Diastereo- and Enantioselective Chemoenzymatic Synthesis of Chiral Tricyclic Intermediate of Anti-HIV Drug Lenacapavir

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ABSTRACT: Despite its great potential, the development and implementation of scalable new-to-nature biocatalytic transformations in the chemoenzymatic synthesis of clinically significant pharmaceuticals still present a considerable challenge. We developed a chemoenzymatic synthesis of very recently developed anti-HIV drug lenacapavir's 5/5/3 fused tricyclic fragment featuring an unusual chiral cyclopropane moiety. Key to this development is a biocatalyst-controlled, fully diastereo- and enantiodivergent cyclopropanation of a highly functionalized vinylpyrazole substrate, granting access to all four possible stereoisomers of lenacapavir cyclopropane. High-throughput experimentation led to the discovery of heme-dependent globins, including nitrous oxide dioxygenase (NOD) and protoglobin (Pgb), as promising cyclopropanation biocatalysts. Directed evolution furnished a highly diastereo- and enantioselective cyclopropanation (up to 99:1 d.r. and 99:1 e.r.). Further developed downstream chemical cyclization afforded the desired lenacapavir 5/5/3 fused tricycle with great stereochemical purity.

INTRODUCTION

As a novel and highly potent long-acting HIV capsid protein inhibitor,[1] lenacapavir (**1**, GS-6207, brand name: Sunlenca, Figure 1A) is able to reduce HIV infection by 96% and was very recently approved by the US Food and Drug Administration (FDA) as an antiretroviral drug for HIV treatment.[2] Due to its outstanding potency, low *in vivo* systemic clearance, slow release kinetics, and minimal cross-resistance with previously approved antiretroviral drugs, lenacapavir presents an excellent opportunity for the treatment of multi-drug resistant HIV.[1] In light of the excellent efficacy, an efficient, sustainable, and selective synthesis of lenacapavir represents an essential goal for the advancement of global health, particularly in developing countries, and has thus captured the attention of philanthropic foundations in the public health domain.[3-9] As a structurally complex small-molecule drug, lenacapavir features an unusual chiral 5/5/3 fused tricyclic fragment ((3b*S*,4a*R*)-**2**) possessing a *cis*-cyclopropane moiety. Gilead Biosciences' disclosed synthetic route towards this 5/5/3 fused tricyclic compound hinges on the use of chiral separation by preparative supercritical fluid chromatography (SFC) or employing chiral starting materials with somewhat reduced synthetic efficiency (Figure 1A).[10–14] Therefore, alternative efficient and stereoselective routes based on catalytic asymmetric (bio)transformations could potentially streamline the production of this clinically significant drug.

Due to their excellent catalytic efficiency, stereoselectivity and sustainability as well as the complementary retrosynthetic logic of biotransformations, the pharmaceutical industry recently started to embrace the power of new biocatalysis tools in the production of active pharmaceutical intermediates (APIs).[15,16] As a testament to this, the Merck process chemistry team recently developed fully biocatalytic syntheses of anti-HIV and immunooncology drugs islatravir (MK-8591) and MK-1454, respectively.[17,18] By merging highly selective biotransformations with versatile chemical synthesis, chemoenzymatic synthesis offers further improved synthetic versatility and efficiency in the manufacturing of APIs.[19–21] Over the past decade, through mechanism-guided enzyme repurposing and evolution,[22,23] new-to-nature biocatalytic reactions which were unknown in nature's vast catalytic repertoire have emerged as powerful tools for synthetic applications.[24] Despite the promise of new-to-nature biocatalysis, the evolution and integration of these newly developed toolkits into the chemoenzymatic synthesis of clinically important therapeutics remains largely underdeveloped.

To further explore the synthetic potential of new-to-nature biocatalysis in pharmaceutical applications, we set out to advance a chemoenzymatic strategy for the stereoselective catalytic synthesis of lenacapavir's chiral 5/5/3 fused tricyclic fragment (**2**) featuring a disubstituted cyclopropane moiety (Figure 1B). Groundbreaking research[25,26] by Arnold,[27–35] Fasan[36–44], and other pioneers[45–53] has elegantly established the efficiency of engineered heme proteins in the stereoselective cyclopropanation of olefins using ethyl diazoacetate (EDA) as the carbene precursor. On the basis of these results, we envisioned a chemoenzymatic strategy featuring an intermolecular biocatalytic cyclopropanation for the stereoselective assembly of this 5/5/3 fused tricycle **2** (Figure 1B and 1C). In particular, we surmised that efficient heme protein catalysts could be identified and further evolved to allow for the diastereo- and enantioselective synthesis of cyclopropane (1*R*,2*S*)-**4** using vinylpyrazole **5**, which could be conveniently prepared by Suzuki-Miyaura

coupling. Furthermore, we postulated that downstream chemical cyclization of (1*R*,2*S*)-**4** under appropriate conditions could effect the formation of ketone (3b*S*,4a*R*)-**3**. Finally, through desulfurative difluorination[54,55], the *gem*-difluoro moiety of (3b*S*,4a*R*)-**2** could be introduced to ketone (3b*S*,4a*R*)-**3** (Figure 1B). Herein, we report the successful implementation of this synthetic plan. Importantly, through the identification and evaluation of heme protein biocatalysts, all the four possible stereoisomers of disubstituted cyclopropane **4** could be accessed with excellent diastereo- and enantiocontrol (Figure 1C). In light of the central role of stereochemistry in medicinal chemistry campaigns,[56,57] these results are expected to allow further study and preparation of related antiviral agents.

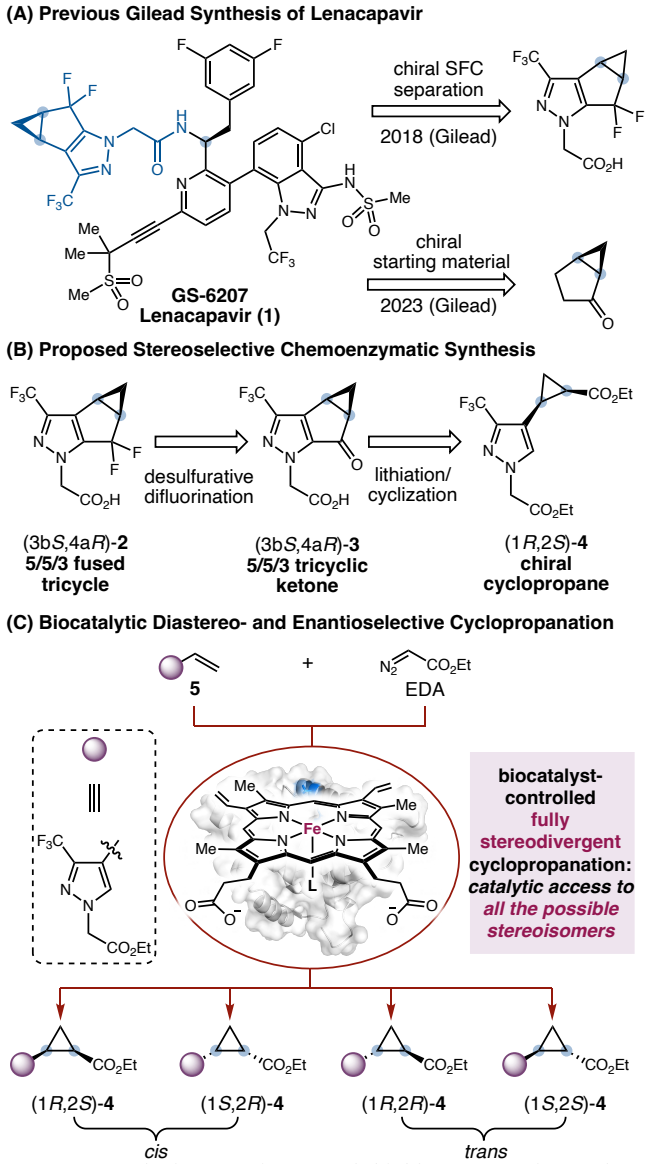
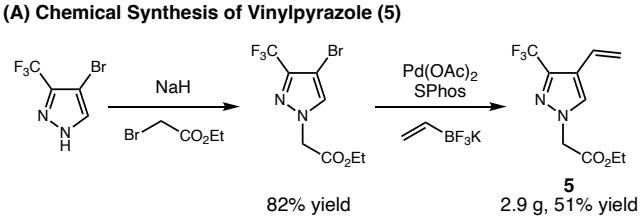
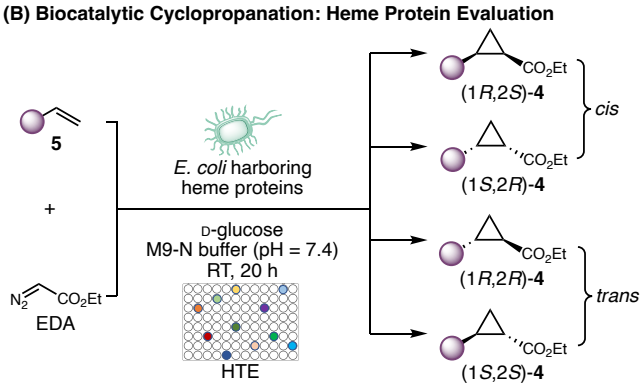


Figure 1. Synthetic strategies towards highly potent anti-HIV drug lenacapavir (**1**). (A) Gilead synthesis of lenacapavir's 5/5/3 fused tricycle; (B) Our proposed stereoselective chemoenzymatic synthesis of lenacapavir's 5/5/3 fused tricycle: retrosynthetic analysis; (C) Biocatalytic diastereo- and enantioselective cyclopropanation to access all the four stereoisomers: key step in our retrosynthetic plan.

RESULTS AND DISCUSSION

To commence this study, we first prepared vinylpyrazole **5** using an *N*-alkylation[58]/Pd-catalyzed Suzuki-Miyaura cross-coupling[59] sequence using the commercially available 4-bromo-3-(trifluoromethyl)-1*H*-pyrazole as the precursor. This sequence could be easily carried out using an SPhos[60]-based Pd catalyst on a 3-gram scale (Figure 2A).





variant	yield (*cis*-**4**)	yield (*trans*-**4**)	e.r. (*cis*-**4**)	e.r. (*trans*-**4**)	d.r. (*cis* : *trans*)
Rma cyt *c*	1.5	10	-	-	14:86
Cj-trHbP	1	7	-	-	24:76
P411-CIS T438S I263F	0.5	13	-	-	39:61
$P450_{rad1}$	2	23	-	-	9:91
$P450_{rad3}$ L267E	24	8	-	-	75:25
*Rma*NOD	1	22	-	77:23	8:92
*Rma*NOD Y32G	2	15	-	50:50	17:83
***Rma*NOD Y32K V97L**	**1**	**87**	**-**	**13:87**	**2:98**
*Ape*Pgb	1	3	-	-	29:71
*Ape*Pgb Y60G	6	6	-	-	57:43
***Ape*Pgb W59S Y60G**	**61**	**3**	**96:4**	**-**	**96:4**

Figure 2. Biocatalytic stereoselective cyclopropanation: towards lenacapavir's 5/5/3 fused tricycle. (A) Preparation of vinylpyrazole substrate **5** via Suzuki-Miyaura cross-coupling. (B) Evaluation of heme proteins for the diastereo- and enantioselective biocatalytic cyclopropanation of **5** via high-throughput experimentation (HTE): selected results.

With this vinylpyrazole substrate **5** in hand, we next evaluated an in-house collection of wild-type and engineered heme proteins, including various cytochromes P450, cytochromes *c*, and various globins, in the form of whole-cell biocatalysts in a 96-well plate format. With many heme proteins examined, the formation of both *trans*-**4** and *cis*-**4** could be observed, although with low yields and low levels of enantioselectivity. Nevertheless, with *Rhodothermus marinus* cytochrome *c* (*Rma* cyt *c*, Uniprot ID: B3FQS5)[61] and *Campylobacter jejuni* truncated hemoglobin (*Cj*-trHbP, Uniprot ID: Q0PB48)[62], *trans*-**4** was formed as the major product. With *Rma* cyt *c*, the cyclopropane product **4** was formed in 10% yield and 16:84 d.r. (*cis*-**4**:*trans*-**4**). Using *Cj*-trHbP, 7% yield and 24:76 d.r. were observed for

4. Additionally, a serine-ligated P450 mutant previously evolved for C–H amination, P411-CIS T438S I263F,[63] provided *trans*-**4** as the major product in 39:61 d.r. and 13% yield. Our previously evolved radical indole dearomatase $P450_{rad1}$[64] also furnished *trans*-**4** in slightly higher yield and diastereoselectivity (23% yield and 9:91 d.r.). Interestingly, another previously evolved radical pyrrole dearomatase $P450_{rad3}$ L267E[64] instead formed the *cis*-**4** as the major isomer although in moderate diastereoselectivity (24% yield and 75:25 d.r.). Among the heme proteins we evaluated, a double mutant derived from *Rhodothermus marinus* nitric oxide dioxygenase (*Rma*NOD, Uniprot ID: D0MGT2), *Rma*NOD Y32K V97L, provided *trans*-**4** as the major product in 87% yield and excellent diastereoselectivity (2:98 d.r.) and enantioselectivity (13:87 e.r.). Furthermore, a double mutant of *Aeropyrum pernix* protoglobin (*Ape*Pgb, Uniprot ID: Q9YFF4), *Ape*Pgb W59S Y60G, provided *cis*-**4** as the major product with excellent diastereoselectivity (96:4 d.r.) and enantioselectivity (96:4 e.r.) (Figure 2B, see Table S1 for more details).

To further enhance the diastereo- and enantioselectivity of this biocatalytic cyclopropanation, using *Ape*Pgb W59S Y60G and *Rma*NOD Y32K V97L as the template, we performed enzyme engineering through site-saturation mutagenesis (SSM) and screening by targeting active-site residues in proximity to the substrate (Figure 3). At the time when we started this project, the structure of *Ape*Pgb was unknown. We thus used AlphaFold2 to generate the structure of *Ape*Pgb W59S Y60G and docked the heme cofactor into this apo protein based on the structure of a homologous protoglobin from *Methanosarcina acetivorans* (*Ma*Pgb, Protein Data Bank (PDB) ID: 3ZJI).[65,66] Guided by this structure and previous engineering efforts with *Ape*Pgb,[30,67–69] SSM libraries of the *Ape*Pgb W59S Y60G variant were created for active-site residues 59, 60, 93, and 145 (Figure 3A, see Table S2-S4 for details). In a similar vein, SSM libraries of *Rma*NOD Y32K V97L[27,30] were generated by randomizing active-site residues 32, 46, 52, and 97 (Figure 3B, see Table S5-S7 for details).

For *Ape*Pgb W59S Y60G, protein engineering efforts (Figure 3C and Figure 4) led to the identification of F145L as the key beneficial mutation. With this added mutation, the resulting triple mutant *Ape*Pgb W59S Y60G F145L provided the *cis*-cyclopropane (1*S*, 2*R*)-**4** in 83% yield (540 ± 20 TTN) with improved diastereo- and enantioselectivity (98:2 d.r. and 99:1 e.r.). Interestingly, the inclusion of another single mutation, F93L, reversed the diastereoselectivity. Using *Ape*Pgb W59S Y60G F93L as the biocatalyst, *trans*-cyclopropane (1*S*,2*S*)-**4** was generated in 62% yield (380 ± 20 TTN), 19:81 d.r., and 95:5 e.r.. Furthermore, starting from *Ape*Pgb W59S Y60G, the introduction of S59L allowed the opposite enantiomer of *cis*-cyclopropane, namely (1*R*,2*S*)-**4**, to form in 71% yield (700 ± 10 TTN), 92:8 d.r., and 10:90 e.r., giving rise to the third stereoisomer of disubstituted cyclopropane **4** with promising enantio- and diastereoselectivity. With *Ape*Pgb W59L Y60G in hand, an additional round of SSM targeting active-site residues 63, 73, 93, and 145 led to *Ape*Pgb W59L Y60G F145G displaying further enhanced enantioselectivity, providing (1*R*,2*S*)-**4** in 83% yield (780 ± 30 TTN), 97:3 d.r., and 1:99 e.r. (Figure 3C and Figure 4, see Table S4 for details). With this result, closely related variants derived from the same *Ape*Pgb furnished three distinct stereoisomers with excellent enantio- and diastereoselectivity, highlighting the power of protein engineering to induce complementary stereochemistry starting from the same protein scaffold. We note that at this stage of research, the absolute stereochemistry of these cyclopropanation products were not fully established. We later determined the relative and absolute stereochemistry of these products through X-ray single crystal diffraction of their derivatives (*vide infra*).

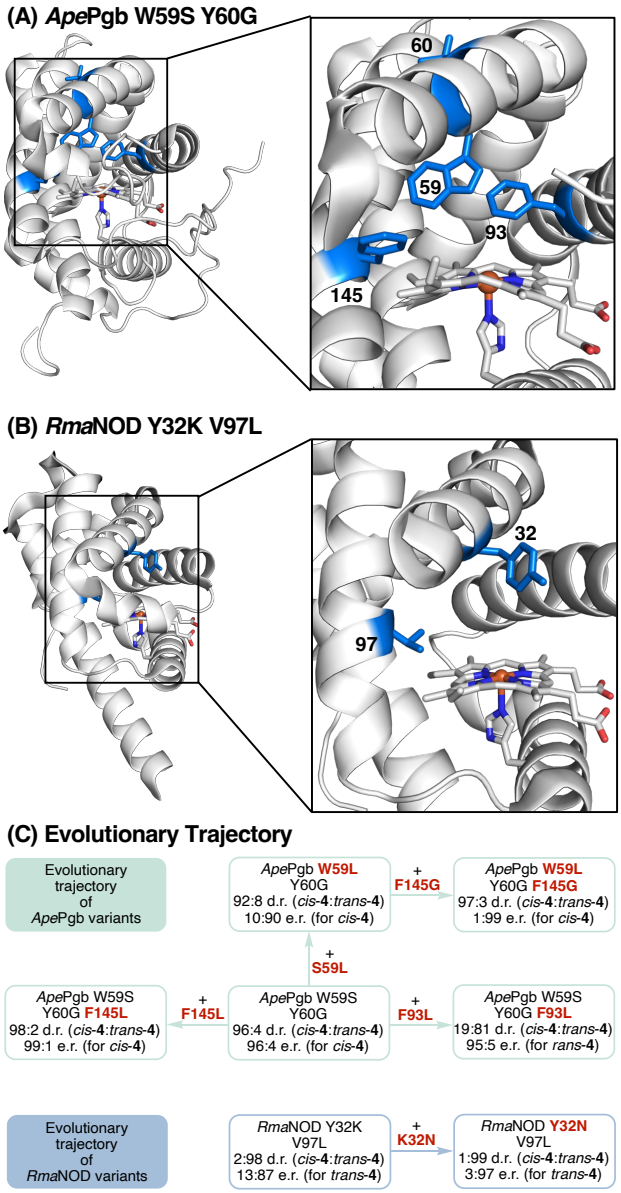


Figure 3. Key active-site residues and evolutionary trajectory for the optimization of heme protein catalysts for stereoselective cyclopropanation. (A) Structure of *Ape*Pgb W59S Y60G; active-site illustration was made based on the crystal structure of a closely related *Ma*Pgb variant (PDB ID: 3ZJI). (B) Structure of *Rma*NOD Y32K V97L; active-site illustration was made based on the crystal structure of *Rma*NOD Q52V variant (PDB ID: 6WK3). (C) Evolutionary trajectory.

Starting from *Rma*NOD Y32K V97L, evaluation of SSM libraries targeting active-site residues 32, 46, 52, and 97 led to the identification of a single mutant, *Rma*NOD V97L Y32N, providing the fourth stereoisomer, (1*R*,2*R*)-**4**, with further enhanced stereoselectivity (92% yield (420 ± 80 TTN), 1:99 d.r., and 3:97 e.r., Figure 3C and Figure 4). Thus, all the four possible stereoisomers of this cyclopropane-based lenacapavir precursor could be readily accessed by heme protein-controlled

stereodivergent biocatalytic cyclopropanation. These results further demonstrated the excellent evolvability and outstanding stereocontrol of heme protein catalysts over carbene transfer processes.

With these optimized biocatalysts in hand, we carried out gram-scale biocatalytic cyclopropanation using *E. coli* whole-cell catalysts (see Supporting Information for details), It was found that at a preparative scale (gram quantities), these biocatalytic cyclopropanation reactions provided similar results as analytical scale biotransformations (Figure 5). Using *Rma*NOD V97L Y32N, *trans*-cyclopropane (1*R*,2*R*)-**4** were obtained in 76% yield (1.02 g), 2:98 d.r., and 4:96 e.r.. Ester saponification with LiOH provided the diacid (1*R*,2*R*)-**6** in 84% isolated yield as a single diastereomer. Importantly, the absolute and relative stereochemistry of (1*R*,2*R*)-**6** were determined by single-crystal X-ray diffraction analysis (Cambridge Crystallographic Data Centre (CCDC) number: 2359512). Similarly, using our engineered protoglobin variant *Ape*Pgb W59S Y60G F145L as the whole-cell biocatalyst, *cis*-cyclopropane (1*S*,2*R*)-**4** was isolated in 77% yield (1.04 g), 98:2 d.r., and 99:1 e.r.. The corresponding diacid (1*S*,2*R*)-**6** was obtained using the same protocol described above in 78% yield as a single diastereomer. The absolute and relative stereochemistry of (1*S*,2*R*)-**6** was also determined by single-crystal X-ray diffraction analysis (CCDC no. 2359513). The stereochemistry of (1*R*,2*R*)-**6** and (1*S*,2*R*)-**6** in turn allowed us to unambiguously assign the stereochemistry of all the biocatalytic cyclopropanation products produced by engineered heme proteins. Importantly, from these results, it could be inferred that the desired stereoisomer (1*R*,2*S*)-**4**, a key proposed intermediate for the chemoenzymatic synthesis of lenacapavir, could be prepared by engineered protoglobin variant *Ape*Pgb W59L Y60G F145G.

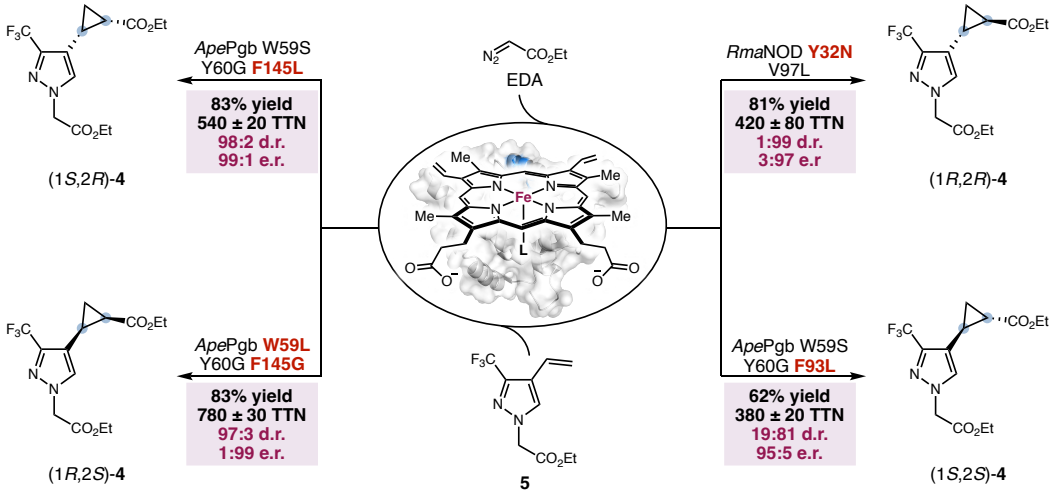


Figure 4. Heme protein-controlled fully diastereo- and enantiodivergent cyclopropanation of vinylpyrazole **5** to access all the four stereoisomers of cyclopropane **4** using engineered *Ape*Pgb and *Rma*NOD.

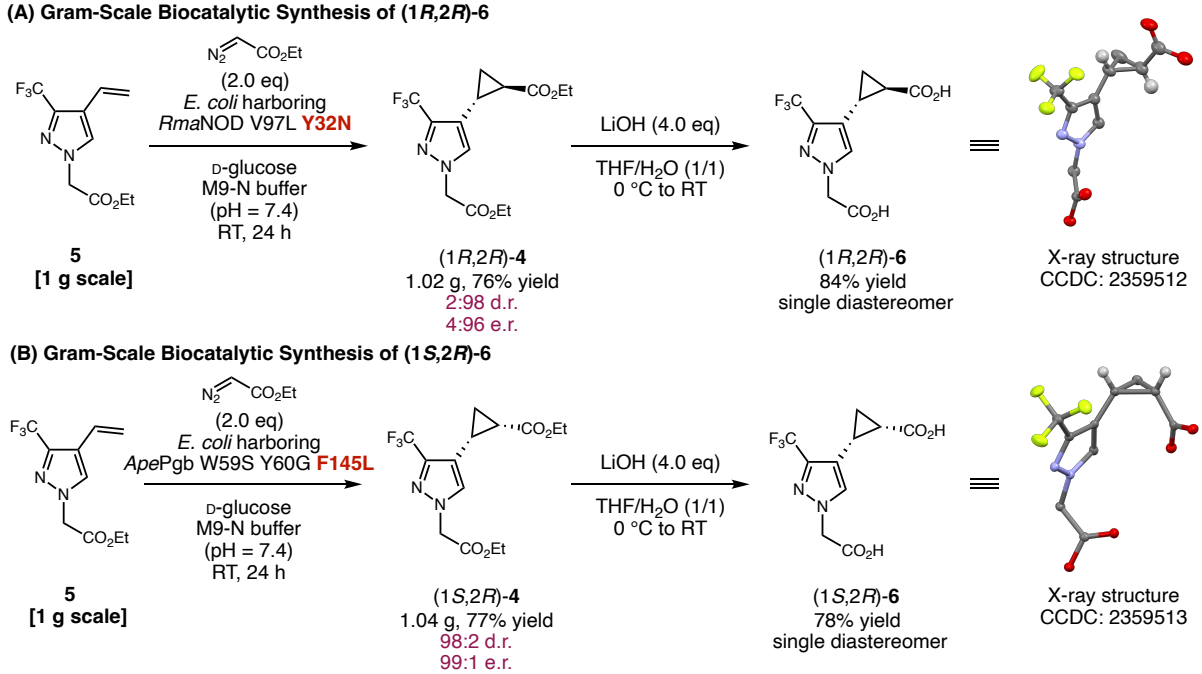


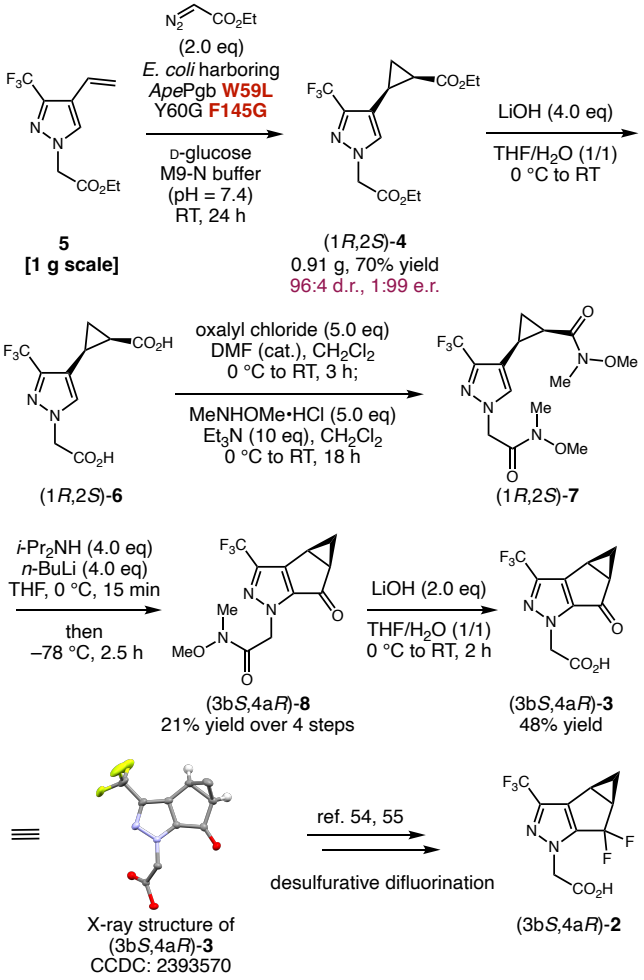
Figure 5. Gram-scale biocatalytic cyclopropanation of **5**. (A) *Rma*NOD V97L Y32N-catalyzed synthesis of (1*R*,2*R*)-**4** and X-ray diffraction analysis for the determination of the stereochemistry of (1*R*,2*R*)-**6** (CCDC no. 2359512). (B) *Ape*Pgb W59S Y60G F145L-catalyzed synthesis of (1*S*,2*R*)-**4** and X-ray diffraction analysis for the determination of the stereochemistry of (1*S*,2*R*)-**6** (CCDC no. 2359513).

The unambiguously established stereochemistry allowed us to complete the proposed chemoenzymatic synthesis of the desired stereoisomer of lenacapavir 5/5/3 fused tricycle. Through whole-cell biotransformation with *Ape*Pgb W59L Y60G F145G, we prepared the desired lenacapavir cyclopropane (1*R*,2*S*)-**4** on a gram scale in 70% isolated yield (0.91 g), 96:4 d.r., and 1:99 e.r.. This diester (1*R*,2*S*)-**4** was then treated with LiOH to afford the diacid (1*R*,2*S*)-**6**. We were able to achieve the proposed cyclization via a Weinreb amide formation/directed *ortho*-metalation (DoM)/cyclization sequence. In this process, the diacid (1*R*,2*S*)-**6** was first converted to the corresponding acyl chloride *in situ* with oxalyl chloride, followed by amidation with *N*,*O*-dimethylhydroxylamine to form the corresponding Weinreb amide (1*R*,2*S*)-**7**. We posited that the Weinreb amide moiety may serve as a dual-function group. First, the presence of this amide may enhance the lithiation activity when **7** was treated with *in-situ* generated lithium diisopropylamide (LDA). Second, this Weinreb amide would allow the facial acyl substitution reaction with the *in-situ* formed pyrazolyllithium intermediate.[6] Indeed, over four steps, the cyclized product (3b*S*,4a*R*)-**8** was formed in 21% yield (an average yield of 68% per step). Hydrolysis of the amide moiety in 5/5/3 fused tricycle **8** under basic conditions using LiOH provided the carboxylic acid (3b*S*,4a*R*)-**3** in 48% yield**,** which could be further converted to the final lenacapavir 5/5/3 fused tricycle (3b*S*,4a*R*)-**2** through desulfurative difluorination.[44,45] We further determined the specific rotation of (3b*S*,4a*R*)-**3** ($[\alpha]_D^{22} = -113.78$ (c = 0.01 g/mL, $CHCl_3$)) and performed single-crystal diffraction analysis (CCDC no. 2393570). These results confirmed that no erosion of stereochemistry occurred during the chemical cyclization sequence, establishing its stereochemical fidelity. Thus, this stereoselective chemical synthesis provided a facile new route to the stereochemically complex lenacapavir 5/5/3 fused tricyclic fragment (Scheme 1). We note that the desulfurative difluorination product of Weinreb amide **8** has the potential to be directly used for the synthesis of lenacapavir (**1**) via amidation in further studies.

CONCLUSION

In conclusion, we developed a novel stereoselective chemoenzymatic synthesis of the 5/5/3-fused tricyclic fragment of lenacapavir, a key intermediate in the manufacturing of this essential anti-HIV drug. Key to the success of our chemoenzymatic synthesis lies in the development of an orthogonal set of heme protein catalysts, allowing for the scalable and fully stereodivergent cyclopropanation of a functionalized vinylpyrazole substrate. Among these engineered heme proteins, a newly evolved protoglobin double mutant *Ape*Pgb W59L Y60G F145G functioned as an effective carbene transferase, allowing the highly diastereo- and enantioselective gram-scale cyclopropanation to prepare the key chiral *cis*-cyclopropane. Combined with a newly devised chemical cyclization sequence, the lenacapavir 5/5/3 fused tricycle was prepared with excellent diastereo- and enantiocontrol without relying on the use of chiral separation or chiral starting materials. This stereoselective chemoenzymatic synthesis provides a rare example of applying very recently developed new-to-nature biocatalytic reactions in the preparation of clinically important pharmaceuticals, demonstrating the potential of newly developed biocatalytic tools in high-value pharmaceutical applications. We expect that the further development of other tailored biocatalytic reactions will ultimately provide efficient and highly stereoselective syntheses of anti-HIV drug lenacapavir possessing three stereogenic centers.

Scheme 1. Completion of the stereoselective chemoenzymatic synthesis of the lenacapavir 5/5/3 fused tricyle (3b*S*,4a*R*)-2: gram-scale biotransformation and downstream stereoretentive chemical synthesis.



ASSOCIATED CONTENT

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Notes

The authors declare no competing financial interest.

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Supporting Information

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

Experimental procedures, DNA and protein sequences, characterization data, HPLC traces, and NMR spectra (PDF)
Crystallographic data for compound (1*R*,2*R*)-**6** (CIF)
Crystallographic data for compound (1*S*,2*R*)-**6** (CIF)
Crystallographic data for compound (3b*S*,4a*R*)-**3** (CIF)
Deposition numbers 2359512 (for (1*R*,2*R*)-**6**), 2359513 (for (1*S*,2*R*)-**6**), and 2393570 (for (3b*S*,4a*R*)-**3**) contain the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service.

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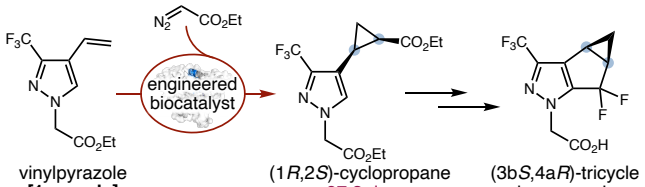
Table of Contents



vinylpyrazole
[1 g scale]

(1*R*,2*S*)-cyclopropane
97:3 d.r.
1:99 e.r.

(3b*S*,4a*R*)-tricycle
lenacapavir
5/5/3 fragment