nature portfolio

Peer Review File

Divergent Evolution of Fungal P450 Monooxygenase Unlocks Simultaneous Access to C12β and C15α Oxyfunctionalization of Steroids

Corresponding Author: Professor Aitao Li

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Wang et al. presents a significant advancement in fungal P450 engineering for regioselective steroid hydroxylation. It is well known that engineering fungal P450 enzymes poses considerable challenges due to the absence of crystal structures, their membrane-bound nature, and the difficulty in achieving efficient heterologous expression. To address these issues, the authors employed a combined SSM and FRISM mutagenesis approach to facilitate the divergent evolution of the fungal P450 CYP68J5_fg. This strategy successfully yielded two variants capable of catalyzing C12β- and C15α-hydroxylation with high selectivity and activity, providing an important example of overcoming the classical trade-off between selectivity and activity in enzyme engineering.
Molecular dynamics simulations revealed that key mutations alter the polarity landscape of the enzyme's active site and reconfigure hydrogen-bonding networks, thereby directing hydroxylation to distinct positions. Additionally, high-density fermentation achieved unprecedented product titers exceeding 10 g/L, which represent the highest yields reported to date for yeast-based steroid hydroxylation. Importantly, these products are valuable intermediates for the synthesis of C12- and C15-functionalized steroid drugs, such as drospirenone, as well as C-nor D-homo derivatives.
Overall, this study provides valuable insights into the precise control of regio- and stereoselectivity through the evolution of CYP68J5_fg mutants. It also highlights the utility of computational simulations in engineering non-structure-dependent P450 enzymes derived from fungi. In addition, the experimental work appears thorough and convincing, and the manuscript is generally well written. I recommend its publication after addressing the following points to enhance clarity, scientific rigor, and impact:

1. Justification of why SSM+FRISM was chosen over established methods like CASTing. Compare library sizes/screening efforts (e.g., Line 153: "small collections" is vague; quantify variant numbers screened).
2. In Table 1, substrate 6 (androstenedione) shows reduced conversion to testosterone, likely due to endogenous reductases. Is it possible to identify which reductase(s) are responsible for this reduction? Additional discussion or experiments addressing this point would strengthen the results.
3. Isolation yields for 1a (45.1%) and 1b (71.6%) differ significantly (Lines 328, 342). Please discuss potential reasons for this discrepancy, such as cytotoxic effects or losses during purification.Some errors and typos:
4. There are some typographical errors and inconsistent terminology:
Line 175: "catalzyed" should be corrected to "catalyzed."
Line 363: "chemocatalytic" should be corrected to "chemoenzymatic." Please use "chemoenzymatic" consistently throughout the manuscript.
Figures 3b and 3c: Please explicitly define "activity"—for example, specify whether it refers to conversion rate, turnover number, or another metric for clarity.
5. The abstract could be slightly shortened to improve conciseness.

Reviewer #2

(Remarks to the Author)
In the manuscript entitled "Divergent Evolution of Fungal P450 Monooxygenase Unlocks Simultaneous Access to C12β and

C15α oxyfunctionalization of Steroids", Wang et al. performed divergent evolution with combined structure-guided site-saturation mutagenesis and focused rational iterative site-specific metagenesis, achieving rather good selectivity for C12beta and C15alpha while simultaneously enhancing catalytic efficiency. the N-oxidation selective potential of P450BM3 for N-heterocyclic compounds. The reason behind the mutations was further uncovered by MD simulations. Importantly, this work represents a first example of the divergent evolution of a fungal P450. The manu. is well-written and the results seem interesting. I suggest a major revision before its publication due to following concerns.

1. The labels of the substrates (at least 1) may be supplied.

2. Is the regioselectivity coupled with the stereoselectivity?

3. I am curious about the performance of W15M4 and W12M4 for substrate 3, 4 5 and 6. Only some have been tested. Why other mutants were used in Table 1?

4. it is quite confusing to use "pose 12" and "pose 15".

5. The binding conformations shown in Figure 4 are not clear. Additionally, the polar H atoms may be shown in all related figures to make them clear.

6. Optimized geometries (it seems key information is missing in Figure S4) and energy profiles obtained with QM are missing.

7. It is confusing to say "To obtain the optimal geometry for the transition state (TS), the calculations were performed using unrestricted density functional theory (UDFT) with the hybrid functional B3LYP as implemented in the Gaussian 16 program package."

8. Instead of 6-31G(d,p)+, it is more common to use 6-31+G(d,p).

9. More details may be supplied for how the binding conformations are obtained. For example, whether the chosen conformation is the most favorable one?

10. What is the reference value (distQM) in Figure 4, the distance between Cpd I and hydrogen atoms or carbon atoms? Why use values of the transition state?

11. The energy difference between doublet and quartet states is 3.2 kcal/mol in supplementary Table 3. However, it is only 0.1 kcal/mol in supplementary Table 4. What is the reason?

12. According to Figure S5b, it is hard to explain the C15 reactivity of WT enzyme.

13. It would be interesting to see what happened at 20 ns in Figure S5C.

Reviewer #3

(Remarks to the Author)
The C12β- or15α-hydroxylated steroids are leading compounds in developing steroid pharmaceuticals and medicines. However, it is a challenging role for regioselective and stereoselective hydroxylation of steroid compounds either by chemical or biological approaches. Cytochrome P450 enzymes are known as an important biological hydroxylase for biosynthesis of steroid hormones in vivo. This manuscript presents a groundbreaking work for engineering fungal P450 CYP68J5_fg to achieve highly selective C12β- and C15α-hydroxylation of steroids. The combination of structure-guided SSM and FRISM mutagenesis successfully overcomes the classic activity-selectivity trade-off, yielding two variants (W12M5 and W15M4) with exceptional regioselectivity (>97%). MD simulations revealed that the key mutations reshape the substrate-binding conformation, thereby directing hydroxylation to distinct positions (C12β or C15α). The industrial relevance is supported by unprecedented titers (up to 14.1 g/L) in P. pastoris fermentation. The manuscript is suitable for publishing in Nature Communications after a further revision.
1) From the perspective of directed evolution, this is a surprisingly smooth job! The first step of directed evolution screening based on SSM strategy is a routine operation, but I am a little concerned about calling the second stage of directed evolution FRISM. From the description in the text, it appears that this is somewhat different from the classic FRISM. The author doesn't even need to perform a few limited tests at the target site, essentially just cross combining the single mutations obtained through the SSM strategy in the first round. Although the results may seem perfect, a more accurate description of this process should still be considered.
2) In line 63, the authors describe that "fungal P450 enzymes demonstrate superior suitability for industrial-scale production", please provide more evidences or references.
3) As shown in Figure 2a, the dehydroxylated product 1c can be formed in this reaction. The direct dihydroxylation of steroids is also very important and interesting (see recent example, Wang et al. Catal. Sci. Technol., 2025, 4170-4178.). It is observed that there is an unknown product by W12M1 in Figure 3d. Is this compound a dihydroxylation product? I am wondered if the author has detected and identified the generation of dihydroxy products during directed evolution. If so, it maybe your next interesting paper.
4) In Table 1, can the selectivity and conversion rate be accurately measured to two decimal places?

5) In line 329, the authors describe that “the low yield of 1a is potentially due to the cytotoxicity of 1a”, please provide the experiment details or references.
6) In line 382 and 383, the authors describe that “the integrated approach of SSM coupled with FRISM strategy effectively modulates selectivity and enhances activity in these fungal-derived P450 enzymes”, to some extent, the approach may just effectively suitable for CYP68J5_fg, it would be better to state more precisely.
7) Except for substrate 6, the other four structurally similar substrates all showed excellent selectivity and conversion rates. Did the author also examine for other substrates? If so, it is best to report the results, even if it is only included in the supporting data. Because natural steroid hydroxylation P450 enzymes often have very strict substrate limitations. Similar results are of great significance for the development of substrate-promiscuity P450 isoenzymes through protein engineerings.
8) English language and style are required to fine spell check, such as “in line 175, catalzyed should be catalyzed; in line 324, W1M4 should be W15M4.”

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All of my concerns have been satisfactorily addressed. The manuscript is now suitable for publication. Congratulations to the authors on this excellent work!

Reviewer #2

(Remarks to the Author)
None.

Reviewer #3

(Remarks to the Author)
I have carefully reviewed the revised manuscript. All of my concerns have been solved. I believe this is a nice work for regio and stereoselective hydroxylation of steroids compounds, thereby providing an efficient enzymatic approach for subsequent development of steroids pharmerceuticals, e.g., combining with chemical catalysis as discussed in the paper. This reviewer suggests to publish the paper as is.

Point-by-point response to the reviewers' comments

Reviewer #1: The manuscript by Wang et al. presents a significant advancement in fungal P450 engineering for regioselective steroid hydroxylation. It is well known that engineering fungal P450 enzymes poses considerable challenges due to the absence of crystal structures, their membrane-bound nature, and the difficulty in achieving efficient heterologous expression. To address these issues, the authors employed a combined SSM and FRISM mutagenesis approach to facilitate the divergent evolution of the fungal P450 CYP68J5_fg. This strategy successfully yielded two variants capable of catalyzing C12β- and C15α-hydroxylation with high selectivity and activity, providing an important example of overcoming the classical trade-off between selectivity and activity in enzyme engineering.

Molecular dynamics simulations revealed that key mutations alter the polarity landscape of the enzyme's active site and reconfigure hydrogen-bonding networks, thereby directing hydroxylation to distinct positions. Additionally, high-density fermentation achieved unprecedented product titers exceeding 10 g/L, which represent the highest yields reported to date for yeast-based steroid hydroxylation. Importantly, these products are valuable intermediates for the synthesis of C12- and C15-functionalized steroid drugs, such as drospirenone, as well as C-*nor*-D-*homo* derivatives.

Overall, this study provides valuable insights into the precise control of regio- and stereoselectivity through the evolution of CYP68J5_fg mutants. It also highlights the utility of computational simulations in engineering non-structure-dependent P450 enzymes derived from fungi. In addition, the experimental work appears thorough and convincing, and the manuscript is generally well written. I recommend its publication after addressing the following points to enhance clarity, scientific rigor, and impact:

1. Justification of why SSM+FRISM was chosen over established methods like CASTing. Compare library sizes/screening efforts (e.g., Line 153: "small collections" is vague; quantify variant numbers screened).

Answer: Thank you very much for your feedback and meticulous review of our manuscript.

Regarding the rationale for selecting SSM+FRISM over CASTing: the SSM+FRISM is a well-established methodology (*J. Am. Chem. Soc.* 2019, 141, 19, 7934-7945; *Mol. Catal.* 2024, 553, 113755) that allows for the generation of a focused library with significantly reduced screening effort. While CASTing (Combinatorial active-site saturation test) is effective, it requires screening a large number of variants. In contrast, our approach enabled us to identify the best variants by screening only 29 total mutants for both the C12*β* and C15*α* hydroxylation of steroids.

Regarding the library sizes and screening efforts for the two methods: In our study, FRISM enabled efficient screening of just 29 mutants for both C12*β* and C15*α* oxyfunctionalizations of steroids. In contrast, the CASTing approach requires screening of 96 mutants (2×2×2×2×2×3=96, 3 times oversampling to ensure≥95% library coverage) for C12*β*-hydroxylation and 324 mutants (6×3×3×2×3=324, 3 times oversampling to ensure≥95% library coverage) for C15*α*-hydroxylation, totaling 420 mutants.

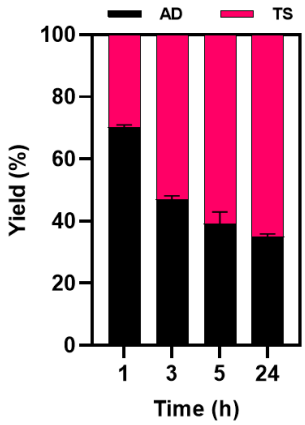
Regarding the term "small collections" in the current line 148-149 (previously line 153), we have added the specific number of screened mutants ("by just screening 29 variants") on line 188 to ensure logical coherence of the manuscript. Furthermore, the term "small collections" of this statement on line 148 has been adjusted.

2. In Table 1, substrate **6** (androstenedione) shows reduced conversion to testosterone, likely due to endogenous reductases. Is it possible to identify which reductase(s) are responsible for this reduction? Additional discussion or experiments addressing this point would strengthen the results.

Answer: We thank the reviewer for this insightful comment.

Extensive studies have characterized yeast-mediated specific 17*β*-reduction of steroids. As demonstrated in the article "Dehydroepiandrosterone (DHEA) metabolism in *Saccharomyces cerevisiae* expressing mammalian steroid hydroxylase

CYP7B: Ayr1p and Fox2p display 17beta-hydroxysteroid dehydrogenase activity (Yeast, 2002, 19(10): 873-86.)", the reductase AYR1 (1-acyl dihydroxyacetone phosphate reductase) can reduce the 17-carbonyl group of DHEA to a hydroxyl group. Our experimental validation confirmed that AYR1 indeed converts androstenedione (AD) to testosterone (TS) (**Supplementary Fig. 9**). Our future work will be focusing on optimizing the yeast chassis (such as to knock out the AYR1 gene) to minimize C17-reduction of steroids. All relevant experiments and statements have been added in both the main text and supplementary information.



Supplementary Figure 9. Bioconversion of 1 mM AD by AYR1 from *P. pastoris* in *E. coli*. Reaction conditions: 3 mL *E. coli* cell lysate containing 1 mM AD, 3% (w/v) glucose, 1 U/mL glucose dehydrogenase (GDH), and 0.2 mM NAD(P)+ cofactor.

3. Isolation yields for **1a** (45.1%) and **1b** (71.6%) differ significantly (Lines 328, 342). Please discuss potential reasons for this discrepancy, such as cytotoxic effects or losses during purification.

Answer: Thank you very much for the comments.

The variance in the isolated yields of **1a** and **1b** can be attributed to the following:

(1) Different catalytic efficiency: the biocatalytic conversion of **1** to **1a** reached approximately 75%, which is lower than its conversion of **1** to **1b** (approximately 90%).

(2) Different isolation approaches: **1a** was purified by column chromatography

because of the large amount of remaining substrate. This approach resulted in substantial loss due to the co-elution of **1** and **1a**, a consequence of their similar polarities. Conversely, the higher conversion rate for **1b** allowed for its efficient isolation through a high-yielding recrystallization.

4. Some errors and typos:

There are some typographical errors and inconsistent terminology:

Line 175: "catalzyed" should be corrected to "catalyzed."

Line 363: "chemocatalytic" should be corrected to "chemoenzymatic." Please use "chemoenzymatic" consistently throughout the manuscript.

Figures 3b and 3c: Please explicitly define "activity"—for example, specify whether it refers to conversion rate, turnover number, or another metric for clarity.

Answer: Thank you for your valuable feedback. We have addressed all the points raised:

1) On the current line 172 (previously Line 175), "catalzyed" has been corrected to "catalyzed".
2) On the current line 370 (previously Line 363), "chemocatalytic" has been replaced with "chemoenzymatic", and this term was used consistently throughout the manuscript.
3) The term "activity" now clearly refers to the conversion rate of steroid substrates, and this clarification has been added in line 155 to the revised manuscript.

5. The abstract could be slightly shortened to improve conciseness.

Answer: Thank you very much for your feedback.

We have streamlined the abstract to keep it concise, and a revised version of the abstract is presented below:

"Steroidal C12β/15α-hydroxylation are pivotal in synthesizing steroid drugs but remain challenging *via* chemical and biological methods. To address this, the fungal P450 monooxygenase CYP68J5_fg underwent structure-guided divergent evolution. Two optimized variants, W12M5 (F107S/Q112R/N295T/V299T/R368K) and W15M4

(Q112C/D126V/V299L/A362M) were created, achieving exceptional selectivity (97.7% for C12*β*- and 99.6% for C15*α*-hydroxylation of progesterone) alongside enhanced catalytic efficiency, effectively overcoming the classic activity-selectivity trade-off. Molecular dynamics simulations revealed that key mutations reorient the substrate by reshaping the binding pocket's polarity and hydrogen-bonding network, enabling hydroxylation at distinct positions. High-density fermentation with engineered *Pichia pastoris* yielded unprecedented titers of 4.6 g/L 12*β*-OH progesterone, 10.9 g/L 15*α*-OH progesterone and 14.1 g/L 15*α*-OH androstenedione. These products serve as key intermediates for streamlined synthesis of C12-/C15-functionalized steroids such as drospirenone and C-*nor*-D-*homo* derivatives. Collectively, this study demonstrates the first successful divergent evolution of a fungal P450 and highlights broad applicability for the scalable synthesis of complex bioactive molecules." (150 words)

Reviewer #2: In the manuscript entitled "Divergent Evolution of Fungal P450 Monooxygenase Unlocks Simultaneous Access to C12β and C15α oxyfunctionalization of Steroids", Wang et al. performed divergent evolution with combined structure-guided site-saturation mutagenesis and focused rational iterative site-specific metagenesis, achieving rather good selectivity for C12beta and C15alpha while simultaneously enhancing catalytic efficiency. The reason behind the mutations was further uncovered by MD simulations. Importantly, this work represents a first example of the divergent evolution of a fungal P450. The manu. is well-written and the results seem interesting. I suggest a major revision before its publication due to following concerns.

1. The labels of the substrates (at least **1**) may be supplied.

Answer: Thank you very much for your feedback and meticulous review of our manuscript.

In Fig. 1e, we have labeled the carbon atom numbering for the model substrate **1** (progesterone, PG). And the specific positions targeted for modifications (C12 and C15) are highlighted in distinct colors in Figs. 4a and 4d.

2. Is the regioselectivity coupled with the stereoselectivity?

Answer: Thank you very much for the comments.

In our study, CYP68J5_fg and its mutants consistently produced either C12β- and/or C15α-hydroxylated products from progesterone and other steroid substrates (canrenone, testosterone, androstenedione, etc.). However, hydroxylated products at either the C12α or C15β position were not observed. Thus, the stereoselectivity inversion or switch was not involved.

3. I am curious about the performance of W15M4 and W12M4 for substrate 3, 4 5 and 6. Only some have been tested. Why other mutants were used in Table 1?

Answer: Thank you for your insightful feedback.

During our initial evaluation of the activities of W15M4 and W12M5 against substrates **2**-**6**, we observed insufficient activity of these variants toward certain

substrates. Considering the sequence-function relationships between the substrates and the enzyme, we ultimately decided to test the activity of all variants (W12M1, W12M2, W12M3, W12M4 and W12M5; W15M1, W15M2, W15M3 and W15M4) against substrates **2**-**6** (**Supplementary Figure 8**). Only the top-performing variants for each of substrates **2**-**6** are presented in Table 1.

4. it is quite confusing to use “pose 12” and “pose 15”.

Answer: Thank you for your comments.

We agree that the original terms “pose 12/15” lacked functional context. “pose 12” designates the substrate-binding conformation that positions C12 of the steroids within catalytic proximity of the heme iron for β-hydroxylation, while “pose 15” refers to the conformation enabling C15α-hydroxylation. To clarify, the revised manuscript now defines these terms parenthetically at first occurrence:

pose 12 (positioning C12 proximal to the heme for β-hydroxylation)

pose 15 (positioning C15 proximal to the heme for α-hydroxylation)

5. The binding conformations shown in Figure 4 are not clear. Additionally, the polar H atoms may be shown in all related figures to make them clear.

Answer: Thank you for these constructive suggestions.

We have revised Fig. 4 accordingly by rotating the view to better expose the substrate-binding pocket. In addition, polar hydrogen atoms have been shown both in Fig. 4 and in all related figures.

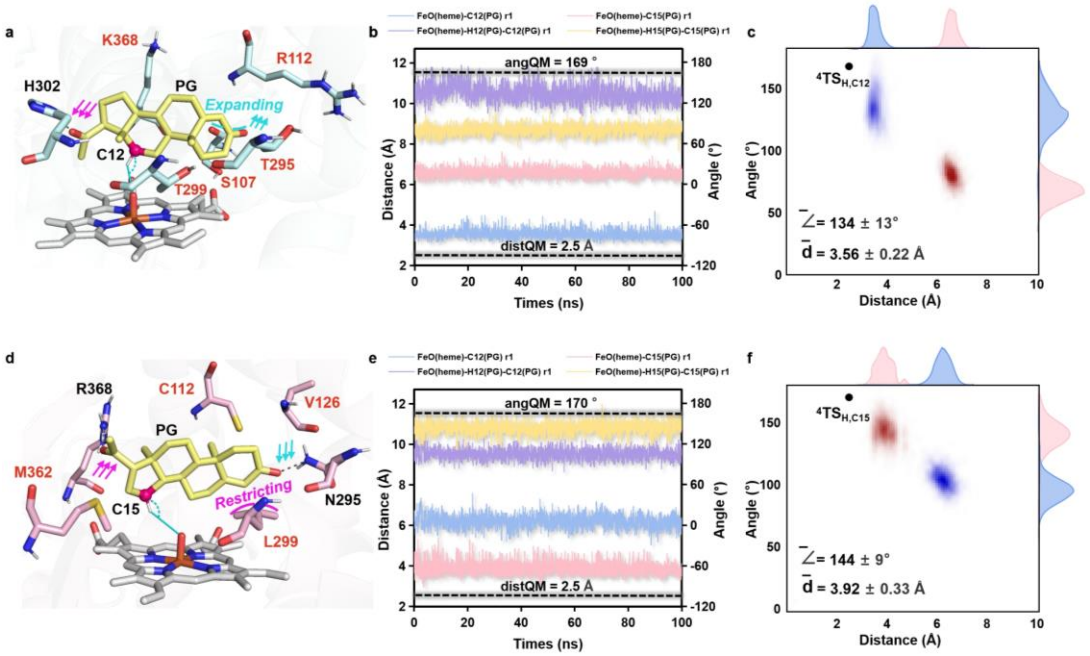
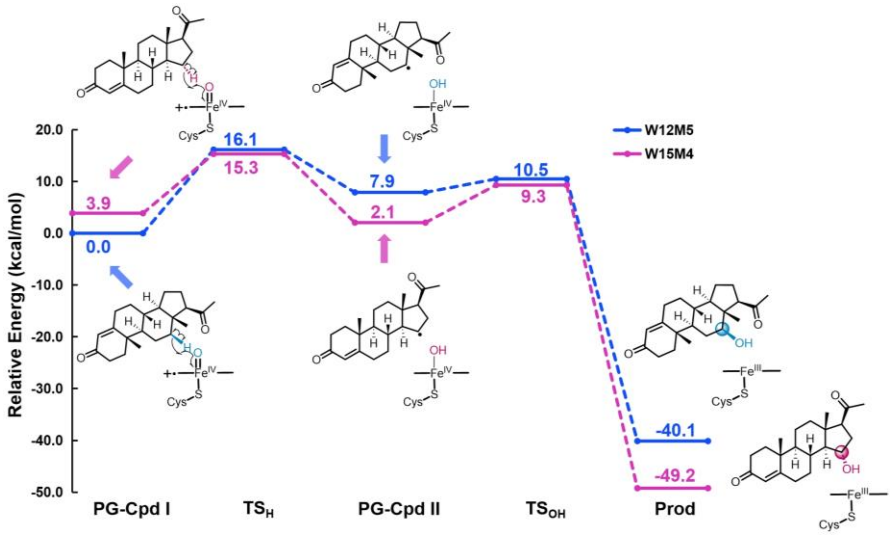


Fig. 4 Mechanism analysis of 1 hydroxylation by the CYP68J5_fg variants. a, **d**, Representative snapshots of the MD simulation revealing the substrate-enzyme binding mode in the active sites of W12M5 (**a**) and W15M4 (**d**). Mutated residues are shown in red. **b**, **e**, Plots of the C12, C15(**1**)···O=Fe distances (y primary axis) and the O(Fe=O)-(**1**)-H(12C, 15C)-(**1**)-C(12, 15) angles (y secondary axis) along the simulation time (x axis) for one of the replicas of W12M5 (**b**) and W15M4 (**e**) (see **Supplementary Figs. 1** and **2** for replicas 2 and 3). **c**, **f**, Conformational population analysis of the prereaction states of W12M5 (**c**) and W15M4 (**f**) in the 100 ns MD simulation trajectories (three joint MD replicas). Quantum mechanics (QM)-predicted distances (distQM) and angles (angQM) of transition state geometry (**Supplementary Figs. 3 and 4**) for C12β-H/C15α-H abstraction are indicated by dashed lines (**b**, **e**) and black dots (**c**, **f**), respectively.

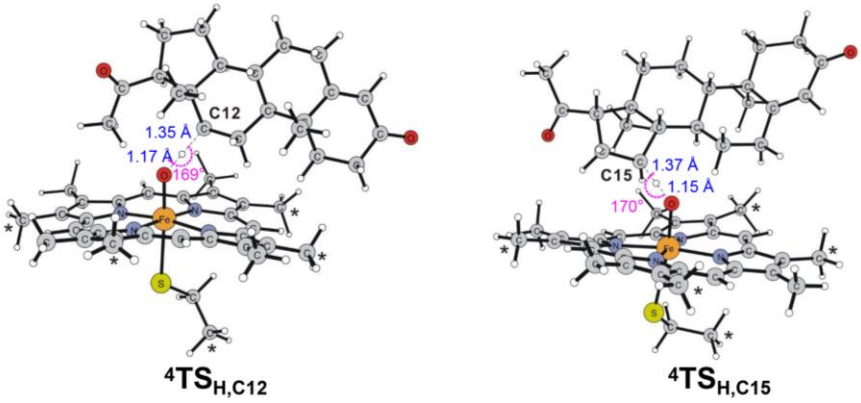
6. Optimized geometries (it seems key information is missing in Figure S4) and energy profiles obtained with QM are missing.

Answer: Thank you for the valuable suggestion.

The QM energy profile for the Cpd I-mediated hydroxylation of progesterone is now provided in **Supplementary Fig. 3**, and the missing key bond lengths/angles have been added to **Supplementary Fig. 4**.



Supplementary Figure 3. Potential energy profiles for the CYP68J5_fg-catalyzed C(12,15)-hydroxylation of 1. Abbreviations indicate the Cpd I and substrate PG complex (**PG-Cpd I**), the TS for hydrogen-atom transfer from PG to heme Fe=O species (**TS$_H$**), the intermediate including Fe-OH s species and the PG radical (**PG-Cpd II**), the TS for hydroxyl radical rebound from Cpd II to PG (**TS$_{OH}$**), and the hydroxylated substrate product intermediate (**Prod**).



Supplementary Figure 4. QM-optimized transition-state structures for C(12,15)-H abstraction in 1. Key geometrical parameters are indicated with dashed lines. Asterisks indicate the atoms fixed in their X-ray positions.

7. It is confusing to say “To obtain the optimal geometry for the transition state (TS), the calculations were performed using unrestricted density functional theory (UDFT) with the hybrid functional B3LYP as implemented in the Gaussian 16 program package.”

Answer: Thank you for the constructive comments.

To eliminate confusion:

(1) Consolidated all computational methods (UDFT/B3LYP, basis sets, CPCM, etc.) into a unified preceding section;

(2) Clarified the logical flow of our computational strategy. The updated content is now: First, the enzyme-substrate complex was optimized in diverse electronic states. The ground state has been found to be in the quartet state (Supplementary Tables 3 and 4), which is consistent with previous research findings [10] demonstrating that the quartet C-H abstraction transition state (TS) consistently exhibits lower energy. Thus, all subsequent calculations were conducted exclusively in the quartet state. P450-mediated hydroxylation involves the hydrogen atom abstraction from the C12/C15 position of PG by Cpd I, followed by a hydroxyl radical rebound. With computational results confirming hydrogen abstraction as the rate-determining step (supplementary Fig. 3), its TS structure was used as a key reference to evaluate the prereactive conformations sampled in MD simulations.

8. Instead of 6-31G(d,p)+, it is more common to use 6-31+G(d,p).

Answer: Thank you for pointing out the inaccuracies in the basis set format.

"6-31G(d,p)+" has been corrected to the standard form "6-31+G(d,p)" in the Supplementary Information.

9. More details may be supplied for how the binding conformations are obtained. For example, whether the chosen conformation is the most favorable one?

Answer: Thank you for raising this important point.

The binding conformations were selected through molecular docking using LeDock software. For docking input files, the PG ligand structure obtained in SDF format from the PubChem database was converted to mol2 format, while the receptor structures, including the predicted CYP68J5_fg and its variants, were in PDB format. Docking poses were clustered based on a Root Mean Square Deviation (RMSD) cutoff of 1.0 Å. The space within 5 Å centered on the heme was designated as the docking

box using the GetBox plugin in PyMOL, which delineate the three-dimensional region where the ligand is expected to bind. The simulation was set to produce 20 distinct binding poses, which were ranked by their predicted binding energy scores. From these energy-ranked poses, catalytically competent conformations were identified by evaluating both the distance and orientation of the target C12-Hβ or C15-Hα bonds relative to the ferryl oxygen of Compound I. Thus, the final selected conformation represents the pose that is both thermodynamically stable (most negative binding energy) and mechanistically viable for C12/C15 hydroxylation. The relevant details have now been added to the "Molecular Docking" section of the Supplementary Information.

10. What is the reference value (distQM) in Figure 4, the distance between Cpd I and hydrogen atoms or carbon atoms? Why use values of the transition state?

Answer: We thank the reviewer for this important clarification.

(1) distQM denotes the QM-predicted distance between Cpd I and carbon atoms at the transition state, i.e., C12, C15(1)···O=Fe. For clarity, these definitions are explicitly illustrated in the caption of Figure 4. Reference values for these distances are provided in Supplementary Fig. 4.

(2) The transition state (TS) geometry serves as the essential reference because it structurally defines the reaction barrier—its energy and arrangement dictate both the rate and selectivity of C–H abstraction. A substrate conformation with bond lengths and angles closer to the TS requires less reorganization energy to reach the barrier, resulting in a lower activation energy and higher reactivity. Thus, proximity to the TS geometry is a direct predictor of reactivity. By comparing distances and angles sampled from MD trajectories (prereactive states) with the QM-optimized TS values, we directly evaluate the enzyme's ability to preorganize the substrate. Conformations near the TS geometry indicate optimal positioning for hydrogen abstraction, enabling us to quantitatively explain and predict regioselectivity and stereoselectivity.

To further emphasize this point, we have now included the following statement in the manuscript: " The close agreement between these structural parameters and the QM-

optimized transition state (TS) for C12 β-H abstraction (^{4}**TS$_{H,C12}$**, **Supplementary Fig. 4**) is consistent with the geometric requirements essential for efficient TS formation and reaction feasibility."

11. The energy difference between doublet and quartet states is 3.2 kcal/mol in supplementary Table 3. However, it is only 0.1 kcal/mol in supplementary Table 4. What is the reason?

Answer: We thank the reviewer for this insightful question regarding the energy differences between the doublet and quartet states in **Supplementary Tables 3 and 4**.

The small difference in the doublet-quartet energy splitting between pose 12 and pose 15 (3.2 vs. 0.1 kcal/mol) is within the expected uncertainty of DFT calculations and does not indicate a fundamental change in electronic structure or reactivity. This variation is attributed to subtle differences in the spatial arrangement and non-covalent interactions between the substrate and Heme in the two binding poses. The weak interaction allows minor conformational adjustments, such as slight changes in distance or orientation (*J. Chem. Theory Comput.* 2012, 8, 5, 1629-1640; *Catal. Sci. Technol.,* 2015,5, 4946-4949; *Annu. Rep. Prog. Chem., Sect. C: Phys. Chem.,* 2006,102, 203-226), to alter the electronic environment around the iron center, thereby modulating the relative stability of the spin states. Fluctuations on the order of a few kcal/mol are computationally common and acceptable (*Theor. Chem. Acc.* 2010, 127, 429-442), whereas larger differences (e.g., >8–10 kcal/mol) would be more unusual and indicative of a fundamental electronic change.

Thus, the observed differences between pose 12 and pose 15 are consistent with minor structural perturbations and do not affect the main conclusions of our work.

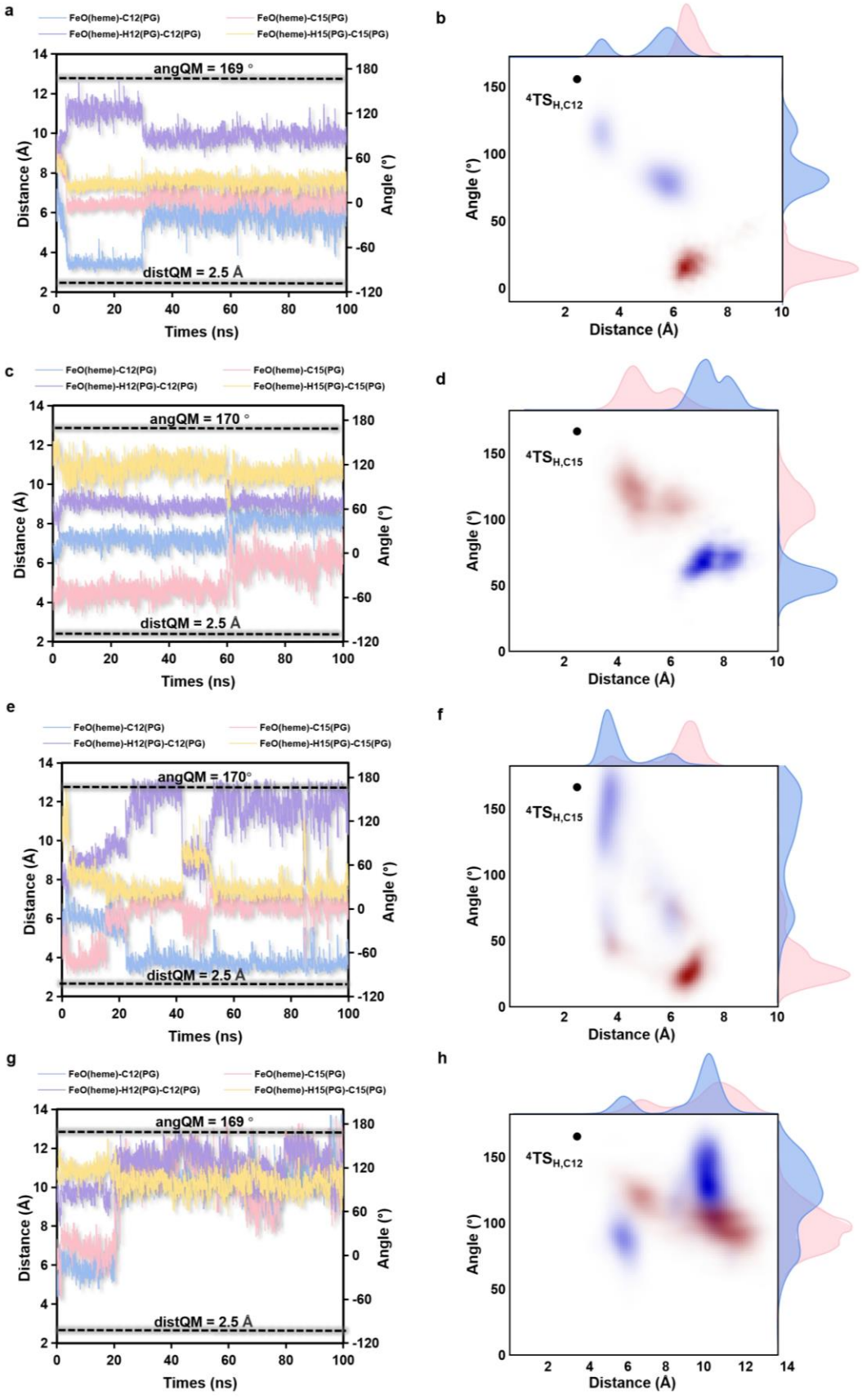
12. According to Figure S5b, it is hard to explain the C15 reactivity of WT enzyme.

Answer: Thank you for raising this important point.

We agree that the original simulation results for the WT enzyme-substrate complex did not adequately account for its C15 reactivity. To address this, we re-ran the MD simulation specifically for the pose 15 binding conformation of CYP68J5_fg with

substrate **1**. Although the majority of the sampled conformations remain non-productive (revised **Supplementary Fig. 6c**), a minor population of poses in the WT enzyme adopts a geometry favorable for C15α-hydroxylation, with distance and angle parameters approaching those required for catalysis.

To better visualize the conformational distribution, we have also included a conformational population analysis in the revised Supplementary Fig. 6, which better illustrates the presence of these catalytically competent states in the WT enzyme.

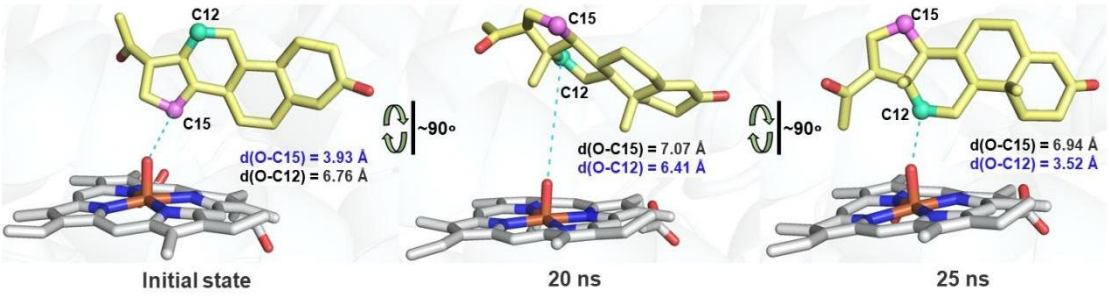

a
FeO(heme)-C12(PG)
FeO(heme)-C15(PG)
FeO(heme)-H12(PG)-C12(PG)
FeO(heme)-H15(PG)-C15(PG)
angQM = 169 °
distQM = 2.5 Å
Distance (Å)
Angle (°)
Times (ns)
b
$^4TS_{H,C12}$
Angle (°)
Distance (Å)
c
FeO(heme)-C12(PG)
FeO(heme)-C15(PG)
FeO(heme)-H12(PG)-C12(PG)
FeO(heme)-H15(PG)-C15(PG)
angQM = 170 °
distQM = 2.5 Å
Distance (Å)
Angle (°)
Times (ns)
d
$^4TS_{H,C15}$
Angle (°)
Distance (Å)
e
FeO(heme)-C12(PG)
FeO(heme)-C15(PG)
FeO(heme)-H12(PG)-C12(PG)
FeO(heme)-H15(PG)-C15(PG)
angQM = 170°
distQM = 2.5 Å
Distance (Å)
Angle (°)
Times (ns)
f
$^4TS_{H,C15}$
Angle (°)
Distance (Å)
g
FeO(heme)-C12(PG)
FeO(heme)-C15(PG)
FeO(heme)-H12(PG)-C12(PG)
FeO(heme)-H15(PG)-C15(PG)
angQM = 169 °
distQM = 2.5 Å
Distance (Å)
Angle (°)
Times (ns)
h
$^4TS_{H,C12}$
Angle (°)
Distance (Å)

Supplementary Figure 6. MD simulations of CYP68J5_fg and its variants in the non-productive position with 1. a, c, e, g, Distances determined between the oxygen atom of the Fe=O and the C(12,15) atom of 1 (x axis) and angles formed by O(Fe=O)-(1)-H(C12,15)-(1)-C(12,15) (y axis) in CYP68J5_fg (Pose 12, 15) (**a, c**), W12M5 (Pose 15) (**e**) and W15M4 (Pose 12) (**g**) along the whole simulation time. **b, d, f, h,** Conformational population analysis of the prereaction states of CYP68J5_fg (**b, d**), W12M5 (**f**) and W15M4 (**h**) in the 100 ns MD simulation trajectories.

13. It would be interesting to see what happened at 20 ns in Figure S5C.

Answer: We appreciate the reviewer's insightful observation.

At around 20 ns in **Supplementary Fig. 5c** (now **Supplementary Fig. 6c**), the substrate undergoes a significant reorientation within the active site of variant W12M5, flipping nearly 90°. By 25 ns, the substrate has flipped almost 180° relative to its initial binding pose and repositioned into a C12-oriented binding mode (**Supplementary Fig. 7**). This spontaneous re-orientation reflects the instability of the C15-bound conformation and confirms that the W12M5 variant specifically promotes the C12 hydroxylation of progesterone. This may be attributed to substrate binding pocket of W12M5 that better accommodate the C12-binding pose while disfavoring orientations for C15-hydroxylation.



Supplementary Figure 7. Representative Snapshots from MD Simulations of the W12M5-1 Complex. Each snapshot illustrates the spatial arrangement of substrate **1** (yellow) relative to the heme iron-oxygen (gray).

Reviewer #3: The C12*β*- or 15*α*-hydroxylated steroids are leading compounds in developing steroid pharmaceuticals and medicines. However, it is a challenging role for regioselective and stereoselective hydroxylation of steroid compounds either by chemical or biological approaches. Cytochrome P450 enzymes are known as an important biological hydroxylase for biosynthesis of steroid hormones in vivo. This manuscript presents a groundbreaking work for engineering fungal P450 CYP68J5_fg to achieve highly selective C12*β*- and C15*α*-hydroxylation of steroids. The combination of structure-guided SSM and FRISM mutagenesis successfully overcomes the classic activity-selectivity trade-off, yielding two variants (W12M5 and W15M4) with exceptional regioselectivity (>97%). MD simulations revealed that the key mutations reshape the substrate-binding conformation, thereby directing hydroxylation to distinct positions (C12*β* or C15*α*). The industrial relevance is supported by unprecedented titers (up to 14.1 g/L) in *P. pastoris* fermentation. The manuscript is suitable for publishing in Nature Communications after a further revision.

1. From the perspective of directed evolution, this is a surprisingly smooth job! The first step of directed evolution screening based on SSM strategy is a routine operation, but I am a little concerned about calling the second stage of directed evolution FRISM. From the description in the text, it appears that this is somewhat different from the classic FRISM. The author doesn't even need to perform a few limited tests at the target site, essentially just cross combining the single mutations obtained through the SSM strategy in the first round. Although the results may seem perfect, a more accurate description of this process should still be considered.

Answer: Thank you very much for your feedback and meticulous review of our manuscript.

Regarding whether the engineering approach employed in the manuscript qualifies as FRISM, we have reviewed the article of FRISM methodology (*J. Am. Chem. Soc.* 2019, 141, 7934-7945; *Mol. Catal.* 2024, 553, 113755) and determined that the method employed in this study aligns with the definition of FRISM (focused rational iterative site-specific mutagenesis).

We began by employing site-saturation mutagenesis (SSM) to identify key residues and their corresponding beneficial mutations, followed by FRISM:

For the screening of C15α-hydroxylated products, SSM first identified four key residues (Q112, D126, V299, A362) and their beneficial mutations (Q112A/M/T/S/C, D126T/V, V299I/L, A362M). Then, FRISM was performed: the variant V299L was selected as a template, and the beneficial mutations (D126T/V, A362M, Q112A/M/T/S/C) were individually introduced to generate 8 double variants. After screening, the optimal double variant V299L/D126V was selected. Next, iterative combinations with mutations A362M and Q112M/A/T/S/C produced 6 triple variants, the subsequent screening led to the identification of the best triple variant V299L/D126V/A362M. Lastly, a final round of combination integrating the mutations Q112A/M/T/S/C generated 5 quadruple variants, and the catalytic efficiency test revealed V299L/D126V/A362M/Q112C to be the best-performing variant.

Similarly, for screening C12β-hydroxylated products, by employing N295T as the initial template, iterative combination with mutations at F107S, Q112R, V299T, and R368K efficiently yielded the optimal quintuple variant F107S/Q112R/N295T/V299T/R368K, after screening only 10 designed variants.

In summary, utilizing the FRISM approach, we constructed a focused yet comprehensive library and successfully identified optimal variants after screening only 29 mutants. This strategy significantly reduced experimental workload and circumvented the need for exhaustive screening of all possible combinatorial mutations.

2. In line 63, the authors describe that “fungal P450 enzymes demonstrate superior suitability for industrial-scale production”, please provide more evidences or references.

Answer: Thank you very much for the comments.

Microbial hydroxylation of steroids is fundamentally mediated by enzymatic catalysis. Current evidence indicates that steroid-hydroxylating enzymes in

filamentous fungi belong to the cytochrome P450 superfamily (KLINGENBERG M. 1958, 75, 2, 376-386.). Numerous fungal industrial strains have been employed in steroid drug synthesis.

Industrial applications of fungal P450-mediated steroid hydroxylation include:

(1) Bayer's process employing *Curvularia lunata* mycelia containing P450 enzymes to catalyze the 11β-hydroxylation of 11-deoxycortisol for hydrocortisone production (Process for the preparation of 11 β-hydroxy steroids. EP19800103478. 1980).

(2) Upjohn's biocatalytic route using *Rhizopus arrhizus* CYP509C12 to convert progesterone to 11α-hydroxyprogesterone, thereby shortening the cortisol synthesis pathway by >10 steps with significant cost reduction (Steroids. 1992, 57, 12, 593-616.).

We have cited these key references in our manuscript.

3. As shown in Figure 2a, the dehydroxylated product **1c** can be formed in this reaction. The direct dihydroxylation of steroids is also very important and interesting (see recent example, Wang et al. Catal. Sci. Technol., 2025, 4170-4178.). It is observed that there is an unknown product by W12M1 in Figure 3d. Is this compound a dihydroxylation product? I am wondered if the author has detected and identified the generation of dihydroxy products during directed evolution. If so, it maybe your next interesting paper.

Answer: Thank you very much for your comments and suggestions.

In Fig. 3d, the unknown product generated from **1** by mutant W12M1 is not the dihydroxylated product **1c**. Compound **1c** was previously identified in our earlier work ("A Fungal P450 Enzyme from *Fusarium graminearum* with Unique 12β-Steroid Hydroxylation Activity." *Appl. Environ. Microbiol.* 2023, 9, 3, e0196322.). Due to its dihydroxylated nature, **1c** exhibits higher polarity than both the C15- and C12-monohydroxylated products, resulting in an earlier retention time during HPLC analysis.

Regarding the unknown product generated by W12M1 in Fig 3d, its structural

characterization was not pursued owing to its low yield (approximately 3.8%) and the fact that it disappeared upon further mutagenesis (not detectable in variants catalyzed reactions).

4. In Table 1, can the selectivity and conversion rate be accurately measured to two decimal places?

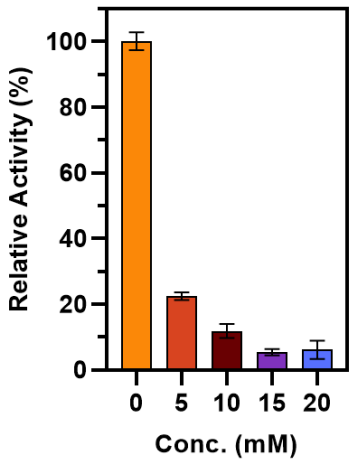
Answer: Thank you very much for the suggestions. We will revise all the data in Table 1 and the main text to maintain precision using one decimal place.

5. In line 329, the authors describe that "the low yield of **1a** is potentially due to the cytotoxicity of 1a", please provide the experiment details or references.

Answer: Thank you for your question.

Considering that the OD_{600} of the culture continued to increase alongside the accumulation of **1a** during the fermentation process, we have tentatively ruled out cytotoxicity as a contributing factor. Therefore, we suspect that the accumulation of **1a** may have caused product inhibition, leading to reduced substrate conversion. To test this hypothesis, we conducted a new experiment, as shown in **Supplementary Fig. 12**. As the concentration of **1a** increases, the relative activity of W12M5 toward substrate **1** progressively decreases, indicating that high concentrations of the product inhibit the catalytic activity of the enzyme toward the substrate.

We have revised the mention of "cytotoxicity" in the main text manuscript accordingly and include this figure in the supplementary information.



Supplementary Figure 12. Relative activity of GS115-W12M5 after 24 h reaction with simultaneous addition of varying concentrations of 1a. The concentrations on the x-axis represent the initial concentrations of compound **1a** (0, 5, 10, 15, 20 mM) added simultaneously with a fixed concentration (5 mM) of substrate **1** at the start of the reaction.

6. In line 382 and 383, the authors describe that "the integrated approach of SSM coupled with FRISM strategy effectively modulates selectivity and enhances activity in these fungal-derived P450 enzymes", to some extent, the approach may just effectively suitable for CYP68J5_fg, it would be better to state more precisely.

Answer: Thank you very much for your feedback and suggestion.

We have revised the sentence in the current line 387 (previously line 382 and 383) by specifying "these fungal-derived P450 enzymes" as "CYP68J5_fg, with potential applicability to other fungal-derived P450 enzymes" to ensure greater accuracy.

7. Except for substrate 6, the other four structurally similar substrates all showed excellent selectivity and conversion rates. Did the author also examine for other substrates? If so, it is best to report the results, even if it is only included in the supporting data. Because natural steroid hydroxylation P450 enzymes often have very strict substrate limitations. Similar results are of great significance for the development of substrate-promiscuity P450 isoenzymes through protein

engineerings.

Answer: Thank you very much for your feedback and suggestions.

In addition to substrates 2-6, we evaluated the enzyme variants against a panel of steroids with greater structural diversity, including lithocholic acid, pregnenolone, 9(10)-dehydronandrolone, estrone, and D-ethylgonendione. While this screen confirmed catalytic potential, all variants exhibited either severely diminished activity or a complete loss of function against these substrates. Consequently, they were excluded from the formal analysis. This initial activity, however, provides a foundation for future protein engineering efforts aimed at improving both catalytic activity and selectivity for structurally complex steroids.

8. English language and style are required to fine spell check, such as “in line 175, catalzyed should be catalyzed; in line 324, W1M4 should be W15M4.”

Answer: Thank you very much for the comments.

We have corrected “catalzyed” to “catalyzed” in the current line 172 (previously line 175) and “W1M4” to “W15M4” in the current line 326 (previously line 324). We have further performed a thorough English language and style review of the manuscript to ensure proper spelling and grammar.