nature portfolio

Peer Review File

Engineered halohydrin dehalogenase mediates remote enantiocontrolled dehalogenative hydroxylation via an unconventional mechanism

Corresponding Author: Dr Nan-Wei Wan

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Jin et al. reports on a new reaction type catalyzed by engineered variants of halohydrin dehalogenase HheC. While halohydrin dehalogenases (HHDHs) typically catalyze the dehalogenation of haloalcohols with formation of cyclic ethers (intramolecular S_N2 reaction), the engineered variants catalyze an intermolecular substitution reaction of ε-haloalcohols with a water molecule attacking the haolgenated carbon atom resulting in halide release and diol formation. These are highly interesting results as the variants employ a different catalytic mechanism than the classic HHDHs. An active-site aspartate functions as a new catalytic residue (similar to other hydrolytic dehalogenases), while the serine and tyrosine of the catalytic triad of classic HHDHs only seem to play a role in substrate binding and orientation. Moreover, the reported reactions are enantioselective, even though the stereocenter is remote from the halogenated carbon atom.
The performed work is very comprehensive and, in my opinion, highly suitable for publication in Nature Communications. However, I have a few points for a further improvement of the manuscript:

1. The authors do not mention how they unambiguously assigned the (*R*)- and (*S*)-enantiomers of the different substrates and products in chiral HPLC. Only for one compound (lactone 1ba) the absolute configuration was assigned by single-crystal X-ray diffraction analysis.

2. Page 7, lines 21-25: It is not surprising that the SM9/D80A variant was completely inactive in all tested reactions as the mutation D80A alone already abolishes HheC activity completely (see https://doi.org/10.1093/emboj/cdg479). The side chain of D80 is important for proton relay from R149 (as part of the catalytic triad) to the solvent in HheC, as partially stated by the authors already. However, they might have overseen that the impact of mution D80A on HheC activity had been reported before. In connection to that (lines 25-28 on page 7), reference 14 is in my opinion not the best one to cite to refer to the previously published role of D80 in HheC.

3. Page 8, lines 90 ff.: It is not unexpected that the combination of beneficial single mutations results in a worse-performing combination mutant, as the effect of mutations is often not additive. Instead, the impact of mutations is always context dependent, i.e. dependent on other amino acids in the surrounding.

4. Page 9, lines 35-37 and Figure 4d: The authors state that their best variants afforded the product (S)-1b with 94-96% ee while leaving the substrate (R)-1a enriched to 91-94% ee. The latter, however, does not fit to the data shown in Figure 4d. Based on this figure, the substrate ee is only around 80%. Please correct.

5. Only one reaction (i.e. using one of the subsrates, 12a, reported in the manuscript) was performed at a substrate concentration higher than 10-20 mM. Even the different preparative-scale reactions only employed substrate concentrations of 10-20 mM (while using 100 mL reaction volume). I would like to understand why. Does the activity or enantioselectivity of the different HheC variants decrease significantly at higher substrate concentrations, except for substrate 12a? Bromide is definitely a better leaving group that chloride. Or is substrate solubility a problem? The authors should elaborate on this in their manuscript to also highlight possible limitations of their approach.

6. With respect to substrate solubility, the authors state in the method description for the large-scale biotransformation employing 120 mM 12a that DMSO was added as cosolvent. In contrast, no DMSO addition is mentioned for reactions employing 10-20 mM haloalcohol substrate. What is the solubility limit of the used substrates in aqueous reaction media? The authors should add this information to their manuscript.

Reviewer #2

(Remarks to the Author)
In this well designed study by Wan and co-workers, new catalytic activity of halohydrin dehalogenase (HHDH) have been revealed, and the enzyme was found to catalyse enantioselective dehalogenative hydroxylation of -haloalcohols to form - diols. This is new biocatalytic reaction not previously described for HHDHs, that are known for their catalytic versatility by catalysing many non-natural reactions.
Screening of a set of wild-type HHDHs as well as HheG and HheC mutants (72 in total) revealed 3 active enzymes. The best variant HheC-L142A that showed low S-enantioslectivity and 19% yield was further engineered via structure guided directed evolution, achieving increased enantioselectivity and activity on a model substrate (1a). Substrate scope was found to be broad on aromatic -haloalcohols mostly concerning activity, however, high enantioselectivity was demonstrated only on 6 substrates while on majority the E value was low to moderate. Finally, this novel biotransformation was demonstrated on 2.3 g of selected substrate.
The mechanism of the reaction has been proposed, involving one-step hydrolytic dehalogenation assisted by aspartate D80 that activate water molecule. Catalytic triade Ser-Try-Arg (S132, Y145, R149) and halide-binding site (N176-Y187) remain of fundamental importance for catalytic activity as confirmed by mutagenesis experiments.
My opinion is that this paper is a valuable contribution to the field of HHDH-catalysed reactions and biocatalysis in general. Overall, this well written manuscript presented a novel idea. Paper is clearly presented and well organized. All the claims in the manuscript are well supported by relevant experiments. I suggest acceptance of this article for publication in Nature Communications after minor revision:
- Page 4, line 47: The sentence “Currently, Dehalogenative…” capital “D” should be corrected
- Page 5, line 13: The sentence “This This…” should be corrected
- Page 8, lines 28-32: The sentence “This evidence indicates the emergence of a novel dehalogenative mechanism” in the SM9 mutant, thereby representing a previous unrecognized catalytic strategy in hydrolytic dehalogenation” is not clear.
- Page 9, line 4: I suggest to remove word “exceptional”
- Page 10, Fig 5: The reaction time should be included in figure caption
- Page 11, line 7: I suggest “high enantioselectivity” instead of “outstanding enantioselectivity”, since it was 96-98%. Outstanding would be >99.9%.
- Page 12, line 81: I suggest providing the range for enantiomeric purities rather then “up to >99% ee” since this particular value is related to only one product

Reviewer #3

(Remarks to the Author)
The manuscript “Engineered halohydrin dehalogenase mediates remote enantiocontrolled dehalogenative hydroxylation via an unconventional mechanism” by Xiao Jin et al. presents a comprehensive experimental and computational study of engineered HHDH mutants and their enzymatic activity toward 5-chloro-1-phenylpentan-1-ol. As a result, an unconventional mechanism of dehalogenative hydroxylation catalyzed by engineered HHDHs is proposed. The authors applied a range of standard biochemical, organic, and computational chemistry methods (SDM, SSM, NMR, HRMS, MD) to support their findings. The study includes interesting discussions, particularly regarding the investigation of the reaction mechanism, with attention to pH dependence and the application of isotopic labeling. However, I find that the novelty and significance of the work are not sufficiently emphasized. In addition, several technical issues are present in the current version of the manuscript. I believe that revision is necessary to improve the article.

Main text:

1. The authors refer to “a limited repertoire of reaction types” in both the abstract and the introduction, however, they do not introduce new reaction types in the present work. The enzymatic conversion of halogen-containing compounds into alcohols or epoxides is not novel or unique and does not broaden “nature’s catalytic repertoire.” Also, flavin-dependent oxygenases, cytochrome P450 oxidases, and dehaloperoxidases are not directly connected to the processes discovered by the authors. I suggest that the authors modify the abstract and introduction to highlight the specific problems they are addressing and the possible applications of their findings. This would help the reader better understand the choice of the main substrate and how the described unconventional mechanism could be applied further.

2. Page 4, lines 3-6: “Our investigation began with systematic evaluation of our HHDH enzyme library using racemic ε-haloalcohol 1a (5-chloro-1-phenylpentan-1-ol) as the model substrate.”
– Which specific halohydrin dehalogenases are included in the term HHDH in this study?
– What does the HHDH enzyme library consist of and how was it constructed?
– What was the rationale for selecting 5-chloro-1-phenylpentan-1-ol as the model substrate?

3. How were the amino acid residues selected for mutation (e.g., W249P, P84G, F186Y, N176Q)? The authors use the term “unexpectedly,” which suggests that a specific strategy may have been applied for choosing not only the positions but the particular substitutions (W to P, P to G, F to Y, N to Q). Please clarify the rationale or methodology used to select these

mutations.

4. I am not fully convinced by the aspartate–arginine dyad hypothesis, as the majority of the mutants did not show activity, not only those with mutations at D80 and R149. Would it be possible that SM9 and its improved variants exhibit catalytic promiscuity, potentially employing different catalytic centers depending on the substrate?

5. Fig. 3a should be corrected: the oxygen atom in the product should be shown in blue, in accordance with the depicted mechanism.

6. Fig. 3g should be corrected: please verify the protonation states of Y145 and R149, as their pKa values are above 10. These residues are most likely protonated at pH 8.5. If protonated states are correct, please check a "+" of the nitrogen of R149 as the nitrogen presently has three covalent bonds.

7. The plus sign in Fig. 3e (+S132A, +N176A) is not necessary, as it does not indicate the type of the wild type but may be misleading to the reader.

Supplementary Information:

1. In the molecular dynamics simulations, thermostat and barostat are not specified.

2. "A representative snapshot of HheC-SM9 was used for docking"
– How was this snapshot chosen?
– Did the authors perform MD simulations for the protein–ligand complex?

3. The authors aim to investigate conformational changes in the protein; however, a 200 ns simulation may not be sufficient for this purpose.

4. RMSD values of all atoms may be less informative than RMSD of the protein backbone or Cα atoms for tracing conformational changes. The authors may consider preparing a figure showing these RMSD values to better illustrate the protein's structural dynamics.

5. Supplementary Table 7:
– What does entry #1 represent?
– Why is (R)-1a3 indicated as a product when its ee varies from 5% to 99%? Wasn't (S)-1a3 present in entries 1, 3, 4, and 5? The same issue applies to Tables 4–9.
– Since the reaction is stereoselective and only the (S)-isomer undergoes transformation, only the (R)-isomer of the starting compound should remain. It would be interesting to understand what determines this specificity, and the authors may provide an explanation based on their MD simulation results.

6. Supplementary Fig. 8 should be revisited. If Asp-132 is protonated, there is no need to show a "–" sign. At physiological pH or higher, however, aspartate is typically deprotonated.

7. Supplementary Fig. 9: The color code is inconsistent for the HOH molecule and the OH group of the product. The arrows indicating stereochemistry of the substituents should be corrected. It would also be helpful to indicate the reaction conditions or provide a reference. Note that Fig. 4c of Ref. 52 suggests leaving a chlorine anion rather than a hydrogen.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors revised their manuscript according to the reviewer comments. However, I still have a few further remarks that need the authors' attention:

1. Assignment of enantiomers in chiral analysis: If I understand the authors correctly, they have assigned the (*R*)- and (*S*)-enantiomers of all substrates and products based on the assumption that their HheC mutant exhibits *S*-selectivity. But how can the authors know that and be sure that this is true for all their substrates? They only confirmed the stereochemistry for one single product by single crystal X-ray diffraction. Can the authors indeed be sure that the enzyme (or the different tested mutants) exhibits *S*-selectivity with each tested substrate?

2. Page 7, lines 5-25: Reading this paragraph again with the adjusted wording regarding mutation D80A, I am wondering if this paragraph is really relevant for the overall manuscript. The authors mention already on page 4 the impact of mutation D80A on the activity of HheC SM9 in the dehalogenative hydroxylation of ε-haloalcohols. Now in the paragraph on page 7, the focus is on the dehalogenative cyclization of β-, γ- and δ-haloalcohols. That HheC WT and mutant SM9 are able to perform this reaction is either already known from previous literature or, in my opinion, not really relevant for the current manuscript. Hence, my suggestion would be to remove this paragraph and to add the information regarding the importance

of residue D80 for HheC activity already on page 4. However, if the authors deem the information in the paragraph on page 7 important for the overall manuscript, they should rewrite it to better highlight the purpose and relevance of these results for the overall manuscript.

3. Page 4, line 91: The authors performed a new MD simulation for 600 ns and adjusted the manuscript descript in the SI accordingly. In the main manuscrit text, however, they still mention the 200 ns MD simulation. This should be corrected.

Reviewer #3

(Remarks to the Author)
The authors have carefully revised their manuscript and answered my questions. I recommend the manuscript for publication.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors replied to and addressed my comments carefully. They also adjusted the paragraph on page 7, however, I am still not fully satisfied with this new version. I suggest the following changes to make it easier for the reader to follow the authors' argumentation:

Lines 5-12: "We subsequently evaluated ... dehalogenation cyclization activity of HheC-WT, mutant SM9 (containing mutation L142A), and mutant SM9/D80A (containing mutations L142A and D80A) against ..."
Line 21: replace "catalyze" by "convert"

After this minor revision, I suggest to accept the manuscript for publication.

Response to Reviewers

Reviewer #1 (Remarks to the Author):

Comments: The manuscript by Jin et al. reports on a new reaction type catalyzed by engineered variants of halohydrin dehalogenase HheC. While halohydrin dehalogenases (HHDHs) typically catalyze the dehalogenation of haloalcohols with formation of cyclic ethers (intramolecular SN2 reaction), the engineered variants catalyze an intermolecular substitution reaction of ε-haloalcohols with a water molecule attacking the haolgenated carbon atom resulting in halide release and diol formation. These are highly interesting results as the variants employ a different catalytic mechanism than the classic HHDHs. An active-site aspartate functions as a new catalytic residue (similar to other hydrolytic dehalogenases), while the serine and tyrosine of the catalytic triad of classic HHDHs only seem to play a role in substrate binding and orientation. Moreover, the reported reactions are enantioselective, even though the stereocenter is remote from the halogenated carbon atom.

The performed work is very comprehensive and, in my opinion, highly suitable for publication in Nature Communications. However, I have a few points for a further improvement of the manuscript.

Answer: We are very grateful to your encouraging comments and have revised the manuscript accordingly to improve its quality.

Question 1: The authors do not mention how they unambiguously assigned the (*R*)- and (*S*)-enantiomers of the different substrates and products in chiral HPLC. Only for one compound (lactone **1ba**) the absolute configuration was assigned by single-crystal X-ray diffraction analysis.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have now clarified in the revised manuscript how the absolute configurations of the (*R*)- and (*S*)-enantiomers for the various substrates and products were assigned. The *S*-enantioselectivity of the enzymatic transformation was unambiguously confirmed by comparison with literature data and by single-crystal X-ray diffraction analysis of lactone **1ba**. The (*R*)- and (*S*)-enantiomers of the different substrates and products in chiral HPLC were assigned by analogy based on this established enantiopreference. Specifically, by comparing the consumption of racemic substrate standards in chiral HPLC analysis, the more rapidly depleted substrate peak was assigned as the *S*-enantiomer, while the less consumed peak corresponded to the *R*-enantiomer. Correspondingly, the major product peak formed was assigned as the *R*-enantiomer, and the minor product peak as the *S*-enantiomer. These details have been added to the "Chromatography analysis" section (Page S2) of the Supplementary Information.

Question 2: Page 7, lines 21-25: It is not surprising that the SM9/D80A variant was completely inactive in all tested reactions as the mutation D80A alone already abolishes HheC activity completely (see https://doi.org/10.1093/emboj/cdg479). The side chain of D80 is important for proton relay from R149 (as part of the catalytic triad) to the

solvent in HheC, as partially stated by the authors already. However, they might have overseen that the impact of mution D80A on HheC activity had been reported before. In connection to that (lines 25-28 on page 7), reference 14 is in my opinion not the best one to cite to refer to the previously published role of D80 in HheC.

Answer: We appreciate your insightful suggestion.

We fully agree that the previously reported role of Asp80 in the catalytic mechanism of HheC should be acknowledged. According to your suggestion, we have revised the text on page 7 to cite the appropriate work (https://doi.org/10.1093/emboj/cdg479: de Jong, R. M. et al. Structure and mechanism of a bacterial haloalcohol dehalogenase: a new variation of the shor-chain dehydrogenase/reductase fold without an NAD(P)H binding site. *EMBO J.* **2003**, 22, 4933-4944), which first demonstrated that mutation D80A completely abolishes HheC activity by disrupting the proton relay from Arg149 to the solvent. We have also updated the reference accordingly to ensure accurate citation (Page 7, lines 20-25).

Question 3: Page 8, lines 90 ff.: It is not unexpected that the combination of beneficial single mutations results in a worse-performing combination mutant, as the effect of mutations is often not additive. Instead, the impact of mutations is always context dependent, i.e. dependent on other amino acids in the surrounding.

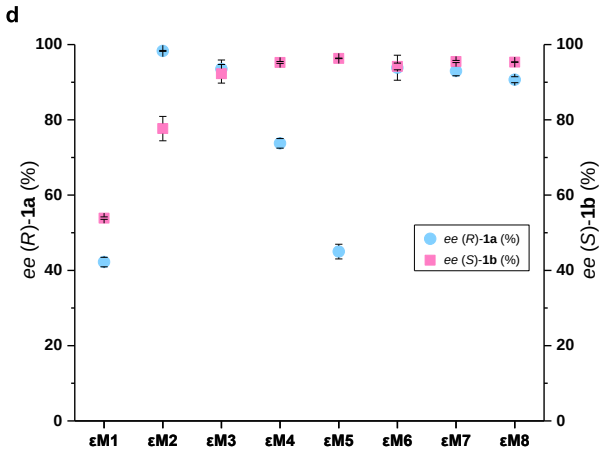
Answer: We appreciate your insightful suggestion.

We fully agree that the impact of mutations is non-additive and always context dependent. According to your suggestion, we have revised the text on page 8 by removing the word “unexpectedly” to better reflect this understanding.

Question 4: Page 9, lines 35-37 and Figure 4d: The authors state that their best variants afforded the product (*S*)-**1b** with 94-96% *ee* while leaving the substrate (*R*)-**1a** enriched to 91-94% *ee*. The latter, however, does not fit to the data shown in Figure 4d. Based on this figure, the substrate *ee* is only around 80%. Please correct.

Answer: We appreciate your insightful suggestion.

We apologize for the mistake in the original figure. According to your suggestion, we have carefully corrected Figure 4d to ensure it now accurately reflects the substrate enantiomeric excess values, which are consistent with the data presented in Supplementary Table 12.



Question 5: Only one reaction (i.e. using one of the subsrates, **12a**, reported in the manuscript) was performed at a substrate concentration higher than 10-20 mM. Even the different preparative-scale reactions only employed substrate concentrations of 10-20 mM (while using 100 mL reaction volume). I would like to understand why. Does the activity or enantioselectivity of the different HheC variants decrease significantly at higher substrate concentrations, except for substrate **12a**? Bromide is definitely a better leaving group that chloride. Or is substrate solubility a problem? The authors should elaborate on this in their manuscript to also highlight possible limitations of their approach.

Answer: We appreciate your insightful suggestion.

We fully agree that bromide is indeed a better leaving group than chloride. In our manuscript, to demonstrate the scalability of the biocatalytic dehalogenation reaction, we selected substrate **12a** for gram-scale preparation. This substrate was chosen because the variant HheC-εM7 exhibited the highest activity toward **12a** among all tested substrates. Both analytical (Supplementary Table 23) and preparative-scale reactions (20 mM, 100 mL; Page S64; Supplementary Information) were completed within 1 h. In contrast, the other substrates required significantly longer reaction times under the same conditions (Supplementary Tables 12–22 and 24–33). For example, substrate **8a** needed 48 h to reach completion (Supplementary Table 19).

In the gram-scale reaction experiment, we increased the substrate concentration to 120 mM (100 mL). Under these conditions, the reaction required approximately 57 h to reach completion (Page S70; Supplementary Information), indicating that elevated substrate concentrations led to reduced enzyme activity and a substantially prolonged reaction time.

According to your suggestion, we have now elaborated on this limitation in the revised manuscript to highlight this limitation of our approach (Page 12, lines 16-21).

"While these results demonstrate the scalability of the biocatalytic method, the reduced enzyme activity at elevated substrate concentration, reflected in the substantially prolonged reaction time, indicates the necessity for further improving enzyme activity to achieve more efficient synthesis."

Question 6: With respect to substrate solubility, the authors state in the method description for the large-scale biotransformation employing 120 mM **12a** that DMSO was added as cosolvent. In contrast, no DMSO addition is mentioned for reactions employing 10-20 mM haloalcohol substrate. What is the solubility limit of the used substrates in aqueous reaction media? The authors should add this information to their manuscript.

Answer: We appreciate your insightful suggestion.

We apologize for the lack of clarity in the original description. In all analytical-scale and preparative-scale biotransformations (10–20 mM), 1% (v/v) DMSO was used as a co-solvent. This concentration was sufficient to ensure substrate solubility under the standard reaction conditions. In the gram-scale reaction (120 mM) with substrate **12a** at an elevated concentration of 120 mM, the DMSO proportion was increased to 2% (v/v) to maintain substrate solubility throughout the reaction.

According to your suggestion, we have now clearly specified the use of DMSO in all biotransformation experiments in the Supplementary Information to for clarity ("Preparation and screening of *E. coli* (HHDH)" section; "Isotope-Labeling experiments section"; "Directed evolution of HheC-SM9 (HheC-εM1)" section; "Preparative-scale biocatalytic enantioselective dehalogenation section").

Reviewer #2 (Remarks to the Author):

Comments: In this well designed study by Wan and co-workers, new catalytic activity of halohydrin dehalogenase (HHDH) have been revealed, and the enzyme was found to catalyse enantioselective dehalogenative hydroxylation of ε-haloalcohols to form ε-diols. This is new biocatalytic reaction not previously described for HHDHs, that are known for their catalytic versatility by catalysing many non-natural reactions.

Screening of a set of wild-type HHDHs as well as HheG and HheC mutants (72 in total) revealed 3 active enzymes. The best variant HheC-L142A that showed low S-enantioslectivity and 19% yield was further engineered via structure guided directed evolution, achieving increased enantioselectivity and activity on a model substrate (**1a**). Substrate scope was found to be broad on aromatic ε-haloalcohols mostly concerning activity, however, high enantioselectivity was demonstrated only on 6 substrates while on majority the E value was low to moderate. Finally, this novel biotransformation was demonstrated on 2.3 g of selected substrate.

The mechanism of the reaction has been proposed, involving one-step hydrolytic dehalogenation assisted by aspartate D80 that activate water molecule. Catalytic triade Ser-Try-Arg (S132, Y145, R149) and halide-binding site (N176-Y187) remain of fundamental importance for catalytic activity as confirmed by mutagenesis experiments. My opinion is that this paper is a valuable contribution to the field of HHDH-catalysed reactions and biocatalysis in general. Overall, this well written manuscript presented a novel idea. Paper is clearly presented and well organized. All the claims in the manuscript are well supported by relevant experiments. I suggest acceptance of this article for publication in Nature Communications after minor revision.

Answer: We are very grateful to your encouraging comments and have revised the manuscript accordingly to improve its quality.

Question 1: Page 4, line 47: The sentence "Currently, Dehalogenative…" capital "D" should be corrected.

Answer: We appreciate your insightful suggestion.

According to your suggestion, the word "Dehalogenative" has now been corrected to lowercase (dehalogenative) as suggested.

Question 2: Page 5, line 13: The sentence "This This…" should be corrected.

Answer: We appreciate your insightful suggestion.

According to your suggestion, the duplication of "This" has been corrected accordingly.

Question 3: Page 8, lines 28-32: The sentence "This evidence indicates the emergence

of a novel dehalogenative mechanism" in the SM9 mutant, thereby representing a previous unrecognized catalytic strategy in hydrolytic dehalogenation" is not clear.
Answer: We appreciate your insightful suggestion.
According to your suggestion, we have now deleted the unclear sentence to avoid any ambiguity.

Question 4: Page 9, line 4: I suggest to remove word "exceptional".
Answer: We appreciate your insightful suggestion.
According to your suggestion, we have removed the word "exceptional" accordingly.

Question 5: Page 11, line 7: I suggest "high enantioselectivity" instead of "outstanding enantioselectivity", since it was 96-98%. Outstanding would be >99.9%.
Answer: We appreciate your insightful suggestion.
According to your suggestion, we have replaced "outstanding enantioselectivity" with "high enantioselectivity" to more accurately reflect the experimental data.

Question 6: Page 12, line 81: I suggest providing the range for enantiomeric purities rather then "up to >99% ee" since this particular value is related to only one product.
Answer: We appreciate your insightful suggestion.
According to your suggestion, we have revised the text to specify the range for enantiomeric purities to more accurately reflect the experimental data (Page 12, line 83).

Reviewer #3 (Remarks to the Author):
Comments: The manuscript "Engineered halohydrin dehalogenase mediates remote enantiocontrolled dehalogenative hydroxylation via an unconventional mechanism" by Xiao Jin et al. presents a comprehensive experimental and computational study of engineered HHDH mutants and their enzymatic activity toward 5-chloro-1-phenylpentan-1-ol. As a result, an unconventional mechanism of dehalogenative hydroxylation catalyzed by engineered HHDHs is proposed. The authors applied a range of standard biochemical, organic, and computational chemistry methods (SDM, SSM, NMR, HRMS, MD) to support their findings. The study includes interesting discussions, particularly regarding the investigation of the reaction mechanism, with attention to pH dependence and the application of isotopic labeling. However, I find that the novelty and significance of the work are not sufficiently emphasized. In addition, several technical issues are present in the current version of the manuscript. I believe that revision is necessary to improve the article.
Answer: We are very grateful to your encouraging comments and have revised the manuscript accordingly to improve its quality.

Main text:
Question 1: The authors refer to "a limited repertoire of reaction types" in both the abstract and the introduction, however, they do not introduce new reaction types in the

present work. The enzymatic conversion of halogen-containing compounds into alcohols or epoxides is not novel or unique and does not broaden "nature's catalytic repertoire." Also, flavin-dependent oxygenases, cytochrome P450 oxidases, and dehaloperoxidases are not directly connected to the processes discovered by the authors. I suggest that the authors modify the abstract and introduction to highlight the specific problems they are addressing and the possible applications of their findings. This would help the reader better understand the choice of the main substrate and how the described unconventional mechanism could be applied further.

Answer: We appreciate your insightful suggestion.

We agree that the original phrasing regarding "a limited repertoire of reaction types" and "nature's catalytic repertoire" was not optimally framed and could be misleading. According to your suggestion, we have revised both the Abstract and Introduction to more accurately reflect the scope and focus of our work. Specifically:

(1) The phrase "a limited repertoire of reaction types" has been replaced with "the narrow range of transformations".

(2) The term "nature's catalytic repertoire" has been replaced with "catalytic diversity of enzymes" (Page 2, lines 24-27).

(3) In addition, the text of "flavin-dependent oxygenases, cytochrome P450 oxidases, and dehaloperoxidases" has been removed from the Introduction section, as these enzymes are not directly connected to the processes described in this study. We believe these changes address your concerns and improve the overall clarity and accuracy of the manuscript.

Question 2: Page 4, lines 3-6: "Our investigation began with systematic evaluation of our HHDH enzyme library using racemic ε-haloalcohol **1a** (5-chloro-1-phenylpentan-1-ol) as the model substrate."

- Which specific halohydrin dehalogenases are included in the term HHDH in this study?
- What does the HHDH enzyme library consist of and how was it constructed?
- What was the rationale for selecting 5-chloro-1-phenylpentan-1-ol as the model substrate?

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have clarified the following points in the revised manuscript and Supplementary Information:

(1) Composition of the HHDH enzyme library: A complete list of all HHDHs screened in this study, including their sources and accession numbers, has been provided in Supplementary Table 1.

(2) Construction of the HHDH enzyme library: The HHDH enzyme library was constructed in our previous studies. A detailed description of the library composition and construction methods has now been included in the "Preparation and screening of *E. coli* (HHDH)" section of the Supplementary Information (Page S2), along with the relevant references for clarity.

(3) Rationale for selecting substrate **1a** (5-chloro-1-phenylpentan-1-ol) as the model ε haloalcohol: It contains a chlorine atom at the ε-position, which allows us to probe the

enzyme's ability to catalyze dehalogenation at a distal site. In addition, the phenyl group provides a strong UV chromophore, facilitating convenient detection and analysis by chiral HPLC. This selection is consistent with our previous studies, in which 3-chloro-1-phenylpropan-1-ol (Nat Commun, 2025, 16, 1170; doi:10.1038/s41467-025-56463-z) and 4-chloro-1-phenylbutan-1-ol (Commun Chem, 2025, 8, 337; doi:10.1038/s42004-025-01713-w) were used as model substrates for γ- and δ-haloalcohols, respectively. The present work extends this systematic approach to ε-haloalcohols.

Question 3: How were the amino acid residues selected for mutation (e.g., W249P, P84G, F186Y, N176Q)? The authors use the term "unexpectedly," which suggests that a specific strategy may have been applied for choosing not only the positions but the particular substitutions (W to P, P to G, F to Y, N to Q). Please clarify the rationale or methodology used to select these mutations.

Answer: We appreciate your insightful suggestion.

In the enzyme engineering experiments, the residues selected for mutation were chosen based on their positions in the active site, as identified from the docking pose of the substrate **1a** with the HheC-SM9 variant.

According to your suggestion, the term "unexpectedly" has been deleted from the manuscript to avoid any ambiguity regarding the mutation selection strategy. In addition, the rationale used to select these mutations has been clarified in the revised manuscript (Page 8, lines 41-52).

Question 4: I am not fully convinced by the aspartate–arginine dyad hypothesis, as the majority of the mutants did not show activity, not only those with mutations at D80 and R149. Would it be possible that SM9 and its improved variants exhibit catalytic promiscuity, potentially employing different catalytic centers depending on the substrate?

Answer: We appreciate your insightful suggestion.

We fully agree that the lack of activity in the majority of mutants, including but not limited to those targeting D80 and R149, suggests that the catalytic mechanism may be more complex than a D80-R149 dyad-dependent model.

As you rightly point out, it is possible that SM9 and its improved variants exhibit catalytic promiscuity, potentially employing different catalytic centers depending on the substrate. In fact, we observed that the SM9 variant retains dehalogenation activity toward β-, γ-, and δ-haloalcohols (substrates **1a1, 1a2, and 1a3**), forming the corresponding epoxides via intramolecular cyclization. This activity is still catalyzed by the native S132-Y145-R149 catalytic triad.

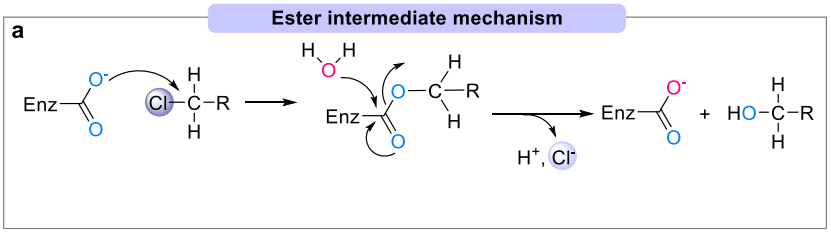
In contrast, for ε-haloalcohol **1a**, which undergoes an intermolecular dehalogenative hydroxylation to form the corresponding diol, we propose that the D80-R149 dyad may function as the catalytic residues. This hypothesis is drawn by analogy to mechanistically similar reactions reported in DL-2-haloacid dehalogenases and limonene-1,2-epoxide hydrolases (Page 8, lines 7-25), and provides a plausible explanation for how SM9 enables this unusual reactivity.

We also fully agree that other residues in the active site also play critical roles, as mutations at many positions led to complete loss of activity. These residues may contribute to substrate binding (S132 and Y145) or halide stabilization (the residues in halide-binding site: P175, N176, Y177, L178, F186, and Y187). Their importance is now more explicitly discussed in the revised manuscript (Page 7, lines 40-43).

Question 5: Fig. 3a should be corrected: the oxygen atom in the product should be shown in blue, in accordance with the depicted mechanism.

Answer: We appreciate your insightful suggestion.

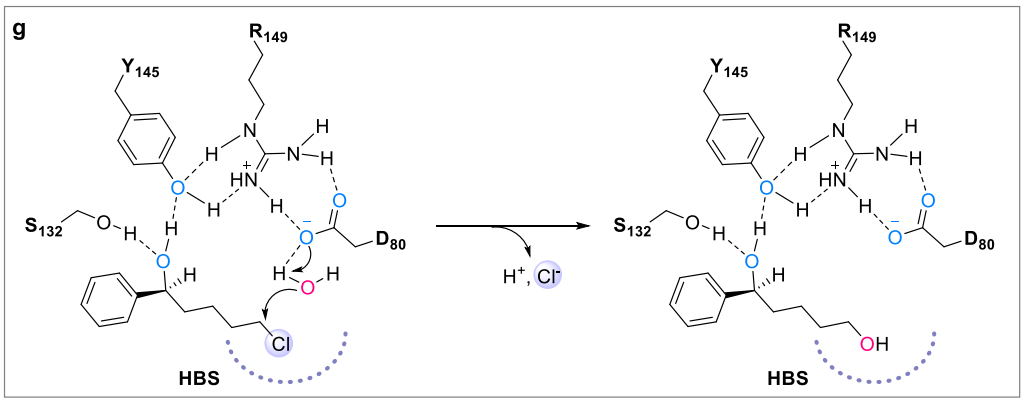
According to your suggestion, we have corrected Fig. 3a by changing the color of the oxygen atom in the product to blue, in accordance with the color scheme used in the depicted mechanism. The revised figure has been updated in the manuscript.



Question 6: Fig. 3g should be corrected: please verify the protonation states of Y145 and R149, as their pKa values are above 10. These residues are most likely protonated at pH 8.5. If protonated states are correct, please check a "+" of the nitrogen of R149 as the nitrogen presently has three covalent bonds.

Answer: We appreciate your insightful suggestion.

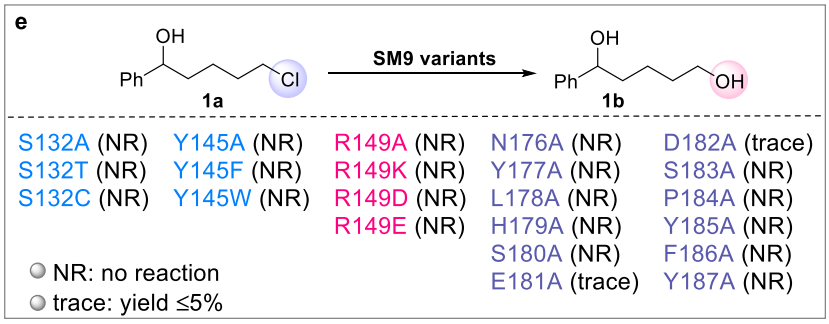
We apologize for the mistake. According to your suggestion, we have revised the Fig. 3g to correctly represent its protonated state with four covalent bonds (including the proton). The figure has been updated accordingly in the revised manuscript.



Question 7: The plus sign in Fig. 3e (+S132A, +N176A) is not necessary, as it does not indicate the type of the wild type but may be misleading to the reader.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have now deleted the plus signs (+) in Fig. 3e to avoid any potential confusion for the reader. The revised figure has been updated in the manuscript.



Supplementary Information:

Question 1: In the molecular dynamics simulations, thermostat and barostat are not specified.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have now specified the thermostat and barostat parameters used in the molecular dynamics simulations. Specifically, the Langevin thermostat was employed for temperature control, and the Berendsen barostat was used for pressure regulation. These details have been added to the "Molecular dynamics simulations" section of the Supplementary Information (Page S3).

Question 2: A representative snapshot of HheC-SM9 was used for docking"

- How was this snapshot chosen?
- Did the authors perform MD simulations for the protein–ligand complex?

Answer: We appreciate your insightful suggestion.

The representative snapshot of HheC-SM9 used for docking was selected after the system reached equilibrium, based on stabilization of the RMSD profile at approximately 30 ns into the molecular dynamics simulation. This indicated that the protein conformation had converged to a stable state suitable for docking studies. We did not perform molecular dynamics simulations on the protein–ligand complex. The docking pose was selected based on the proposed catalytic mechanism illustrated in Fig. 3g. This mechanistic rationale guided the docking orientation and placement of the substrate within the active site.

According to your suggestion, these clarifications have now been added to the "Docking study" section in the Supplementary Information (Page S5).

Question 3: The authors aim to investigate conformational changes in the protein; however, a 200 ns simulation may not be sufficient for this purpose.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have extended the molecular dynamics simulations to 600 ns to better sample the conformational landscape of the protein. The updated results have been incorporated into the revised manuscript (Page 4, lines 93-95) and Supplementary Information (Supplementary Fig. 1, 2, and 3). We believe the extended simulations provide a more basis for understanding the structural dynamics.

Question 4: RMSD values of all atoms may be less informative than RMSD of the protein backbone or Cα atoms for tracing conformational changes. The authors may consider preparing a figure showing these RMSD values to better illustrate the protein's structural dynamics.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have now provided the RMSD values of the Cα atoms to better illustrate the protein's structural dynamics. The corresponding figure has been updated in the Supplementary Information (Supplementary Fig. 1).

Question 5: Supplementary Table 7: What does entry #1 represent? Why is (*R*)-**1a3** indicated as a product when its ee varies from 5% to 99%? Wasn't (*S*)-**1a3** present in entries 1, 3, 4, and 5? The same issue applies to Tables 4–9. Since the reaction is stereoselective and only the (*S*)-isomer undergoes transformation, only the (*R*)-isomer of the starting compound should remain. It would be interesting to understand what determines this specificity, and the authors may provide an explanation based on their MD simulation results.

Answer: We appreciate your insightful suggestion.

We apologize for the lack of clarity in the Supplementary Table 7. Below we address each point:

(1) Entry #1 represents a cell control reaction carried out using *E. coli* cells in the absence of the HheC enzyme. We have now added this clarification to the table legend.

(2) We initially intended to denote the remaining substrate enantiomer after kinetic resolution as (*R*)-**1a3**, since the active biotransformation reactions exhibited *S*-enantioselectivity.

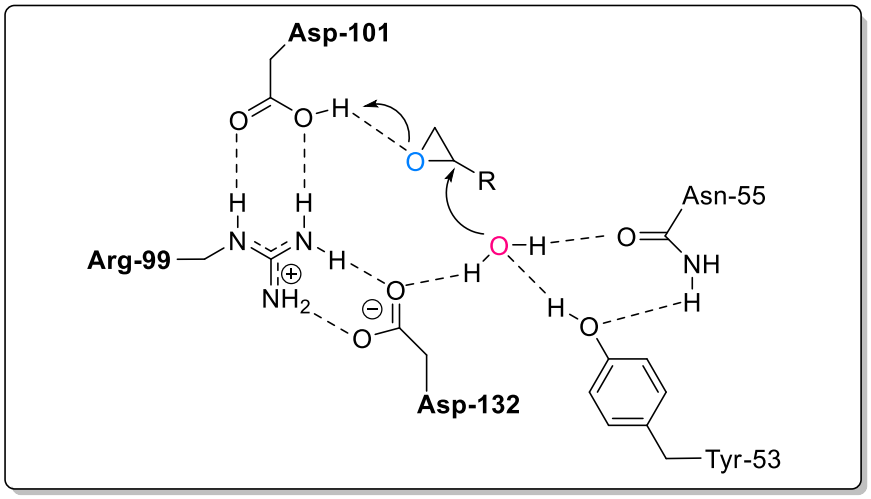
According to your suggestion, we have now revised the table scheme by using an asterisk (*) to indicate the stereocenter of **1a3** and have specified the *R* or *S* configuration directly within the *ee* values for both **1a3** and **1c3**. This modification provides a clearer representation of the reaction outcomes and has been applied consistently to Tables 4–9.

(3) In Table 7, we specifically investigated the dehalogenative cyclization activity of HheC-WT, HheC-SM9, HheC-SM9+D80A, and HheC-SM9+D182A toward δ-haloalcohol **1a3**. We fully agree that understanding the molecular basis of this enantioselectivity is of great interest. The enantioselectivity for the dehalogenative cyclization of δ-haloalcohol **1a3** has been investigated in our previous study (Commun Chem, 2025, 8, 337; doi:10.1038/s42004-025-01713-w). In that work, we elucidated how residues in the halide-binding site and *R*-group binding site control the binding orientation of the substrate enantiomers, thereby dictating the observed enantiopreference.

Question 6: Supplementary Fig. 8 should be revisited. If Asp-132 is protonated, there is no need to show a "–" sign. At physiological pH or higher, however, aspartate is typically deprotonated.

Answer: We appreciate your insightful suggestion.

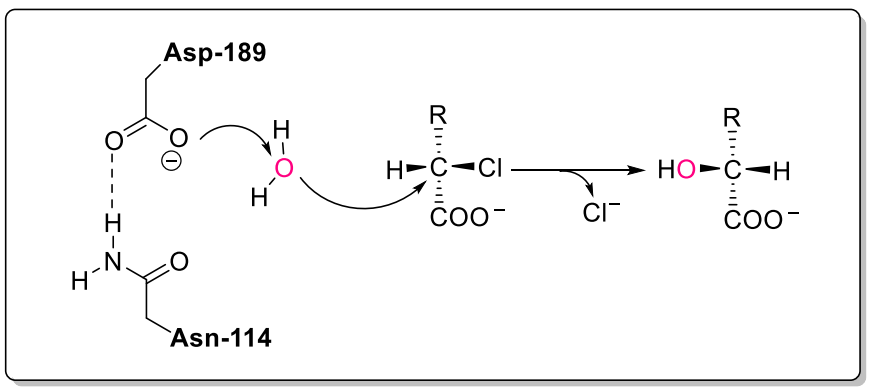
We apologize for the mistake. According to your suggestion, we have revised Supplementary Fig. 8 by removing the proton from Asp-132 and retaining the negative charge to accurately reflect its deprotonated state at physiological pH. In addition, the reaction conditions have now been included in the figure legend, along with a reference (EMBO J, 2023, 22, 2583-2592; doi: 10.1093/emboj/cdg275). The corrected figure has been updated in the Supplementary Information.



Question 7: Supplementary Fig. 9: The color code is inconsistent for the HOH molecule and the OH group of the product. The arrows indicating stereochemistry of the substituents should be corrected. It would also be helpful to indicate the reaction conditions or provide a reference. Note that Fig. 4c of Ref. 52 suggests leaving a chlorine anion rather than a hydrogen.

Answer: We appreciate your insightful suggestion.

We apologize for the mistake. According to your suggestion, we have revised the Supplementary Fig. 9 accordingly. The color code for the HOH molecule and the OH group of the product has been corrected to ensure consistency. The arrows indicating the stereochemistry of the substituents and the leaving group (Cl^-) have been revised in accordance with the Ref. 52 in manuscript (J Mol Biol, 2008, 378, 284-294; doi: 10.1016/j.jmb.2008.02.035). In addition, the reaction conditions have now been included in the figure legend, along with a reference (J Bacteriol, 1992, 174, 1932-1940; doi:10.1128/jb.174.6.1932-1940.1992). The corrected figure has been updated in the Supplementary Information.

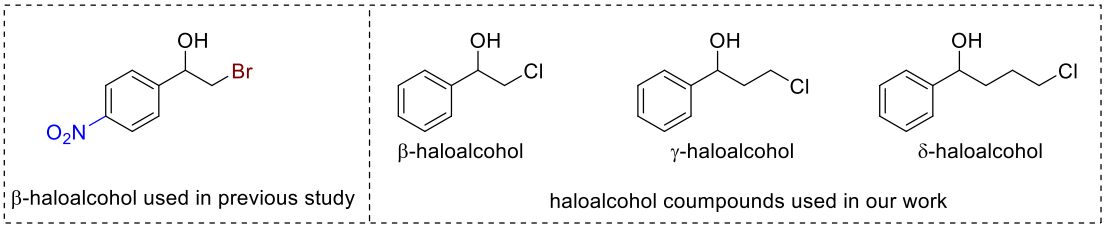


Response to Reviewers

Reviewer #1 (Remarks to the Author):
Comments: The authors revised their manuscript according to the reviewer comments. However, I still have a few further remarks that need the authors' attention.
Answer: We are very grateful to your encouraging comments and have revised the manuscript accordingly to improve its quality.

Question 1: Assignment of enantiomers in chiral analysis: If I understand the authors correctly, they have assigned the (*R*)- and (*S*)-enantiomers of all substrates and products based on the assumption that their HheC mutant exhibits *S*-selectivity. But how can the authors know that and be sure that this is true for all their substrates? They only confirmed the stereochemistry for one single product by single crystal X-ray diffraction. Can the authors indeed be sure that the enzyme (or the different tested mutants) exhibits *S*-selectivity with each tested substrate?
Answer: We appreciate your insightful suggestion.
Assigning the absolute configuration of chiral products by analogy, based on reaction stereoselectivity, is an acceptable approach in asymmetric catalysis, particularly for analyzing the absolute configuration of newly synthesized chiral compounds. This method has been employed in both enzymatic asymmetric synthesis (*Nat. Commun.*, 2026, 17, 646; *J. Am. Chem. Soc.*, 2019, 141, 25, 9798–9802; *Nat. Synth.*, 2024, 3, 1340–1348) and chemical asymmetric synthesis studies (*Nat. Commun.*, 2025, 16, 6078; *Nature*, 2018, 562, 105–109; *J. Am. Chem. Soc.*, 2026, doi:10.1021/jacs.6c00454).

Question 2: Page 7, lines 5-25: Reading this paragraph again with the adjusted wording regarding mutation D80A, I am wondering if this paragraph is really relevant for the overall manuscript. The authors mention already on page 4 the impact of mutation D80A on the activity of HheC SM9 in the dehalogenative hydroxylation of ε-haloalcohols. Now in the paragraph on page 7, the focus is on the dehalogenative cyclization of β-, γ- and δ-haloalcohols. That HheC WT and mutant SM9 are able to perform this reaction is either already known from previous literature or, in my opinion, not really relevant for the current manuscript. Hence, my suggestion would be to remove this paragraph and to add the information regarding the importance of residue D80 for HheC activity already on page 4. However, if the authors deem the information in the paragraph on page 7 important for the overall manuscript, they should rewrite it to better highlight the purpose and relevance of these results for the overall manuscript.
Answer: We appreciate your insightful suggestion.
A previous study (*EMBO J.*, 2003, 22, 4933-4944) demonstrated that the D80A mutation completely abolishes HheC activity by disrupting the proton relay from Arg149 to the solvent, using the β-haloalcohol *para*-nitro-2-bromo-1-phenylethanol as a substrate. However, this substrate is structurally different from our tested β-haloalcohol, 2-chloro-1-phenylethan-1-ol. Furthermore, no other studies have reported whether the D80A mutant exhibits dehalogenation activity toward γ- and δ-haloalcohols.



In the original paragraph (Page 7, lines 5-25), we aimed to investigate the importance of residues A142 and D80 in the HheC-SM9 mutant for dehalogenative activity. Our results revealed that introducing the L142A mutation into HheC-WT largely maintained its activity toward β- and δ-haloalcohols, while reduced its activity toward γ-haloalcohol. In contrast, introducing the D80A mutation into SM9 completely abolished its activity toward β-, γ-, and δ-haloalcohols. These results indicate that the L142A mutation confers upon HheC the ability to catalyze ε-haloalcohols without losing its original dehalogenative cyclization activity. On the other hand, residue D80 is not only important for the dehalogenative cyclization of β-, γ-, and δ-haloalcohols, but also crucial for the dehalogenative hydroxylation of ε-haloalcohols.

Nevertheless, we acknowledge that the original paragraph may not have clearly conveyed our research intent. Following your suggestion, we have now rewritten this paragraph to better highlight the purpose and relevance of these results for the overall manuscript. The revised paragraph is as follows:

"We subsequently evaluated the importance of residues A142 and D80 in HheC-SM9 mutant by assessing the dehalogenation cyclization activity of HheC-WT, SM9, and SM9/D80A against β-, γ-, and δ-haloalcohols (**1a1**, **1a2**, **1a3**), which yield the corresponding epoxide (**1c1**), oxetane (**1c2**), and tetrahydrofuran (**1c3**), respectively (Fig. 3d, see Supplementary Tables 5–7 for details). The results indicated that while HheC-WT exhibited activity toward the three tested substrates, it was inactive toward the longer-chain ε-haloalcohol substrate **1a** in our initial screening. Additionally, the SM9 mutant maintained dehalogenation activity across all three substrates, albeit with notably diminished efficiency observed specifically for γ-halohydrin **1a2**. These results suggest that the L142A mutation confers upon HheC the ability to catalyze ε-haloalcohols without losing its original dehalogenative cyclization activity. However, the SM9/D80A mutant was completely inactive toward all tested substrates. Residue D80, previously shown to be essential for proton delivery in the dehalogenative cyclization reaction by shuttling a proton to the solvent after Y145 deprotonation, was found to be equally indispensable for the dehalogenative hydroxylation pathway. "

Question 3: Page 4, line 91: The authors performed a new MD simulation for 600 ns and adjusted the manuscript descript in the SI accordingly. In the main manuscrit text, however, they still mention the 200 ns MD simulation. This should be corrected.

Answer: We appreciate your insightful suggestion.

We apologize for not updating the MD simulation duration in the main manuscript. According to your comment, we have now corrected the simulation time from 200 ns to 600 ns in the revised manuscript.

Reviewer #3 (Remarks to the Author):

Comments: The authors have carefully revised their manuscript and answered my questions. I recommend the manuscript for publication.

Answer: We are very grateful to your encouraging comments.

Response to Reviewers

Reviewer #1 (Remarks to the Author):

Comments: The authors replied to and addressed my comments carefully. They also adjusted the paragraph on page 7, however, I am still not fully satisfied with this new version. I suggest the following changes to make it easier for the reader to follow the authors' argumentation.

Answer: We are very grateful to your encouraging comments and have revised the manuscript accordingly to improve its quality.

Question: Lines 5-12: "We subsequently evaluated ... dehalogenation cyclization activity of HheC-WT, mutant SM9 (containing mutation L142A), and mutant SM9/D80A (containing mutations L142A and D80A) against ..."

Line 21: replace "catalyze" by "convert".

Answer: We appreciate your insightful suggestion.

Following the reviewer's further suggestions, we have revised the corresponding sentence as follows:

"We subsequently evaluated the dehalogenation cyclization activity of HheC-WT, mutant SM9 (containing mutation L142A), and mutant SM9/D80A (containing mutations L142A and D80A) against β-, γ-, and δ-haloalcohols (**1a1**, **1a2**, **1a3**), which yield the corresponding epoxide (**1c1**), oxetane (**1c2**), and tetrahydrofuran (**1c3**), respectively."

Additionally, the word "catalyze" has been replaced with "convert" as suggested.

The manuscript “Engineered halohydrin dehalogenase mediates remote enantiocontrolled dehalogenative hydroxylation via an unconventional mechanism” by Xiao Jin et al. presents a comprehensive experimental and computational study of engineered HHDH mutants and their enzymatic activity toward 5-chloro-1-phenylpentan-1-ol. As a result, an unconventional mechanism of dehalogenative hydroxylation catalyzed by engineered HHDHs is proposed. The authors applied a range of standard biochemical, organic, and computational chemistry methods (SDM, SSM, NMR, HRMS, MD) to support their findings. The study includes interesting discussions, particularly regarding the investigation of the reaction mechanism, with attention to pH dependence and the application of isotopic labeling. However, I find that the novelty and significance of the work are not sufficiently emphasized. In addition, several technical issues are present in the current version of the manuscript. I believe that revision is necessary to improve the article.

Main text:

1. The authors refer to “a limited repertoire of reaction types” in both the abstract and the introduction, however, they do not introduce new reaction types in the present work. The enzymatic conversion of halogen-containing compounds into alcohols or epoxides is not novel or unique and does not broaden “nature’s catalytic repertoire.” Also, flavin-dependent oxygenases, cytochrome P450 oxidases, and dehaloperoxidases are not directly connected to the processes discovered by the authors.
   I suggest that the authors modify the abstract and introduction to highlight the specific problems they are addressing and the possible applications of their findings. This would help the reader better understand the choice of the main substrate and how the described unconventional mechanism could be applied further.

2. Page 4, lines 3-6: “Our investigation began with systematic evaluation of our HHDH enzyme library using racemic ε-haloalcohol 1a (5-chloro-1-phenylpentan-1-ol) as the model substrate.”
   – Which specific halohydrin dehalogenases are included in the term HHDH in this study?
   – What does the HHDH enzyme library consist of and how was it constructed?
   – What was the rationale for selecting 5-chloro-1-phenylpentan-1-ol as the model substrate?

3. How were the amino acid residues selected for mutation (e.g., W249P, P84G, F186Y, N176Q)? The authors use the term “unexpectedly,” which suggests that a specific strategy may have been applied for choosing not only the positions but the particular substitutions (W to P, P to G, F to Y, N to Q). Please clarify the rationale or methodology used to select these mutations.

4. I am not fully convinced by the aspartate–arginine dyad hypothesis, as the majority of the mutants did not show activity, not only those with mutations at D80 and R149. Would it be possible that SM9 and its improved variants exhibit catalytic promiscuity, potentially employing different catalytic centers depending on the substrate?

5. Fig. 3a should be corrected: the oxygen atom in the product should be shown in blue, in accordance with the depicted mechanism.

6. Fig. 3g should be corrected: please verify the protonation states of Y145 and R149, as their pKa values are above 10. These residues are most likely protonated at pH 8.5. If protonated states are correct, please check a "+" of the nitrogen of R149 as the nitrogen presently has three covalent bonds.

7. The plus sign in Fig. 3e (+S132A, +N176A) is not necessary, as it does not indicate the type of the wild type but may be misleading to the reader.

Supplementary Information:

1. In the molecular dynamics simulations, thermostat and barostat are not specified.

2. "A representative snapshot of HheC-SM9 was used for docking"
   – How was this snapshot chosen?
   – Did the authors perform MD simulations for the protein–ligand complex?

3. The authors aim to investigate conformational changes in the protein; however, a 200 ns simulation may not be sufficient for this purpose.

4. RMSD values of all atoms may be less informative than RMSD of the protein backbone or Cα atoms for tracing conformational changes. The authors may consider preparing a figure showing these RMSD values to better illustrate the protein's structural dynamics.

5. Supplementary Table 7:
   – What does entry #1 represent?
   – Why is (R)-1a3 indicated as a product when its ee varies from 5% to 99%? Wasn't (S)-1a3 present in entries 1, 3, 4, and 5? The same issue applies to Tables 4–9.
   – Since the reaction is stereoselective and only the (S)-isomer undergoes transformation, only the (R)-isomer of the starting compound should remain. It would be interesting to understand what determines this specificity, and the authors may provide an explanation based on their MD simulation results.

6. Supplementary Fig. 8 should be revisited. If Asp-132 is protonated, there is no need to show a "–" sign. At physiological pH or higher, however, aspartate is typically deprotonated.

7. Supplementary Fig. 9: The color code is inconsistent for the HOH molecule and the OH group of the product. The arrows indicating stereochemistry of the substituents should be corrected. It would also be helpful to indicate the reaction conditions or provide a reference. Note that Fig. 4c of Ref. 52 suggests leaving a chlorine anion rather than a hydrogen.