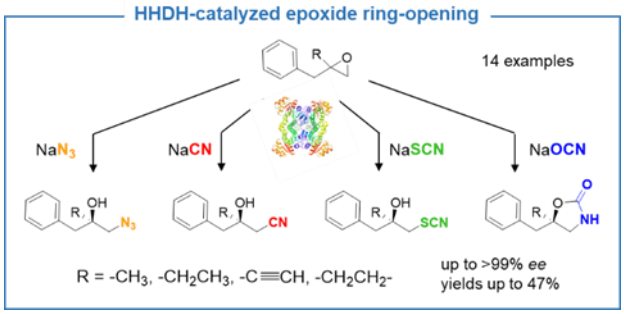
Biocatalytically Generated Library of Chiral Building Blocks Containing Quaternary Stereocenter

Emina Mehić, Robert J. Kolman, Irena Dokli, and Maja Majerić Elenkov*

Ruđer Bošković Institute, Division of Organic Chemistry and Biochemistry, Bijenička cesta 54, 10000 Zagreb, Croatia

Supporting Information Placeholder

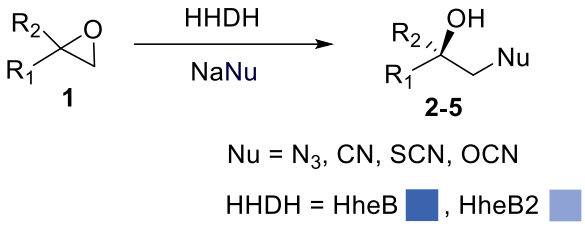


ABSTRACT: A biocatalytic strategy for the preparation of a small library of compounds containing a quaternary chiral center is described. By applying halohydrin dehalogenases, 4 racemic 2,2-disubstituted epoxides were converted in the presence of 4 nucleophiles to 14 chiral products in yields of 21-47% and 74->99% *ee*. The obtained set of building blocks, which hold diverse functional groups, can be modified to many high-value organic molecules for use in medicinal chemistry and other areas.

The chirality of biologically active molecules is an important aspect in the research of new potential drugs.[1] Using chirality, medicinal chemists achieve greater complexity of the three-dimensional structures and better selectivity, thus increasing drug safety. As a consequence, over half of FDA-approved drugs are chiral. Chiral building blocks are among the most important precursors and intermediates in the synthesis of new bioactive compounds, therefore, it is important to develop enantioselective methods that can be used to prepare molecules of a specific chirality.[2–4] The industry mainly depends on established chemical synthesis methods, but it is increasingly adopting biocatalysis, which uses enzymes as catalysts.[2,5] The real power of the biocatalysts lies in their ability to generate chirality in the molecule thanks to their excellent stereoselectivity, due to which the manipulation of protecting groups can be avoided in the synthetic process.[3,6] For example, nucleophilic ring-opening of epoxides catalysed by halohydrin dehalogenases (HHDHs) can produce a number of chiral building blocks containing 2-3 functional groups per molecule.[7–10] It has been established that azide, cyanide, nitrite, cyanate and thiocyanate ions are accepted nucleophiles by several HHDHs.[11,12] It enables the synthesis of various molecules, which can further be synthetically modified in different ways due to the presence of several functional groups (e.g. hydroxy, azido, nitro, cyano etc.).[10,13–18] The versatility of HHDHs makes them attractive catalysts for use in synthesis. Besides, HHDHs are easily available in large quantities, they are stable proteins, easy to use and do not require cofactors. Reactions are carried out under mild conditions that are not harmful to the environment. Many HHDHs have been identified in the nature so far, some of them showing desirable catalytic properties.[19–23] The power of HHDHs as biocatalysts has been demonstrated in many recent synthetic applications, most of which are related to the preparation of compounds containing tertiary stereocenters.[10,16,24–26]

In a recent study, we have found that HHDHs from the group B are highly enatioselective towards the sterically demanding 2,2-disubstituted epoxides when applying sodium azide as a nucleophile,[22] indicating that HHDHs could be suitable catalysts for the synthesis of multifunctionalised chiral building blocks having quaternary stereocenters. In this work, we show that enzymes HheB from *Corynebacterium* sp. and HheB2 from *Mycobacterium* sp. indeed catalyse epoxide ring-opening reaction with excellent enantioselectivity and catalytic efficiency with a variety of nucleophiles and diverse substrates.

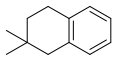
The reaction was initially evaluated with a set of five differently substituted benzyloxirane derivatives, bearing methyl (**1a**), ethyl (**1b**), spirocyclic scaffold (**1c**), ethynyl (**1d**) and trifluoromethyl (**1e**) group as a second substituent. After preliminary testing, substrate **1e** was withdrawn from further biocatalytic study, due to very low hydrolytic stability (Figure S1) and modest catalytic activity. Epoxides **1a**-**1d** were screened with selected enzymes, HheB and HheB2 in the form of cell-free extract (cfe) and with the synthetically most attractive nucleophiles (azide, cyanide, thiocyanate and cyanate ions) (Figure 1).



SUBSTRATES:

1a: R_1 = Bn, R_2 = CH_3

1b: R_1 = Bn, R_2 = C_2H_5

1c: $R_1 + R_2$ = 

1d: R_1 = Bn, R_2 = C≡CH

1e: R_1 = Bn, R_2 = CF_3

Conditions: 2 mL scale, 5 mM epoxide, 5 mM NaNu, 5% DMSO, Tris-SO_4 buffer (50 mM, pH 7.5), HheB or HheB2 (cell-free extract), 25°C. Reaction time was 5 min for NaN_3 and 60 min for other nucleophiles. See Supporting Information for specific details (Section 5).

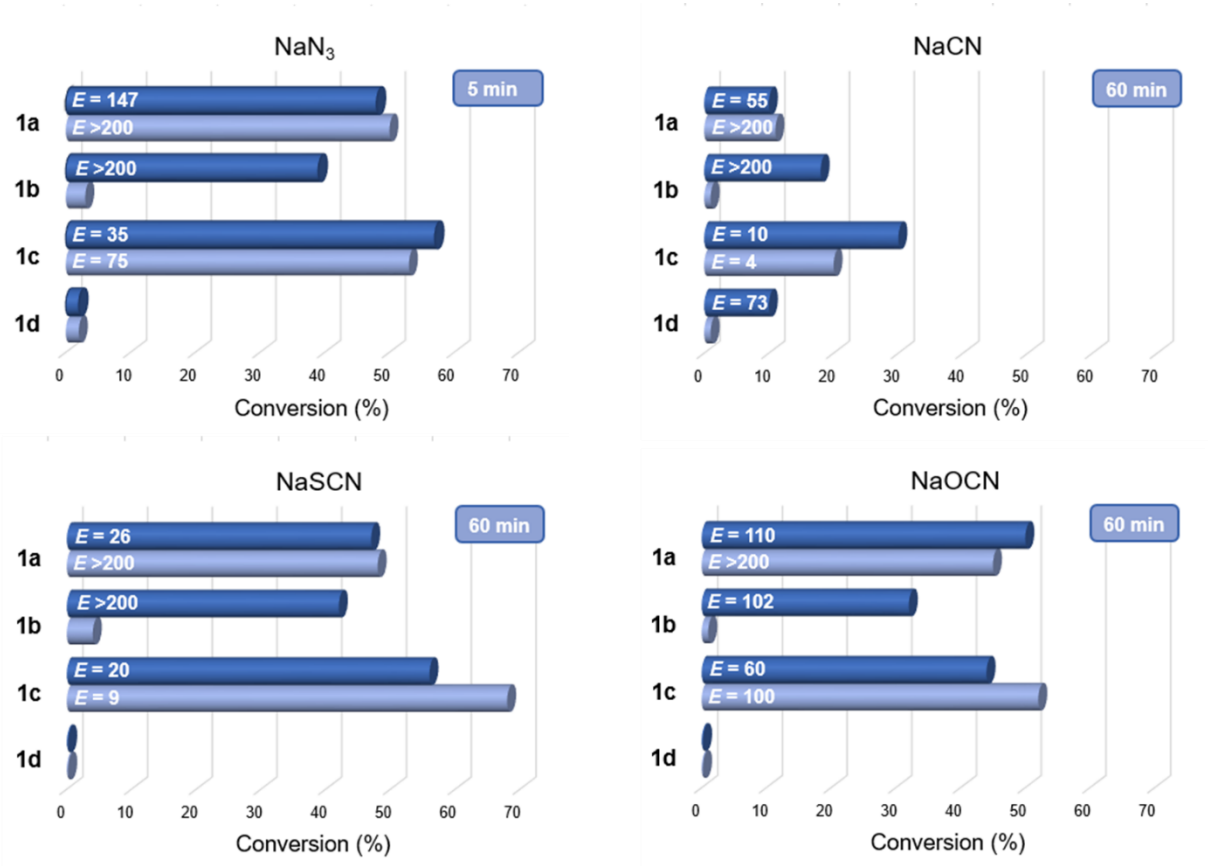


Figure 1. Substrate, enzyme and nucleophile screening. Conversions and *E*-values for the ring-opening reaction of substrates **1a-1d** using four different nucleophiles and two recombinant HHDHs.

Experiments were performed at analytical scale, 5 mM substrate concentration and 1 equivalent of the nucleophile. Different behaviour in terms of activity and enantioselectivity could be observed when comparing nucleophile performance, as well as substrates (Figure 1). Azide ion stands as the most efficient among all nucleophiles, giving the highest reaction rate. Reactions with cyanide ion are on the other hand the slowest, and very often do not reach complete conversion. This might be partly due to product inhibition by the formed hydroxynitrile, which HHDHs seem to be particularly susceptible to.[27]

For further scale-up optimisation, one enzyme, either HheB or HheB2, was selected for each substrate based on catalytic performance (activity and enantioselectivity from the screening experiments, Figure 1, Table S1). Because almost every biocatalytic reaction requires customized conditions, different catalyst loadings, substrate concentrations, substrate/nucleophile ratios, and temperatures were evaluated (Tables S2-S5). Majority of the reactions could be smoothly performed at substrate concentration of 50 mM, using isolated enzyme, and 0.5 to 1.0 equivalent of the nucleophile in buffered media. As mentioned, the highest challenge was cyanide-mediated ring-opening reactions in which β-hydroxy nitriles are formed. By using cfe containing

HheB or HheB2, we couldn't achieve appreciable yields, even below 50 mM substrate concentrations. It was shown that within this group of enzymes exists a very complex enzyme kinetics in which substrates (epoxide and nucleophile), product and by-products might inhibit the HHDHs, which significantly reflects on product yields.[28] The use of enzyme in the form of whole-cells might be a solution to these challenges, since the presence and protective nature of the cell membrane helps stabilize the enzyme and can enable enzyme applications under harsh reaction conditions.[29] To compare the performance of the enzyme in different forms (cfe, wet cells, and lyophilised cells), experiments were set at 50 mM of **1a** and 1 equivalent of NaCN (Figure 2). The same amount of HheB2 was applied, first in the form of cfe and wet cells at 25 °C (Figure 2a), then at five-degree higher temperature (30 °C), where we compared performance of wet and lyophilised cells (Figure 2b).

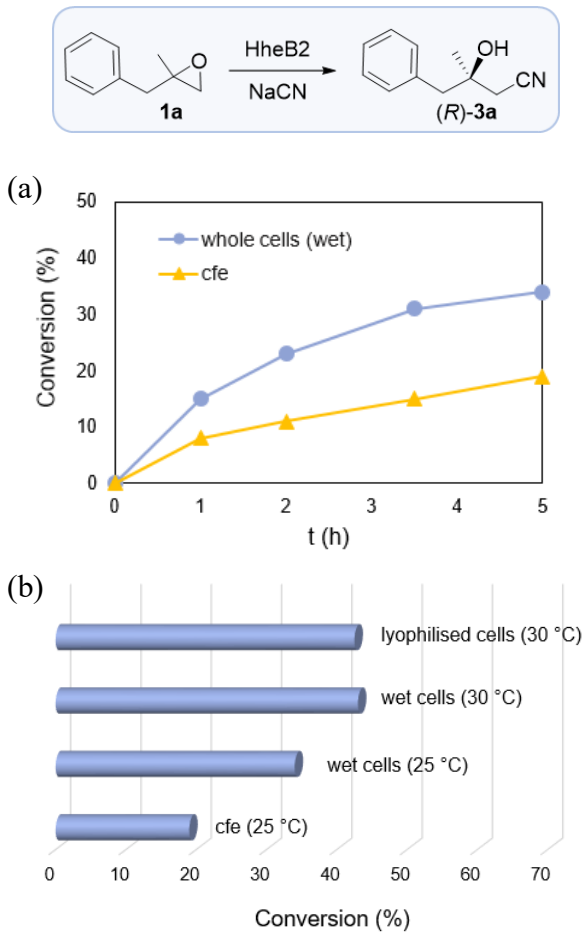


Figure 2. Conversions using different forms of the biocatalyst. Reactions were performed using HheB2, 50 mM of **1a** and 1 equiv of NaCN. (a) Time course of the conversions using cell-free extract (cfe) and wet cells at 25 °C. (b) Total conversions after 5 h from the same experiments and additional performed at 30 °C with wet and lyophilised cells. Details can be found in Supporting Information (section 6.1.).

We observed a much better enzyme activity in the form of whole cells compared to isolated enzyme, and no difference between wet and dry (lyophilised) cells. In fact, we doubled the yield using cells at 30 °C compared to cfe at 25 °C. The cell membrane may act as a mass transport barrier, slowing down the reaction rate, which does not seem to be the case here with highly lipophilic substrates, but rather protects the enzyme from the harmful environment. In addition, the use of whole cells avoids the need for cell lysis and reduces the catalyst cost, and therefore seems the better option.

The most challenging substrate was **1d**, where no activity was observed with cyanate and thiocyanate ions, and very low with azide and cyanide (Figure 1). Therefore, the same approach using the whole cell catalyst was applied. Azidolysis and cyanolysis reactions were successfully accomplished, using cell density of 10-15 g_{cdw}/L, while transformations with the same amount of isolated enzyme were inefficient (Table S5).

Building on these results, we have performed the synthesis of 14 diverse chiral compounds at 1 mmol scale starting from 4 different racemic epoxides (Scheme 1). High enantiomeric excess of 94->99% *ee* was obtained for compounds **2a**-**2d**, **3a**, **3b**, **4a**, **4b** and **5a**-**5c**, and moderate for **3c**, **3d** and **4c** (74-90% *ee*). The activity and enantioselectivity of the applied HHDHs underlines the usefulness of biocatalysts for the synthesis of optical pure alcohols and oxazolidinones with quaternary stereocenter. This methodology seems applicable for epoxides substituted with methyl and ethyl group, and also ethynyl with some limitation in regards to nucleophile. Spiroepoxide **1c** is an excellent substrate in terms of activity, however enantioselectivity was found to be dependent on the nucleophile. Although detailed kinetic analyses were not performed, based on our recent research,[27] and observations with diverse substrates, we assume that both enantiomers of epoxide **1c** have high affinity towards the active site. To some extent, reactions could be optimised to achieve higher product *ee* by simple keeping the nucleophile up to 0.5 eq. In this way, mainly (*R*)-**1c** is converted, while (*S*)-**1c** remains. This was quite evident in the case of azide ions, where in the presence of 1.5 eq. of NaN_3, both enantiomers of **1c** are quickly converted to nearly racemic azido alcohol **2c** (20% *ee*), while the reaction stops approaching 50% conversion when 0.5 eq. was used, in favour of product (*R*)-**2c** of 95% *ee* (Figure 3). A similar trend was observed with cyanate and thiocyanate, while in the case of cyanide, due to the slow reaction rate, both substrate enantiomers were consumed, giving only enantioenriched product (74% *ee* at 21% conversion), regardless of the amount of the nucleophile used.

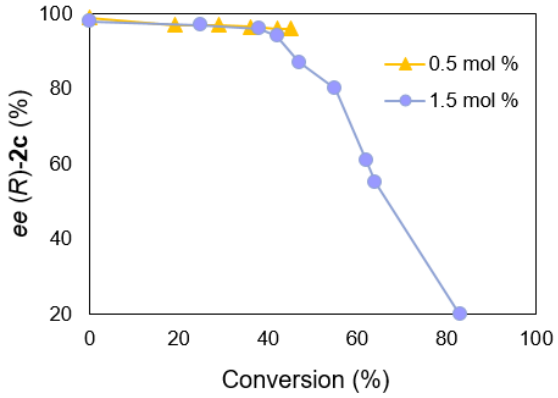
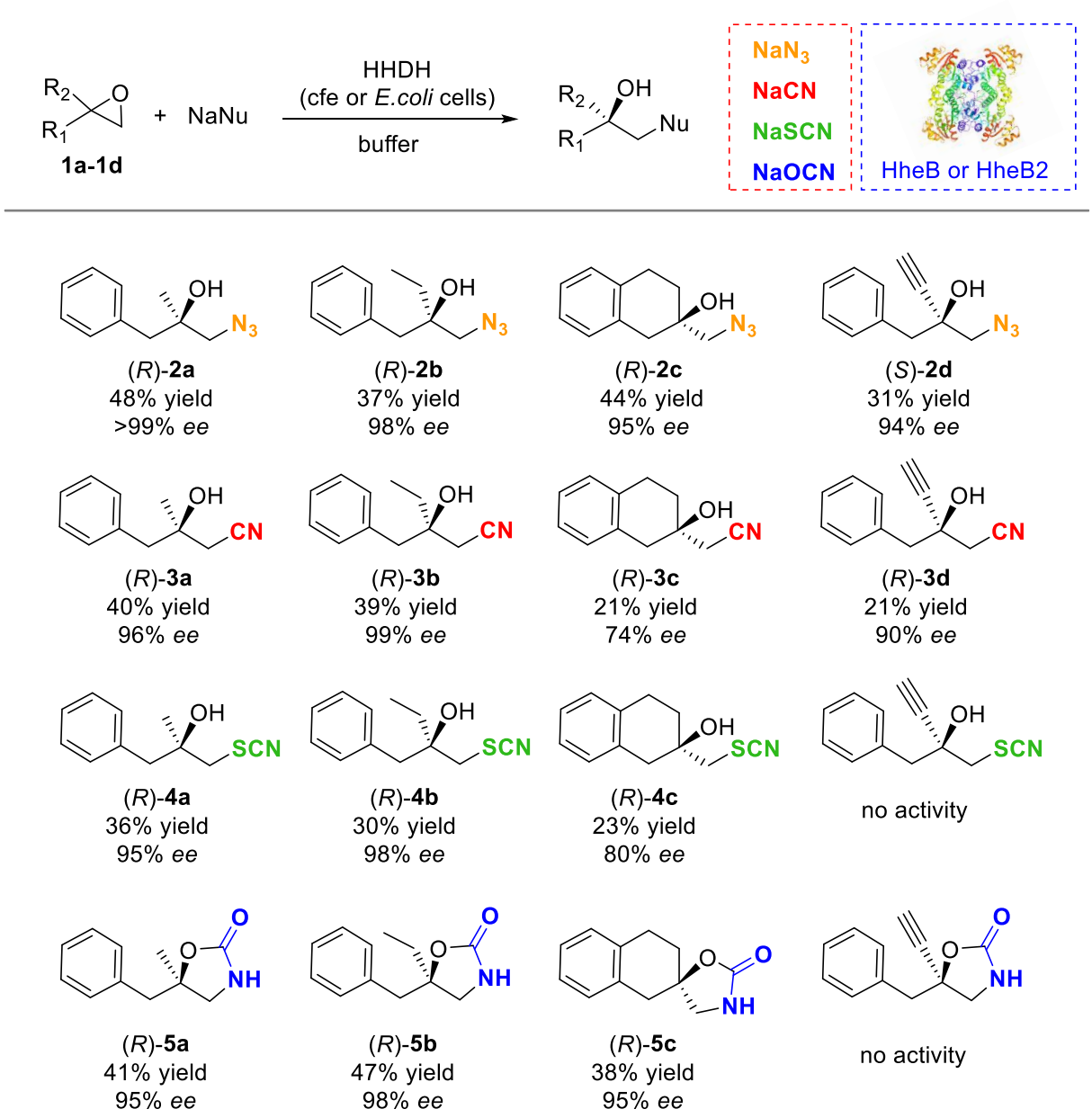


Figure 3. Correlation of conversion and enantiomeric excess of azido alcohol (*R*)-**2c** produced from *rac*-**1c** by using HheB2 and different amounts of NaN_3, 0.5 and 1.5 equivalent (mol %).

Scheme 1. Products obtained in the nucleophilic ring-opening reaction of epoxides **1a-1d** catalysed by HHDHs.[a]



[a] Reaction conditions: epoxide (1.0 mmol, 50 mM), nucleophile 0.5-1.0 equivalent, HheB or HheB2 as a cell-free extract or whole cells, Tris-SO_4 buffer, 24-30 °C, 1000 rpm. Details are given in Supporting Information, section 6.3.

In conclusion, small chiral molecules that serve as building blocks, play an important role in the discovery of new active pharmaceutical ingredients (API). Due to their three-dimensional structures, they have more superior properties compared to flat aromatic compounds. Quaternary stereocenters, in particular, contribute greatly to the three-dimensionality of the molecule. However, synthetic challenges in building quaternary stereocenters are the bottleneck for their implementation in drug discovery. Here, we demonstrated that HHDH-enzymes might be a good option when synthesis of such molecules is considered. Through the enantio- and regioselective nucleophilic epoxide ring-opening reaction, the products were obtained in yields of 21-47% and 74->99% *ee*. Additionally, this approach offers a synthetic route to a small library of chiral building blocks holding diverse functionalities. Further modifications through 2-3 consecutive chemical transformations may lead to diverse libraries of highly complex compounds with direct application in medicinal chemistry. Enzyme engineering certainly offers opportunity to extend this methodology to a more diverse substrate range.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details; GC and HPLC chromatograms; NMR spectra; supplementary tables (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: majeric@irb.hr; orcid.org/0000-0001-6884-0928

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was financially supported by CatPharma (KK.01.1.1.04.0013), a project co-financed by the Croatian Government and the European Union through the European Regional Development Fund – the Competitiveness and Cohesion Operational Programme. We would like to acknowledge the use of the equipment provided by CroBioPro (KK.01.1.1.01.0002). We thank Mrs Mirjana Čičak for valuable technical assistance.

REFERENCES

(1) Silvestri, I. P.; Colbon, P. J. J. The Growing Importance of Chirality in 3D Chemical Space Exploration and Modern Drug Discovery Approaches for Hit-ID. *ACS Med. Chem. Lett.* **2021**, *12*, 1220–1229.

(2) Devine, P. N.; Howard, R. M.; Kumar, R.; Thompson, M. P.; Truppo, M. D.; Turner, N. J. Extending the Application of Biocatalysis to Meet the Challenges of Drug Development. *Nat. Rev. Chem.* **2018**, *2*, 409–421.

(3) Rossino, G.; Robescu, M. S.; Licastro, E.; Tedesco, C.; Martello, I.; Maffei, L.; Vincenti, G.; Bavaro, T.; Collina, S. Biocatalysis: A Smart and Green Tool for the Preparation of Chiral Drugs. *Chirality* **2022**, *34*, 1403–1418.

(4) Ramsden, J. I.; Cosgrove, S. C.; Turner, N. J. Is It Time for Biocatalysis in Fragment-Based Drug Discovery? *Chem. Sci.* **2020**, *11*, 11104–11112.

(5) Simić, S.; Zukić, E.; Schmermund, L.; Faber, K.; Winkler, C. K.; Kroutil, W. Shortening Synthetic Routes to Small Molecule Active Pharmaceutical Ingredients Employing Biocatalytic Methods. *Chem. Rev.* **2022**, *122*, 1052–1126.

(6) Winkler, C. K.; Schrittwieser, J. H.; Kroutil, W. Power of Biocatalysis for Organic Synthesis. *ACS Cent. Sci*. **2021**, *7*, 55–71.

(7) Majerić Elenkov, M.; Hoeffken, H. W.; Tang, L.; Hauer, B.; Janssen, D. B. Enzyme-Catalyzed Nucleophilic Ring Opening of Epoxides for the Preparation of Enantiopure Tertiary Alcohols. *Adv. Synth. Catal.* **2007**, 349, 2279–2285.

(8) Hasnaoui, G.; Lutje Spelberg, J. H.; De Vries, E.; Tang, L.; Hauer, B.; Janssen, D. B. Nitrite-Mediated Hydrolysis of Epoxides Catalyzed by Halohydrin Dehalogenase from *Agrobacterium Radiobacter* AD1: A New Tool for the Kinetic Resolution of Epoxides. *Tetrahedron Asymmetry* **2005**, *16*, 1685–1692.

(9) Majerić Elenkov, M.; Tang, L.; Meetsma, A.; Hauer, B.; Janssen, D. B. Formation of Enantiopure 5-Substituted Oxazolidinones through Enzyme-Catalysed Kinetic Resolution of Epoxides. *Org. Lett.* **2008**, *10*, 2417–2420.

(10) Ma, R.; Hua, X.; He, C.-L.; Wang, H.-H.; Wang, Z.-X.; Cui, B.-D.; Han, W.-Y.; Chen, Y.-Z.; Wan, N.-W. Biocatalytic Thionation of Epoxides for Enantioselective Synthesis of Thiiranes. *Angew. Chem. Int. Ed*. **2022**, e202212589.

(11) Hasnaoui-Dijoux, G.; Majerić Elenkov, M.; Lutje Spelberg, J. H.; Hauer, B.; Janssen, D. B. Catalytic Promiscuity of Halohydrin Dehalogenase and Its Application in Enantioselective Epoxide Ring Opening. *ChemBioChem* **2008**, *9*, 1048–1051.

(12) Findrik Blažević, Z.; Milčić, N.; Sudar, M.; Majerić Elenkov, M. Halohydrin Dehalogenases and Their Potential in Industrial Application – A Viewpoint of Enzyme Reaction Engineering. Adv. Synth. Catal. **2021**, *363*, 388–410.

(13) Tian, S.; Ge, X.; Yan, Q.; Li, M.; Huang, Q.; Zhang, X.; Ma, M.; Chen, B.; Wang, J. B. Directed Evolution of Stereoselective Enzymes Meets Click Reactions: Asymmetric Synthesis of Chiral Triazoles Using a Cu(I)-Compatible Halohydrin Dehalogenase. *Green Synth. Catal*. **2024**, https://doi.org/10.1016/j.gresc.2024.01.001

(14) Zhang, F. R.; Wan, N. W.; Ma, J. M.; Cui, B. D.; Han, W. Y.; Chen, Y. Z. Enzymatic Kinetic Resolution of Bulky Spiro-Epoxyoxindoles via Halohydrin Dehalogenase-Catalyzed Enantio- And Regioselective Azidolysis. *ACS Catal*. **2021**, *11*, 9066–9072.

(15) Yang, X.; Zhao, X.; Luo, X.; Sun, F.; Bocola, M.; Verma, N.; Hong, Z.; Yang, H.; Chen, H. A Self-Regulated Process from Bench to Pilot Production of Non-Natural Heterocycles Using Halohydrin Dehalogenases. *ChemCatChem* **2024**, e202400095.

(16) Wang, Z. X.; Fu, H. K.; Da, X. Y.; Wang, H. H.; Cui, B. D.; Han, W. Y.; Chen, Y. Z.; Wan, N. W. Biocatalytic Synthesis of Metaxalone and Its Analogues. *Org. Lett*. **2023**, *25*, 5049–5054.

(17) Xu, Q.; Huang, K.-S.; Wang, Y.-F.; Wang, H.-H.; Cui, B.-D.; Han, W.-Y.; Chen, Y.-Z.; Wan, N.-W. Stereodivergent Synthesis of Epoxides and Oxazolidinones via the Halohydrin Dehalogenase-Catalyzed Desymmetrization Strategy. *ACS Catal*. **2022**, *12*, 6285–6293.

(18) Molinaro, C.; Guilbault, A. A.; Kosjek, B. Resolution of 2,2-Disubstituted Epoxides via Biocatalytic Azidolysis. *Org. Lett*. **2010**, *12*, 3772–3775.

(19) Van Hylckama Vlieg, J. E. T.; Tang, L.; Lutje Spelberg, J. H.; Smilda, T.; Poelarends, G. J.; Bosma, T.; Van Merode, A. E. J.; Fraaije, M. W.; Janssen, D. B. Halohydrin Dehalogenases Are Structurally and Mechanistically Related to Short-Chain Dehydrogenases/Reductases. *J. Bacteriol*. **2001**, *183*, 5058–5066.

(20) Schallmey, A.; Schallmey, M. Recent Advances on Halohydrin Dehalogenases—from Enzyme Identification to Novel Biocatalytic Applications. *Appl. Microbiol. Biotechnol*. **2016**, 100, 7827–7839.

(21) Koopmeiners, J.; Diederich, C.; Solarczek, J.; Voß, H.; Mayer, J.; Blankenfeldt, W.; Schallmey, A. HheG, a Halohydrin Dehalogenase with Activity on Cyclic Epoxides. *ACS Catal*. **2017**, *7*, 6877–6886.

(22) Mehić, E.; Hok, L.; Wang, Q.; Dokli, I.; Miklenić, M. S.; Findrik Blažević, Z..; Tang, L.; Vianello, R.; Majerić Elenkov, M. Expanding the Scope of Enantioselective Halohydrin Dehalogenases – Group B. *Adv. Synth. Catal*. **2022**, *364*, 2576–2588.

(23) Solarczek, J.; Kaspar, F.; Bauer, P.; Schallmey, A. G-Type Halohydrin Dehalogenases Catalyze Ring Opening Reactions of Cyclic Epoxides with Diverse Anionic Nucleophiles. *Chem. Euro. J*. **2022**, *28*, e202202343.

(24) Guan, X. E.; Miao, R. P.; Hua, X.; Jin, X.; Deng, G. Z.; Cui, B. D.; Han, W. Y.; Wan, N. W.; Chen, Y. Z. Biocatalytic Enantioselective Synthesis of Chiral β-Hydroxy Nitriles Using Cyanohydrins as Cyano Sources. *ACS Catal*. **2023**, *13*, 13597–13606.

(25) Wan, N.; Zhou, X.; Ma, R.; Tian, J.; Wang, H.; Cui, B.; Han, W.; Chen, Y. Synthesis of Chiral 5-Aryl-2-Oxazolidinones via Halohydrin Dehalogenase-Catalyzed Enantio- and Regioselective Ring-Opening of Styrene Oxides. *Adv. Synth. Catal*. **2020**, *362*, 1201–1207.

(26) Dokli, I.; Brkljača, Z.; Švaco, P.; Tang, L.; Stepanić, V.; Majerić Elenkov, M. Biocatalytic Approach to Chiral Fluoroaromatic Scaffolds. *Org. Biomol. Chem*. **2022**, *20*, 9734–9741.

(27) Milčić, N.; Sudar, M.; Marić, A. K.; Kos, K.; Majerić Elenkov, M.; Findrik Blažević, Z. HHDH-Catalyzed Synthesis of Enantioenriched Fluorinated β-Hydroxy Nitrile–Process Advances through a Reaction Engineering Approach. *Ind. Eng. Chem. Res.* **2024**, 63, 7051−7063.

(28) Milčić, N.; Sudar, M.; Dokli, I.; Majerić Elenkov, M.; Findrik Blažević, Z. Halohydrin Dehalogenase-Catalysed Synthesis of Enantiopure Fluorinated Building Blocks: Bottlenecks Found and Explained by Applying a Reaction Engineering Approach. *React. Chem. Eng*. **2023**, *8*, 673-686.

(29) Lin, B.; Tao, Y. Whole-Cell Biocatalysts by Design. *Microb. Cell Fact.* **2017**, *16*, 106.