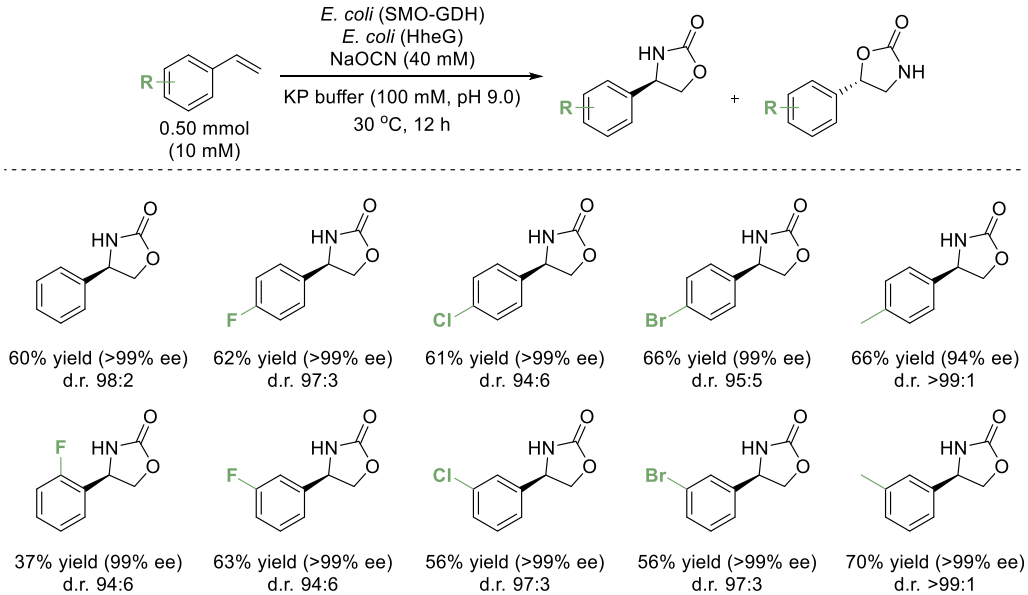


Fig. 47 Aminohydroxylation of styrenes for the synthesis of oxazolidinones

Wu *et al.* established a biocatalytic cascade for the stereoselective hydroxyazidation of alkenes by integrating styrene monooxygenase (SMO) with HHDHs.[84] In this approach, SMO first

converted the alkene substrates into the corresponding chiral epoxides, which subsequently underwent regioselective azide-mediated ring opening catalyzed by either engineered HheCM variant (P84V/F86P/T134A/N176A)[26] or HheG. Selection of the HHDH determined the regioisomer formed, enabling the synthesis of either primary or secondary 1,2-azidoalcohols in yields of up to 97% and with enantiomeric excesses exceeding 99% ee (Fig. 48). The authors further demonstrated the synthetic utility of this cascade by coupling it with a Cu-catalyzed azide–alkyne cycloaddition, providing a one-pot chemoenzymatic route to chiral β-hydroxytriazoles from simple alkene precursors (Fig. 49).

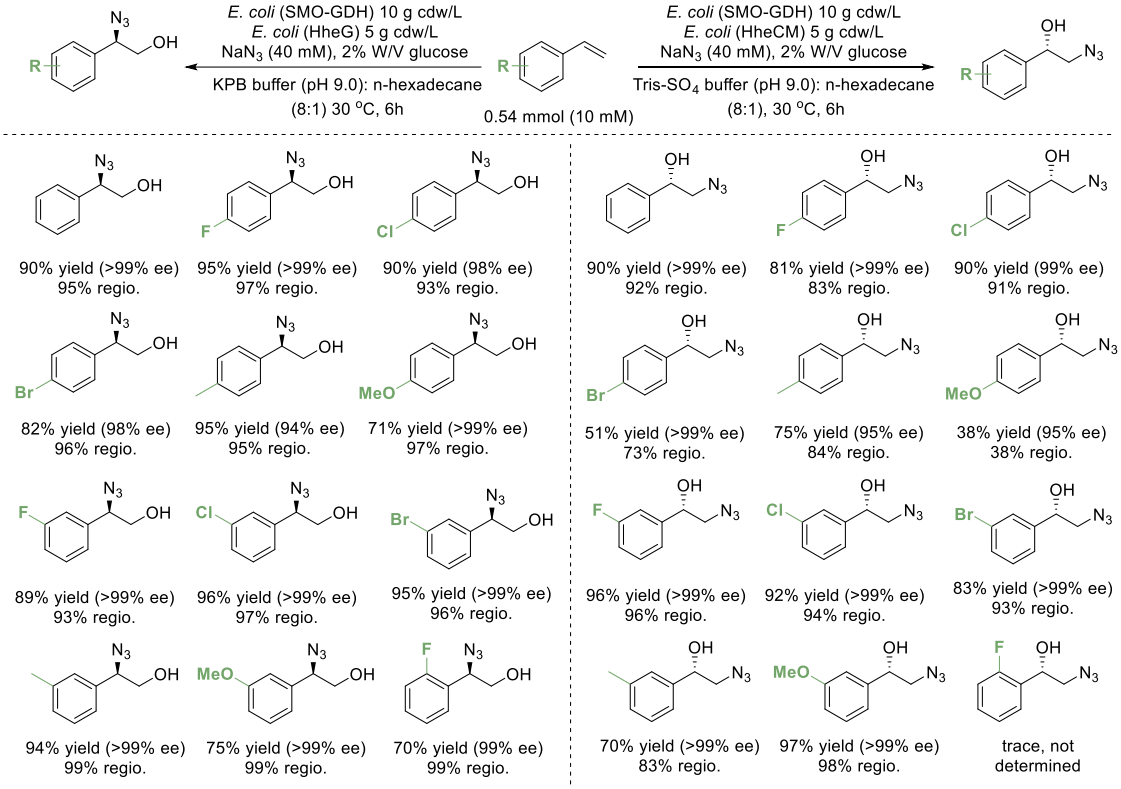


Fig. 48 Dual-enzyme cascade strategy for hydroxyazidation of alkenes

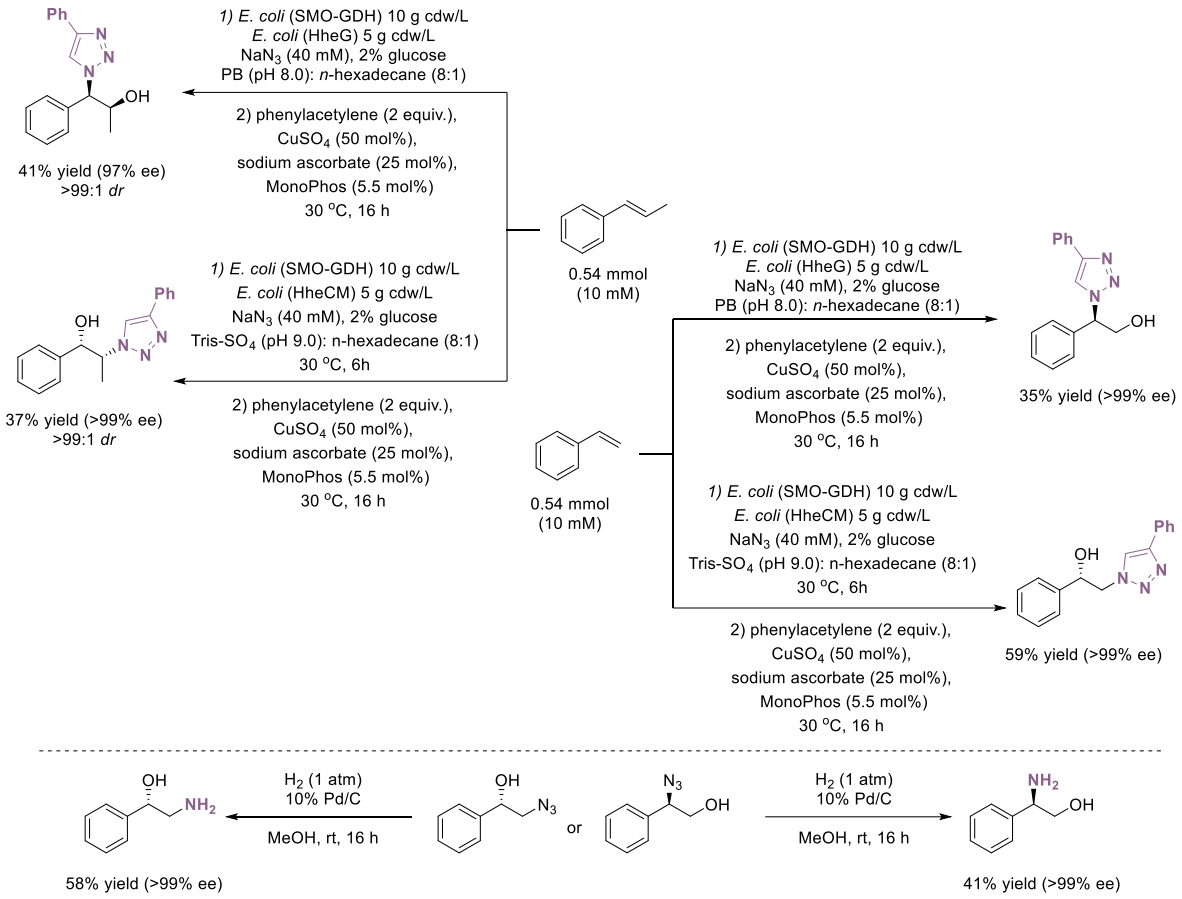


Fig. 49 One-pot chemo-enzymatic route to chiral β-hydroxytriazoles from alkenes

Miao *et al.* introduced a dual-enzyme cascade for the enantiocomplementary synthesis of chiral 3-hydroxy-3-hydroxymethyloxindoles by combining the complementary activities of HHDH and epoxide hydrolase.[85] In this strategy, the HheC-SM8 (T134S) variant selectively resolved racemic spiroepoxyoxindoles through nitrite-mediated ring opening, while the remaining epoxide enantiomer was converted by an epoxide hydrolase via enantiospecific hydrolysis. The coordinated action of both enzymes enabled the efficient preparation of either (*R*)- or (*S*)-3-hydroxy-3-hydroxymethyloxindoles, affording isolated yields of up to 50% with enantiomeric excesses exceeding 99%.

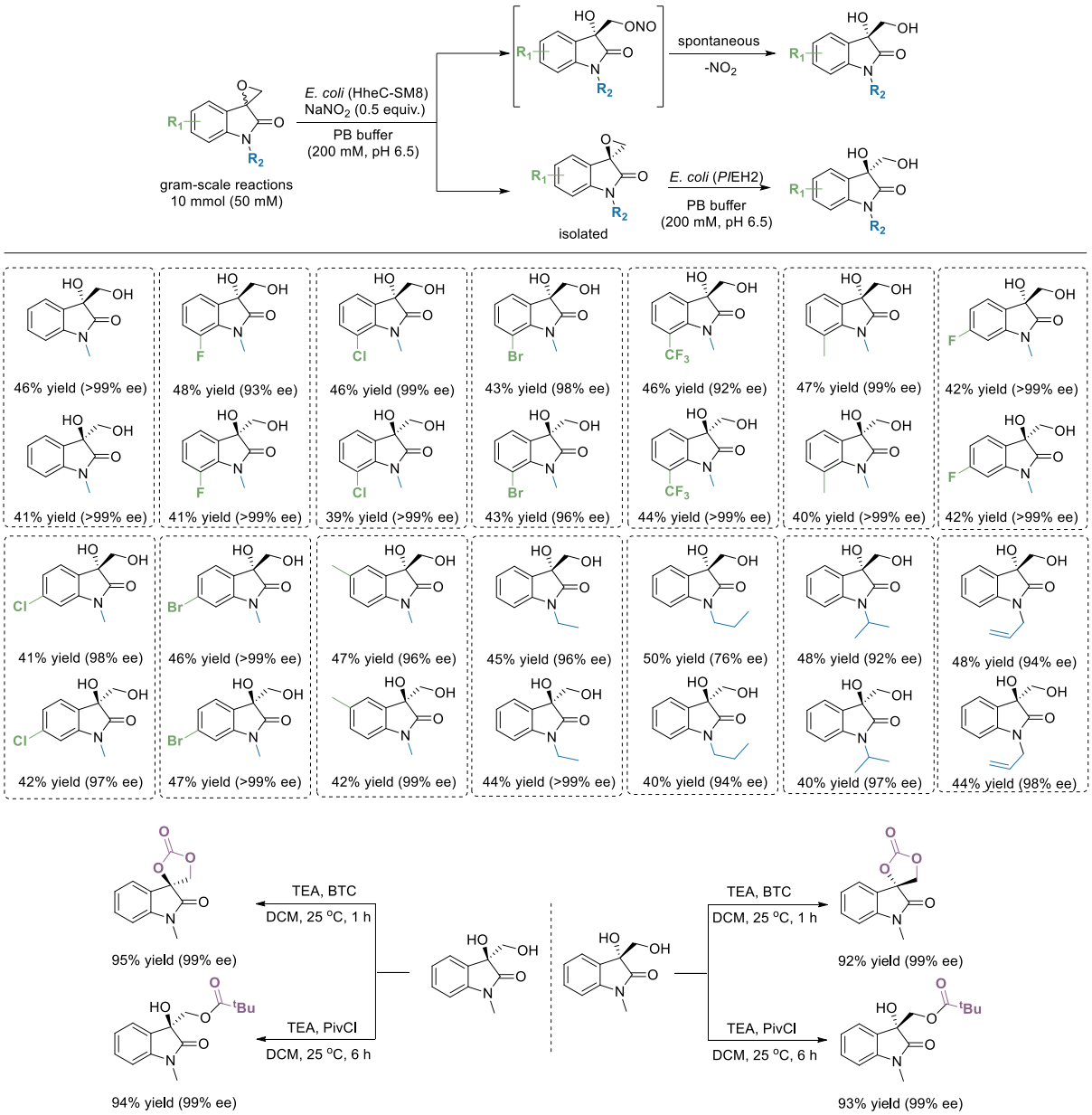


Fig. 50 Enantiocomplementary synthesis of chiral 3-hydroxy-3-hydroxymethyloxindoles

Fu *et al.* developed a biocatalytic one-pot cascade for the synthesis of both enantiomers of 3-hydroxy-γ-butyrolactone starting from prochiral 1,3-dichloropropanol.[86] The cascade comprises stereoselective HHDH-catalyzed dehalogenation–cyanation followed by non-stereoselective nitrilase-catalyzed hydrolysis–cyclization. TMSCN was used as a cyanide source. Two HHDH variants were engineered: *S*-selective HHDHn3–SM2 (N179M/I181V) and *R*-selective HheD15-RM3 (N161A/F169Y). Nitrilase NIT4 variant was found to have excellent catalytic performance with complete substrate conversion. Two-cell biocascade systems, SM2/NIT4 and RM3/NIT, were constructed. However, a single-cell system coexpressing both enzymes proved more efficient than a two-cell cascade, providing higher yields while requiring less biocatalyst. The synthesis of (*S*)-3-hydroxy-γ-butyrolactone was performed on a 1 g scale, using a single-cell *E. coli* (SM2-NIT4) in 80% yield and with >99% ee.

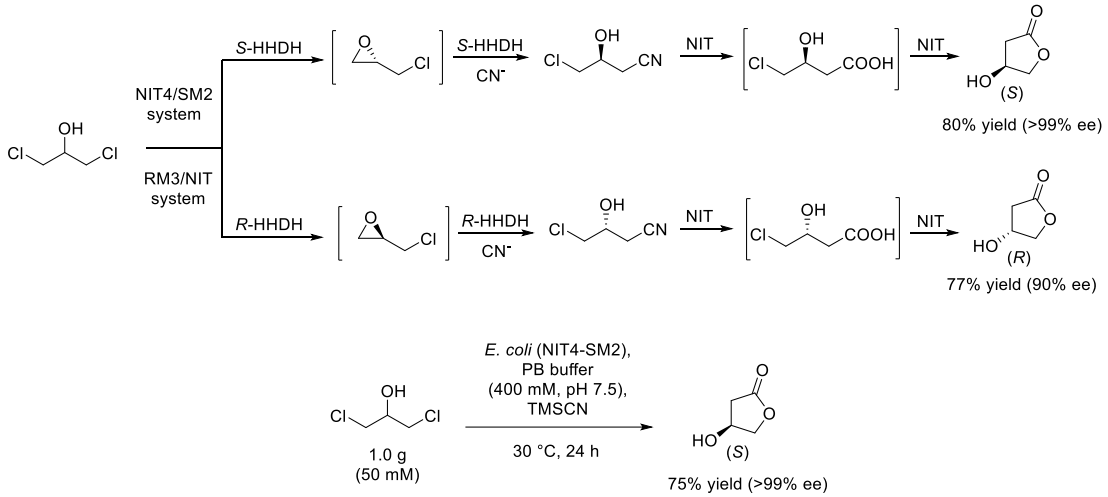


Fig 51. Biocatalytic cascade for the synthesis of (*S*) and (*R*)-3-hydroxy-γ-butyrolactone

4.3. Chemo-enzymatic cascade reactions

A chemo-enzymatic cascade reaction combines traditional chemical catalysts (like transition metals or organocatalysts) with enzymes in a single sequence to perform complex, multi-step syntheses in one pot, minimizing waste, lowering costs, and improving stereochemical control compared to running each step separately.[87]

Building on the pioneering work of Campbell-Verduyn *et al.*, who combined HheC-C153S-catalyzed enantioselective epoxide ring opening with a Cu(I)-catalyzed azide–alkyne cycloaddition in a one-pot process,[88] Szymański *et al.* further expanded this concept by developing a four-step cascade for the synthesis of enantiopure β-hydroxytriazoles from readily available α-haloketones.[89] To accomplish this, recombinant designer cells were constructed that co-expressed alcohol dehydrogenases and HHDHs with complementary stereochemical preferences. CT cells contained the R-selective AdhT and HheC, whereas BL cells expressed the S-selective AdhL together with HheBGP1. By selecting the appropriate whole-cell catalyst, both enantiomers of the target β-hydroxytriazoles could be obtained with excellent enantiomeric purity (Fig. 52).

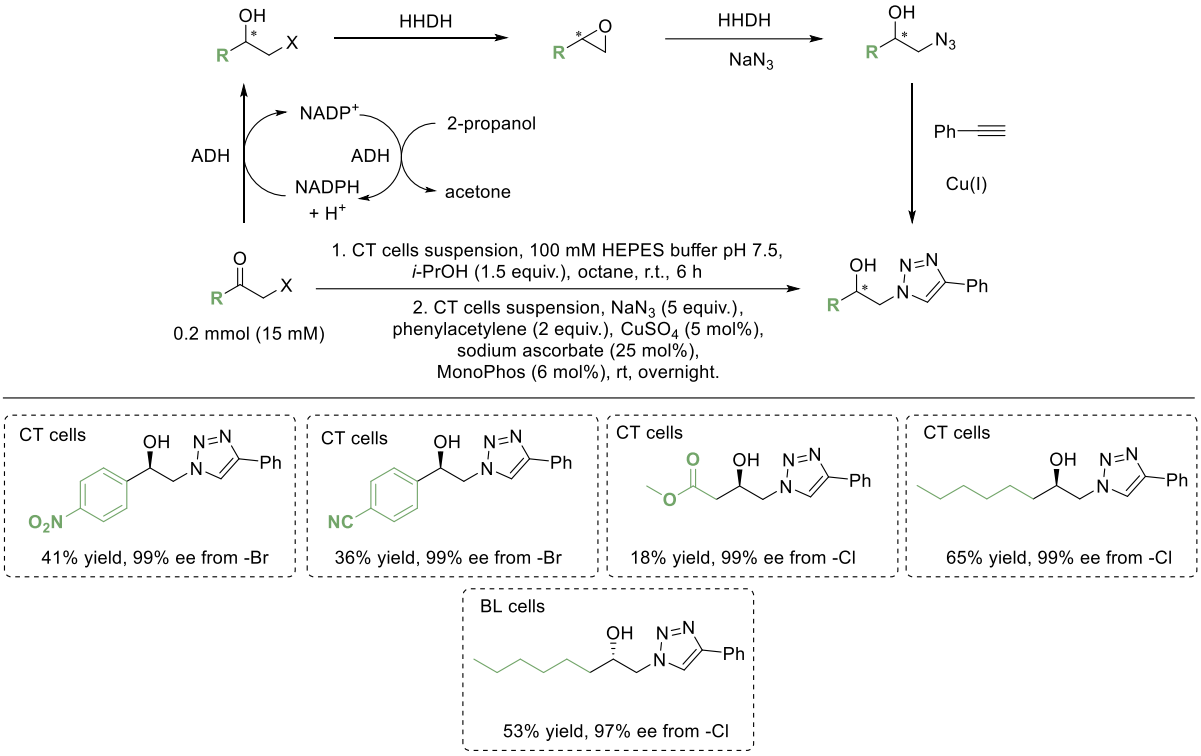


Fig. 52 Preparation of β-hydroxytriazoles from α-haloketones

Calderini *et al.* compared two complementary cascade strategies for the synthesis of chiral aliphatic non-terminal azido alcohols, both relying on HHDH-catalyzed regioselective epoxide ring opening but differing in the method used to generate the chiral epoxide intermediate.[90-91] One approach employed the biocatalytic styrene monooxygenase StyAB, whereas the other used the Shi asymmetric epoxidation catalyst. In both cases, the resulting epoxides were converted to the corresponding azido alcohols by HHDH-catalyzed nucleophilic ring opening, establishing two stereogenic centers in a single sequence. The StyAB–HHDH cascade afforded products with excellent regioselectivity and enantiomeric purity (up to 99% ee), while the chemoenzymatic Shi–HHDH approach provided more moderate enantioselectivities of 56–61% ee (Fig. 53).

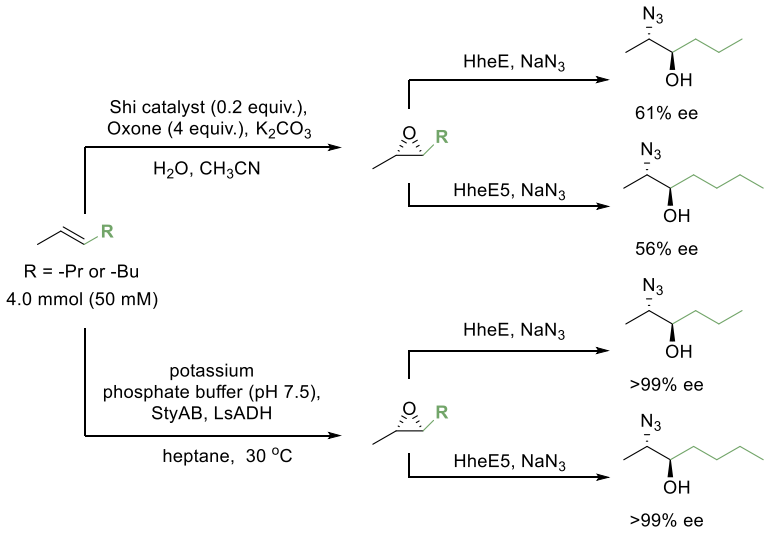


Fig. 53 Two (chemo)-enzymatic cascades for the synthesis of non-terminal azido alcohols

Tian *et al.* employed directed evolution to generate two HheG variants with complementary stereopreferences, GZ-2 (Y18G/N198L/F200W) and GZ-1 (M14L/Y154T/I104V/N196A/T195G/S207F), for the asymmetric ring opening of epoxides with azide ion.[92] The engineered enzymes were integrated into a two-step, one-pot cascade in which the HHDH-catalyzed transformation was followed by a Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC). This approach enabled the synthesis of a range of optically active triazoles in 32–54% yield with enantiomeric excesses of up to >99% ee (Fig. 54).

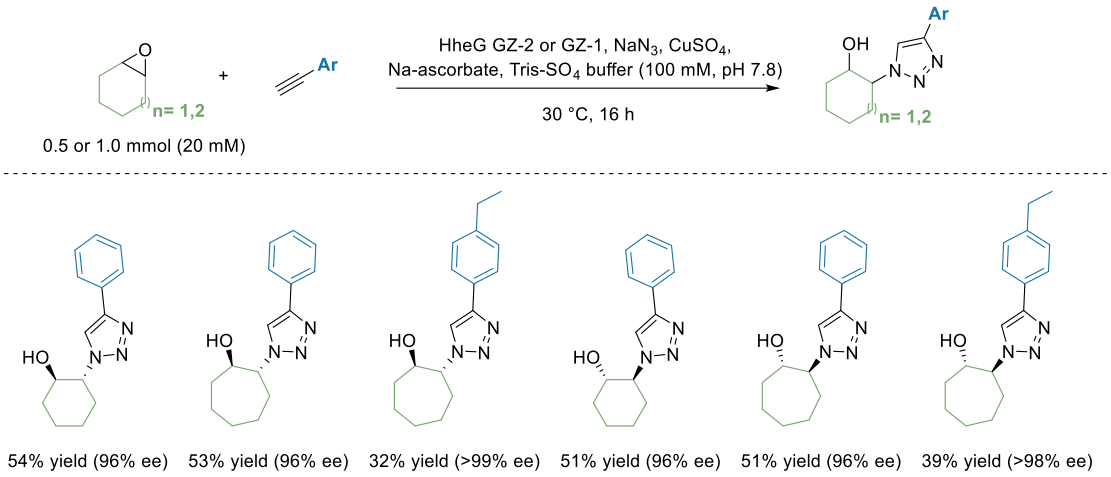


Fig. 54 ERO with azide followed by CuAAC reaction for the synthesis of triazoles

5. Dynamic kinetic resolution strategy

The quantitative conversion of racemic mixtures into enantiomerically pure compounds remains a significant challenge in asymmetric synthesis. Dynamic kinetic resolution (DKR) is one strategy that can be employed for deracemization.[93] This approach combines kinetic resolution (KR) with the continuous *in situ* racemization of the less reactive substrate enantiomer. Effective DKR processes require mild racemization conditions, which can be achieved using either an (organo)metal catalyst or an enzyme.

Haak *et al.* developed a chemo-enzymatic DKR for racemic β-haloalcohols, yielding the corresponding enantioenriched epoxides in a one-pot process in 51–67% yield and 90–99% ee (Fig. 55).[94] The HheC variant C153S/W249F catalyzes the asymmetric ring-closure step, while the residual haloalcohol enantiomer is continuously racemized by an iridacycle catalyst, which is among the most efficient catalysts reported for the racemization of β-haloalcohols.

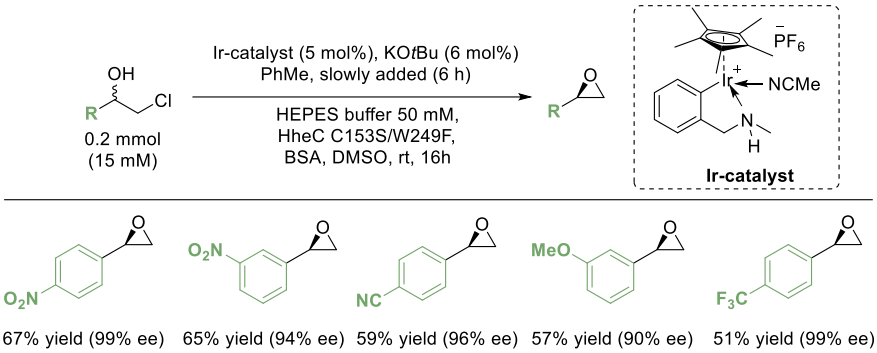


Fig. 55 Chemo-enzymatic DKR of racemic β-haloalcohols

Mikleušević *et al.* demonstrated a highly efficient and simple DKR protocol for the preparation of oxazolidinones using a single enzyme.[95] DKR is achieved through HheC-catalyzed enantioselective ring opening of a racemic epoxide with sodium cyanate (NaOCN) in the presence of catalytic amounts of bromide ions. The bromide ions promote *in situ* racemization of the epoxide, thereby enabling DKR; in their absence, the reaction proceeds as a conventional kinetic resolution. Under these conditions, highly enantioenriched 5-substituted 2-oxazolidinones were obtained. The DKR was carried out on a 0.5 g scale using rac-2-bromomethyl-2-methyloxirane and a cell-free extract containing HheC. Complete substrate conversion was achieved within 5 h, and the desired product was isolated in 87% yield with >99% ee (Fig. 56).

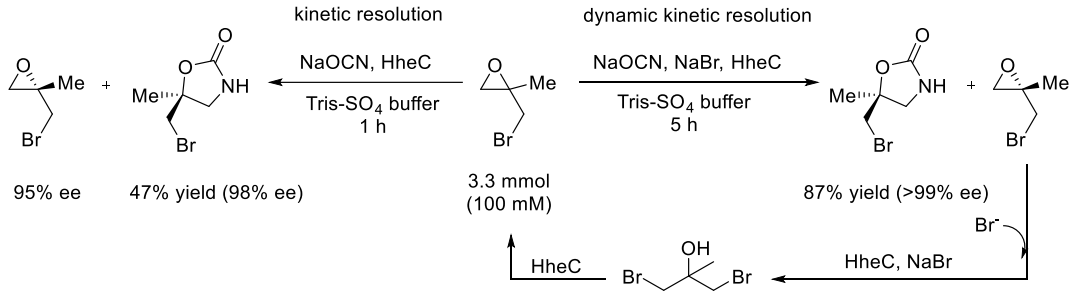


Fig 56. DKR protocol for the synthesis of oxazolidinones

6. Novel reactions

6.1. Formation and ring-opening of oxetanes

Until recently, HHDHs had only been reported to reversibly convert epoxides. However, in recent research, Li and Yuan *et al.* engineered and identified key variants of the HheC enzyme capable of catalyzing the dehalogenation of γ-haloalcohols (via HheC M1–M3) and the ring-opening of oxetanes (via HheC M4–M5), enabling access to both (*R*)- and (*S*)-configured products with high stereoselectivity.[17] Analytical-scale reactions were performed across a broad substrate scope, achieving conversions of up to 49% and products with ee up to 99%. Synthetic applicability was demonstrated through the dehalogenation of 3-chloro-1-phenylpropan-1-ol using HheC M3 variant (P84L/T134A) and the ring-opening of 2-phenyloxetane using HheC M4 variant (P84L/T134A/W139P). Products were isolated in good

yields (34 and 30% respectively) and excellent enantioselectivity (up to 99% ee). Several useful post-modification reactions were also demonstrated (Fig. 57).

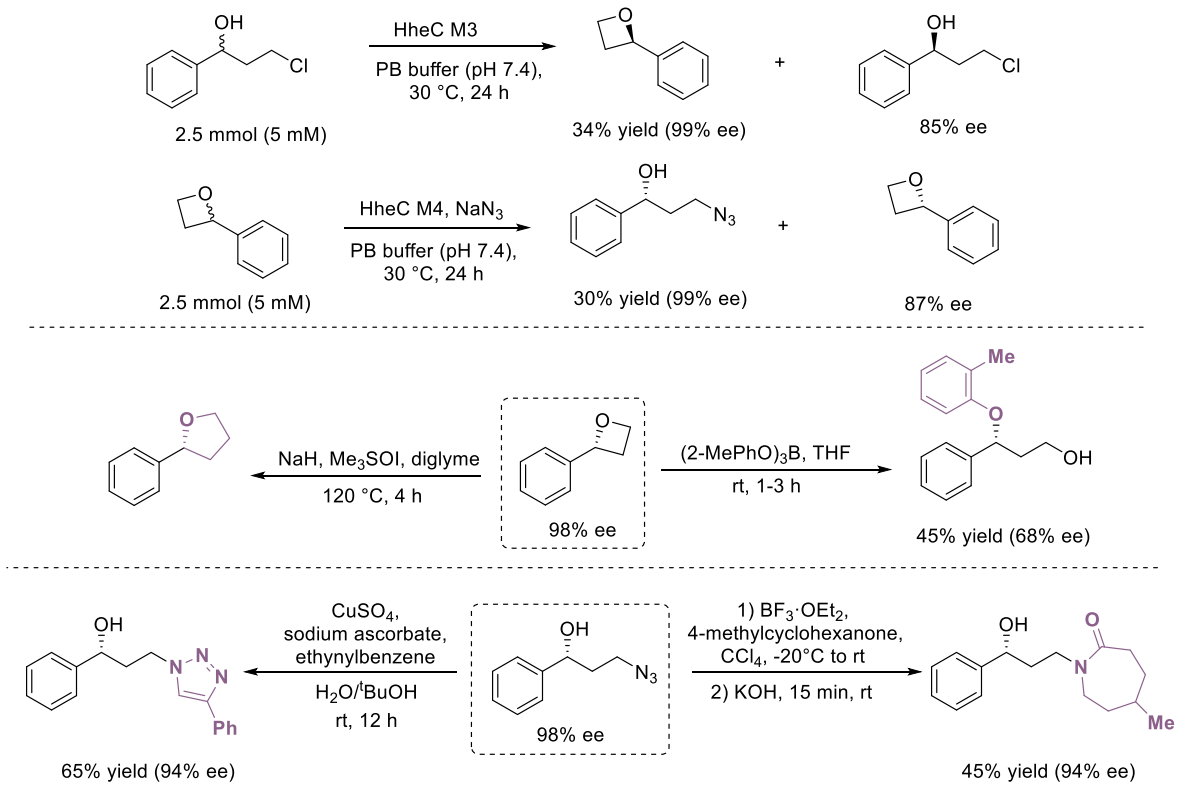


Fig. 57 Dehalogenation of 3-chloro-1-phenylpropan-1-ol and the ring-opening of 2-phenyloxetane

Hua and Wang *et al.* have identified and engineered HheD8 enzyme for enantioselective formation and ring-opening of oxetanes using azide, cyanide, or nitrite ions as nucleophiles.[96] Variants HheD8-M4 (A69F/M124P/R127G/R182W), HheD8-M7 (A69F/M124P/R127L/R182W), and HheD8-M3 (A69F/M124P/R127G) were used in reactions that were conducted with excellent enantioselectivity, over broad substrate scopes, thereby enabling the preparative-scale synthesis of chiral oxetanes (Fig. 58) and chiral γ-substituted alcohols (Fig. 59) in good yields. Biocatalytic dehalogenation and ring-opening were carried out in a cascade and independently on a multigram scale (Fig. 60). Additionally, these chiral products were successfully transformed into various valuable chiral compounds (Fig. 61).

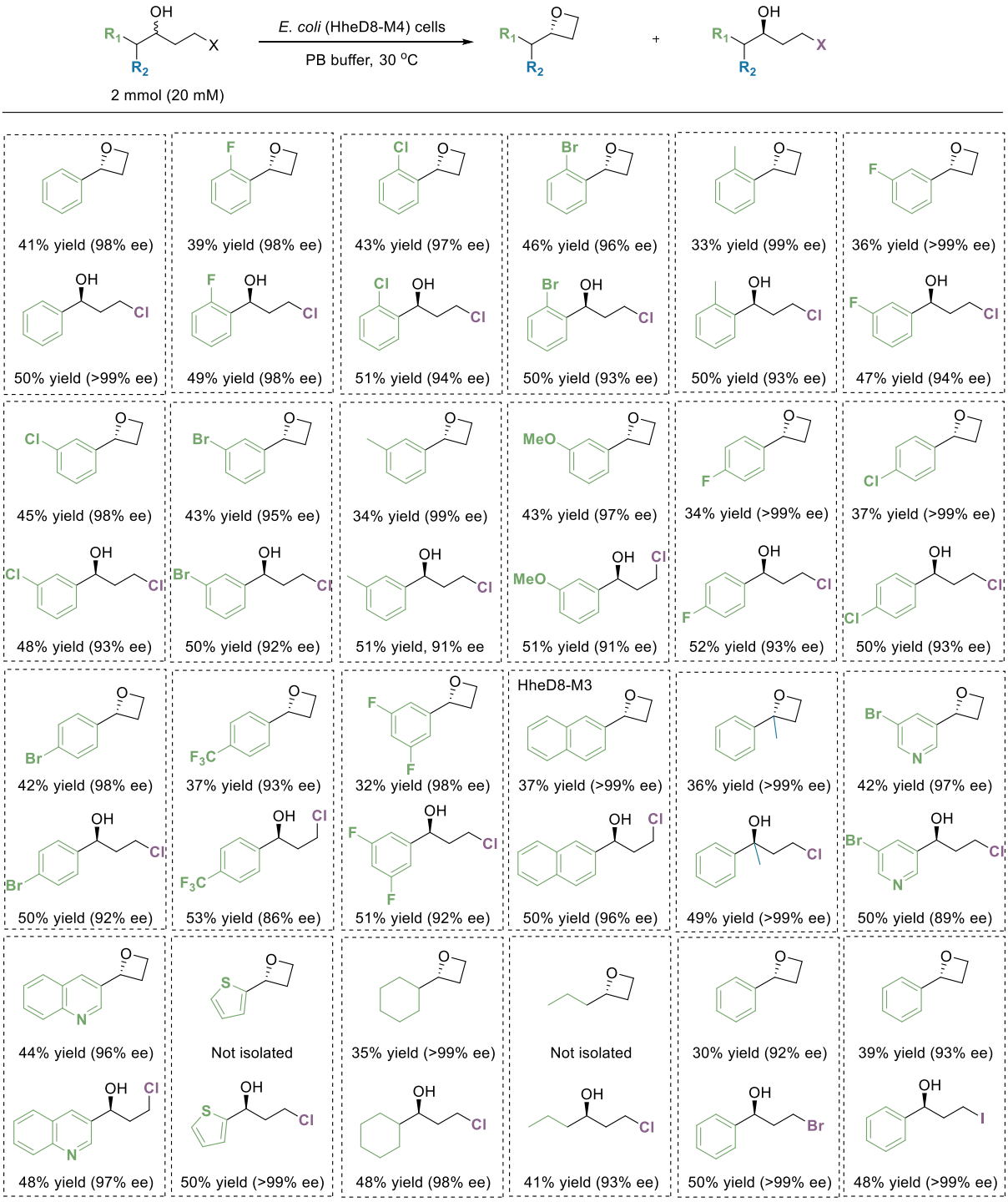


Fig. 58 Enantioselective dehalogenation of γ-haloalcohols

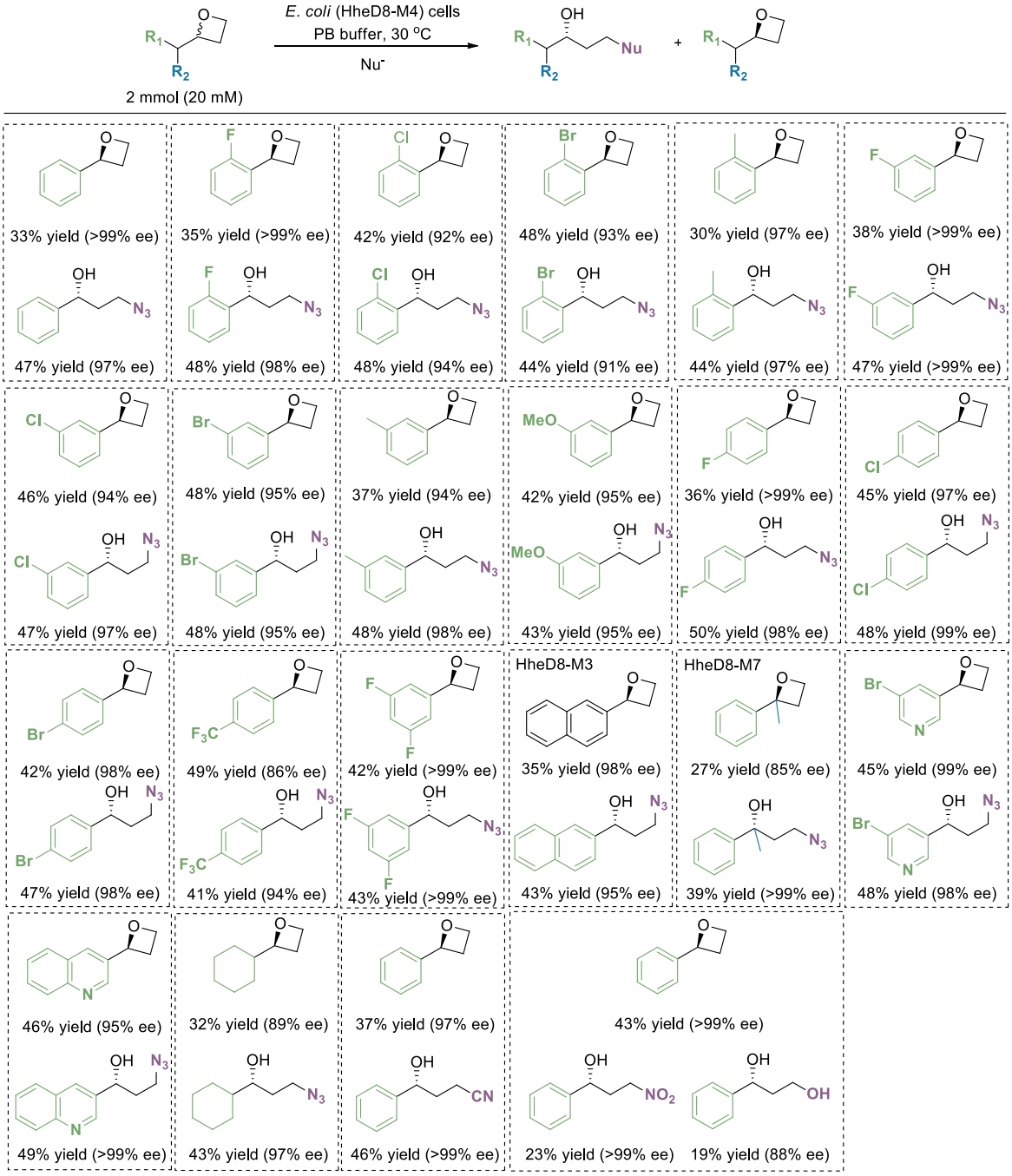


Fig. 59 Enantioselective ring-opening of oxetanes

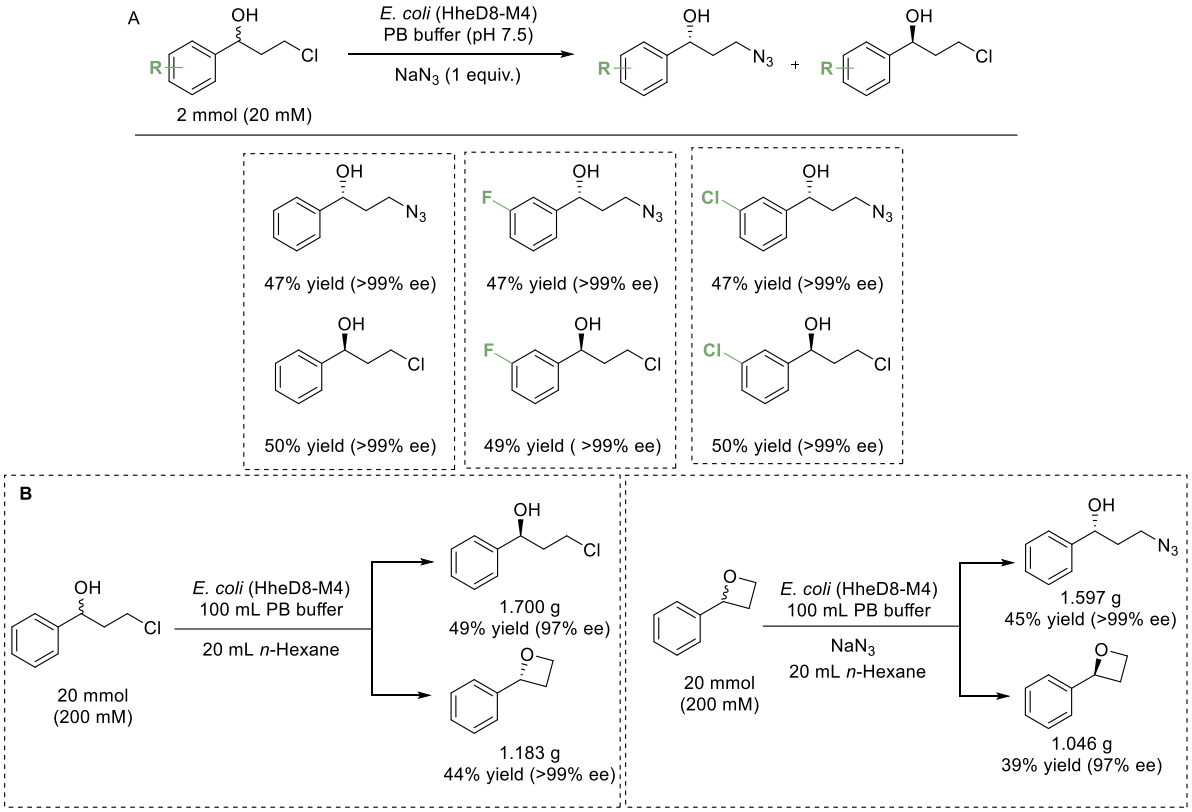


Fig 60. Biocatalytic dehalogenation and ring-opening: in a cascade (A); independently on a multigram scale (B)

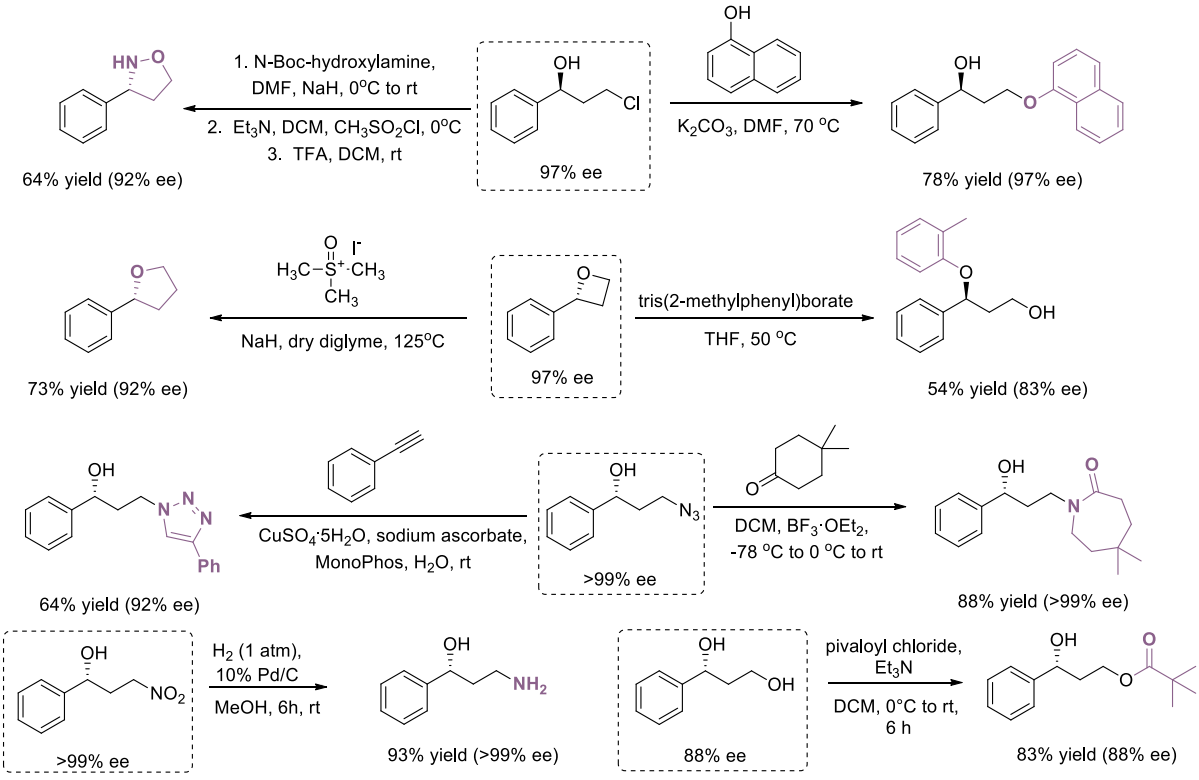


Fig 61. Synthetic applications of chiral oxetanes and γ-substituted alcohols

Da *et al.* developed an enantioselective desymmetrization of prochiral oxetanes using an engineered HHDH, HheG-M14 (I104F/Y18F/T106Y/V105I/L103W).[97] Azidolysis of 3-substituted oxetanes was performed on a preparative scale and provided a range of chiral (*R*)-γ-azido alcohols in >80% isolated yield and high enantiopurity (all >99% ee) (Fig. 62). The practical applicability was demonstrated through gram-scale synthesis and product derivatization (Fig. 63).

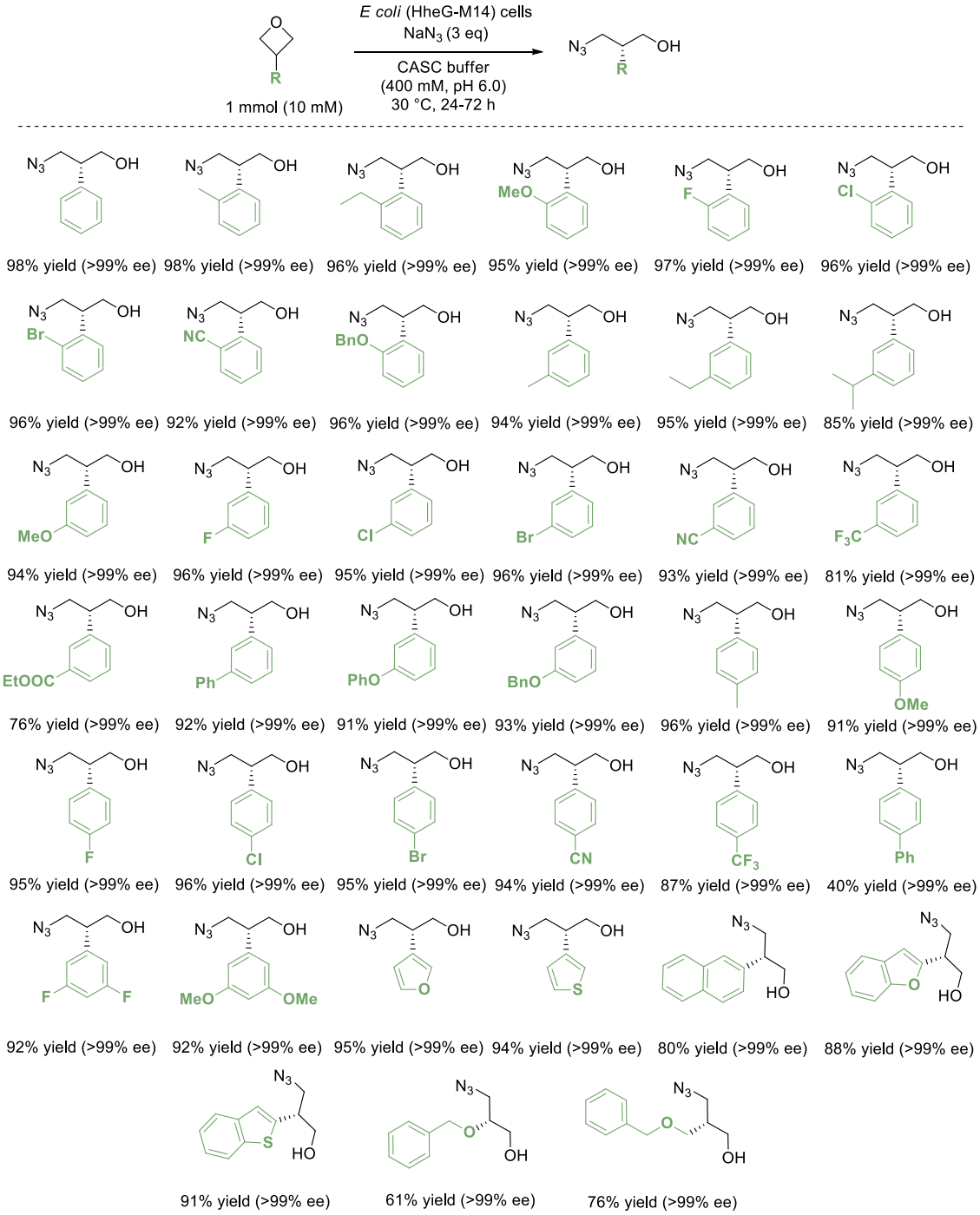


Fig 62. Desymmetrization of prochiral oxetanes through azidolysis

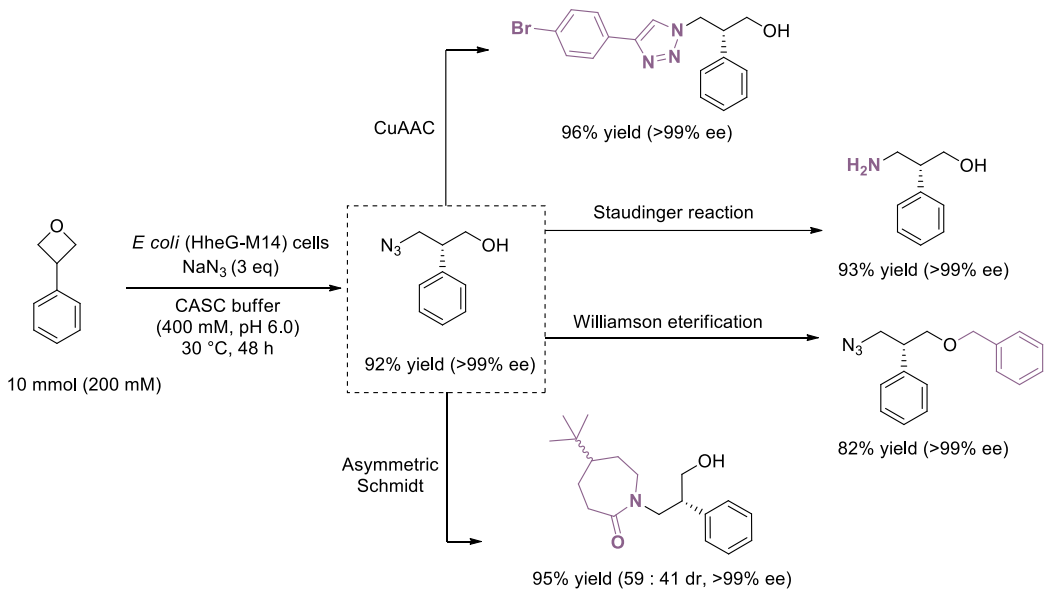


Fig 63. Gram-scale desymmetrization of 3-phenyloxetane and derivatization of product azidoalcohol

6.2. Synthesis of tetrahydrofurans

In 2024, Hu *et al.* demonstrated the potential of HheC from *Agrobacterium radiobacter* AD1 for the enzymatic synthesis of tetrahydrofuran derivatives through intramolecular cyclization of δ-haloalcohols. However, the wild-type enzyme exhibited poor catalytic efficiency and stereoselectivity. Structure-based engineering of HheC led to two stereocomplementary variants, T134M and P84L/W139A, which enabled the kinetic resolution of δ-haloalcohols with excellent selectivity, affording tetrahydrofuran products in up to 99% ee (*E* >200). The reactions were performed on analytical scale.[18]

Wang and Hua *et. al.* used HheC variants: HheC-QM1 (P84V/F86P/T134A/N176A)[26] and HheC-SM11 (F86W) for enantiocomplementary synthesis of a broad range of chiral aryl-, heterocyclic-, alkyl-, and disubstituted chiral δ-haloalcohols (Fig. 64) and tetrahydrofurans (Fig. 65) with good yields (up to 50%) and high optical purities (up to >99% *ee*) on a preparative scale.[98] Notably, the system operates at high substrate loadings (up to 185 g/L), highlighting its potential for industrial application. Subsequent derivatization of the obtained chiral δ-haloalcohols afforded a variety of valuable products (Fig 66).

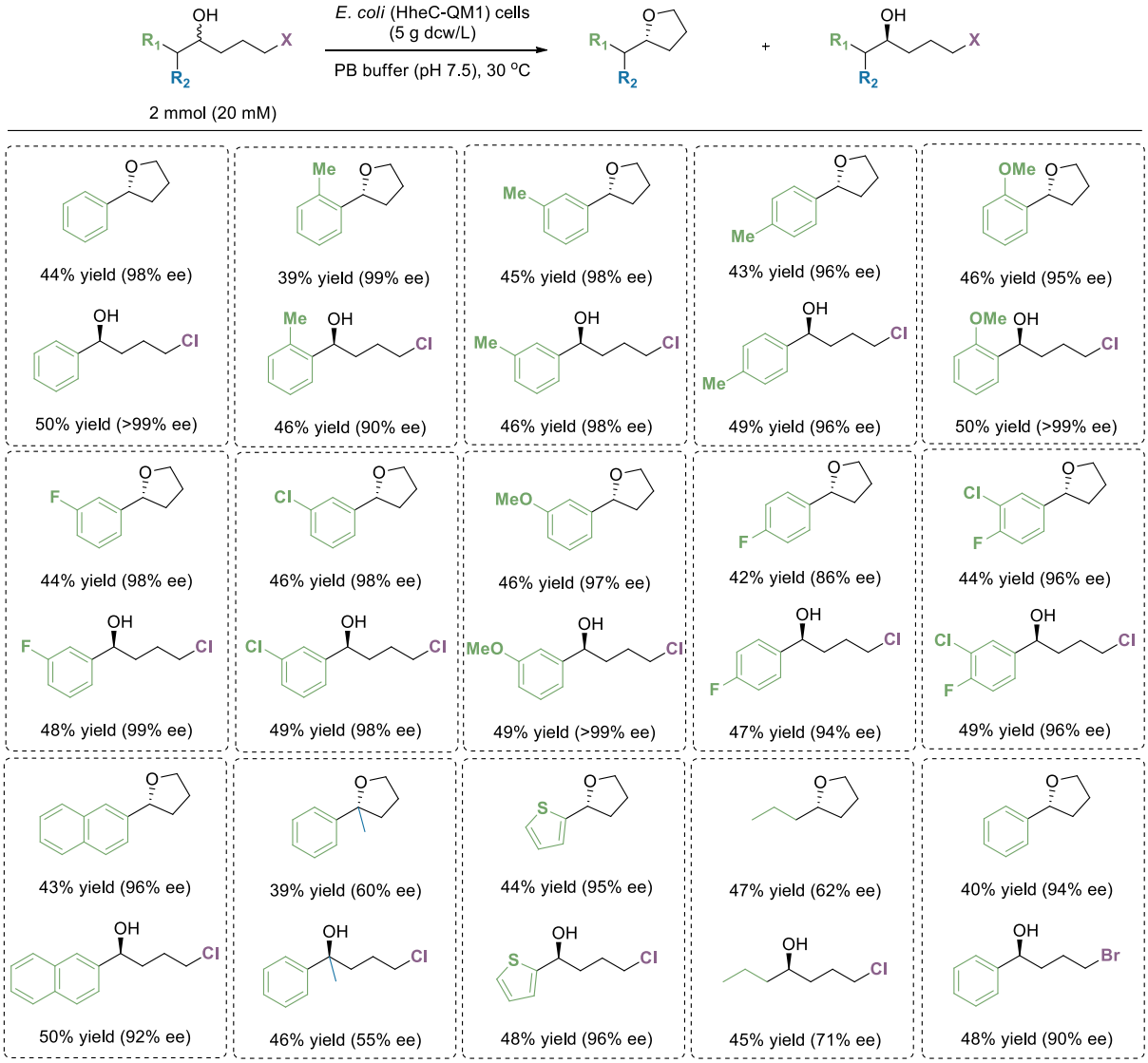


Fig. 64 Synthesis of chiral δ-haloalcohols and tetrahydrofurans using HheC-QM1 variant

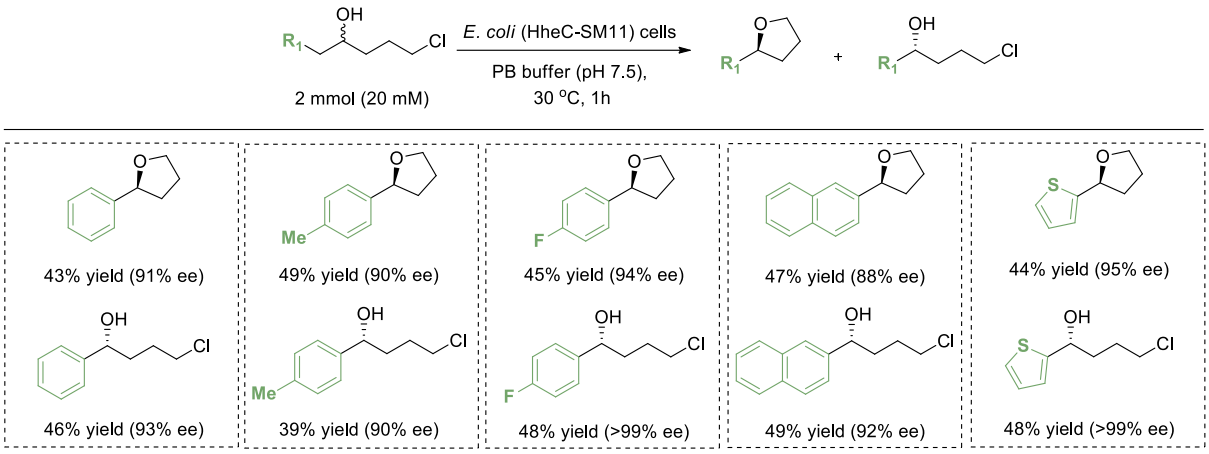


Fig. 65 Synthesis of chiral δ-haloalcohols and tetrahydrofurans using HheC-SM11 variant

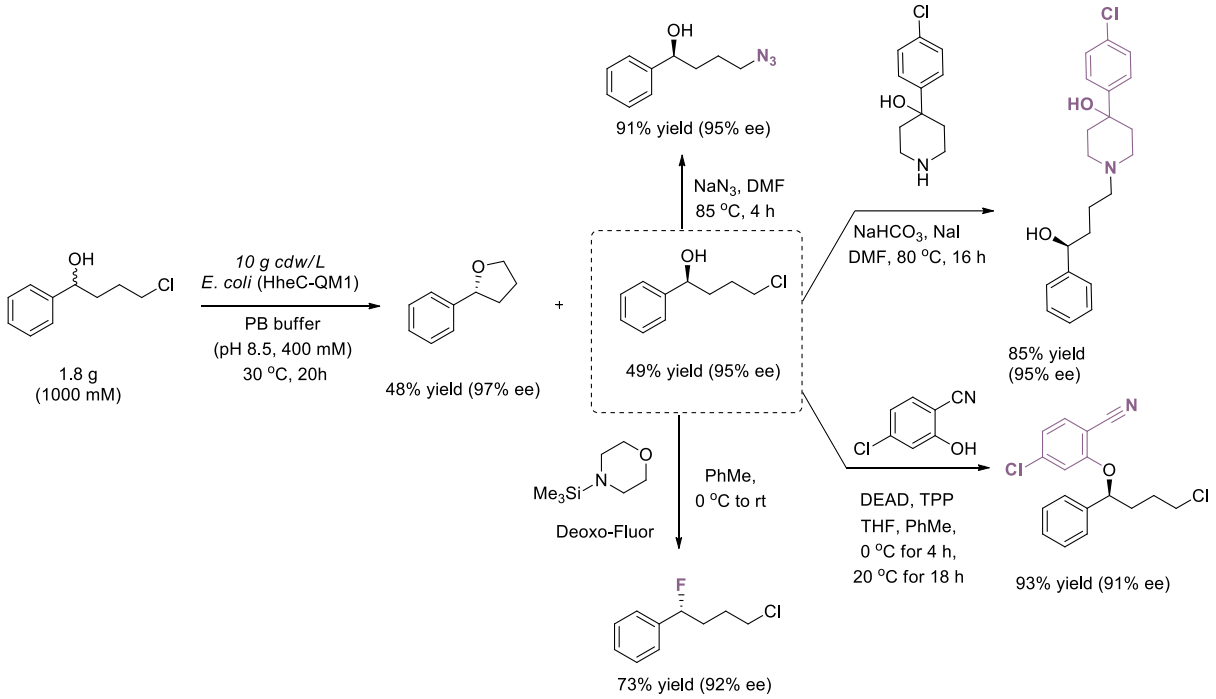


Fig. 66 Gram-scale synthesis and synthetic transformations of (*R*)-2-phenyltetrahydrofuran and (*S*)-4-chloro-1-phenylbutan-1-ol

6.3. Kinetic resolution of ε-haloalcohols and formation of ε-diols

Jin *at al.* recently reported a kinetic resolution of ε-haloalcohols and formation of ε-diols using HheC variants: εM6 (L142A/W249P/P84G/N176Q), εM7 (L142A/W249P/P84G/F186Y), and εM8 (L142A/W249P/P84G/N176Q/F186Y).[19] In contrast to the usual HHDH reactivity, this reaction proceeds *via* a remote intermolecular dehalogenative hydroxylation mechanism, in which the halogen is displaced by a hydroxyl group derived from water rather than from the parent haloalcohol. An Asp–Arg dyad was identified through mechanistic investigations as the catalytic motif responsible for this unconventional dehalogenation pathway. Several ε-haloalcohols and ε-diols were successfully prepared in 45-49% isolated yield (90-98% ee), and 42-49% yield (88-96% ee) respectively (Fig. 67). Reaction was demonstrated on a multigram-scale, and the obtained chiral products were transformed to several valuable synthetic building blocks.

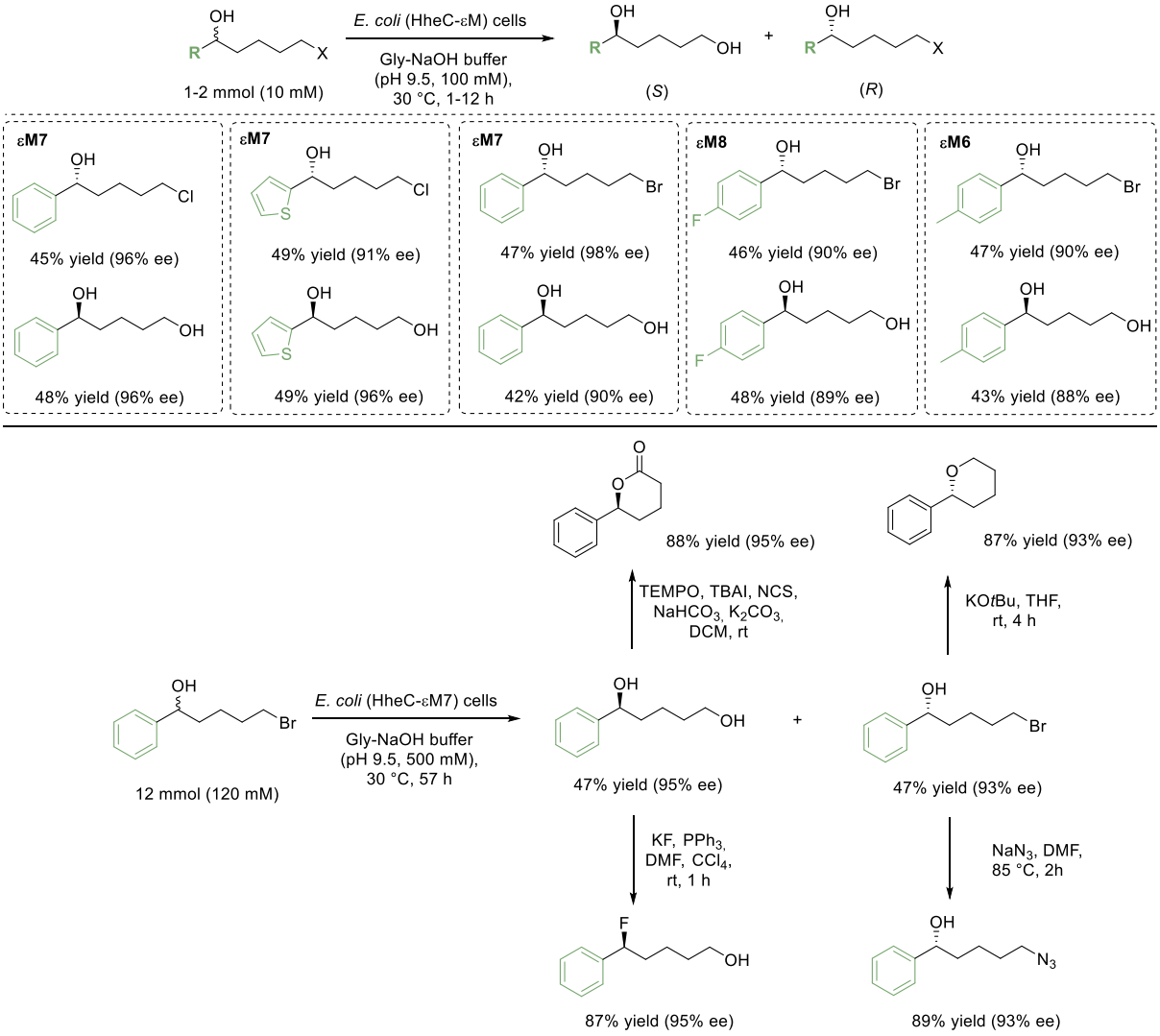


Fig. 67 Kinetic resolution of ε-haloalcohols with formation of ε-diols

7. Conclusion and outlook

Halohydrin dehalogenases have established themselves as multifunctional biocatalysts for organic synthesis, offering unique catalytic capabilities that complement traditional chemical methods. They display great promiscuity including a broad substrate scope as well as the ability to catalyze more than one type of reaction. Their ability to catalyze highly regio- and enantioselective dehalogenation and epoxide ring-opening reactions with a wide range of nucleophiles, as well as recently discovered synthesis of oxetanes and tetrahydrofuranes, enables the efficient synthesis of valuable chiral compounds under mild and environmentally friendly conditions. Synthesis of multifunctionalised chiral molecules, often containing quaternary stereocenter is notable feature of HHDHs, and makes them particularly attractive for the preparation of pharmaceutical intermediates, fine chemicals, and other high-value molecules. Previous successes for compound such as statin side-chain building block,[73] have demonstrated the application of highly engineered HHDH in multistep syntheses for pharma industry. Although, enantioselective HHDHs are rare in nature, it is evident that protein engineering can provide enzymes of high and complementary

selectivity.[18][26][66][72][92][99-100] Furthermore, activity and stability have also been addressed successfully by engineering methods.[27-33] For example, Arabnejad *et. al.* used computational tools to construct a virtual library of 253 variants, from which 29 stabilizing point mutations were confirmed by experiments.[28] The 12 most effective mutations were combined to obtain a remarkably stable HheC variant that exhibited strongly improved stability in the presence of organic solvents as well as thermostability. Despite recent progress, synthetic application of HHDHs is still a relatively young research area, and many challenges still need to be addressed. One issue is the tolerance of enzymes to high concentrations of substrates, products and reaction intermediates, which may lead to enzyme inhibition and/or deactivation.[21-22] With only a few exceptions, [19][51][54][66][71][97-98] preparative-scale reactions reported to date have been conducted at low substrate concentrations, typically ranging from 5 to 50 mM. In a case study by Milčić *et. al*. describing conversion of *rac*-4-fluorostyrene oxide to (*S*)-3-(4-fluorophenyl)-3-hydroxypropanenitrile by HheC variant, five distinct sources of inhibition were identified within a single reaction system: substrate inhibition, product inhibition (both enantiomers of the β-hydroxynitrile), by-product inhibition (diol), and co-solvent inhibition (DMSO).[21] Substrate inhibition becomes apparent at epoxide concentrations above 50 mM, which are concentrations required for an economically viable industrial process. Another limitation of performing reactions at higher epoxide concentrations is that spontaneous hydrolysis of epoxide becomes significant, leading to reduced product yields through the formation of the corresponding diol. Spontaneous hydrolysis is also promoted by prolonged reaction times and elevated temperatures. In addition, the resulting diols can also act as competitive inhibitors. To minimise the impact of chemical side reactions and increase the rate of enzymatic transformation, lower substrate concentrations and higher enzyme loadings are typically used. Moreover, HHDH-catalysed biotransformations reported to date have been performed in batch systems, leaving room for process optimisation to further explore the potential of these systems. Reaction engineering can be guided by a detailed understanding of reaction kinetics and enzyme operational stability. From the other hand, enzyme engineering can provide a route to improve resilience toward reagents, thereby improving catalyst performance and increasing space-time yields. Together, advances in reaction and enzyme engineering are expected to facilitate the implementation of HHDHs in future large-scale biocatalytic processes.

CRediT authorship contribution statement

Danijel Glavač: Conceptualization, Writing – review & editing. Maja Mjerić Elenkov: Conceptualization, Writing – review & editing. Irena Dokli: Conceptualization, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Data availability

No data was used for the research described in the article

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