Supporting Information for

Vitreoscilla hemoglobin: A natural carbene transfer catalyst for diastereo- and enantioselective synthesis of nitrile-substituted cyclopropanes

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Materials and methods.

Materials. All the chemicals and reagents were purchased from commercial suppliers (Sigma Aldrich, Bide Pharmatech, Aladdin, Energy Chemical, TCI) and used without any further purification, unless otherwise stated. Reinheitszahl (RZ) value of commercially available hemoproteins verified to be greater than 2.7, A280/A260 is 1.72-1.93. Ni-NTA Superflow resin obtained from Solarbio. E. coli BL21(DE3) Competent Cell, Spin Miniprep, and Gel Extraction Kits were all obtained from Tiangen. Silica gel chromatography purifications were carried out using AMD Silica Gel 60 230- 400 mesh. Thin Layer Chromatography (TLC) and preparative TLC were carried out using Merck Millipore TLC silica gel 60 F254 glass plates. Proton nuclear magnetic resonance (1H NMR) spectra were recorded on a 400 MHz spectrometer in CDCl3. Chemical shifts for protons are reported in parts per million downfield from tetramethylsilane (TMS) and are referenced to residual protium in the NMR solvent (CHCl3 = δ 7.26 ppm). NMR data are presented as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant in Hertz (Hz), integration. Mass spectra were recorded on the Bruker MicrOTOF Q II and an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific, San Jose, CA, U.S.A.) coupled with HESI ion source. The experiments were performed in triplicate, and all data were obtained based on the average values. The experiments were performed triplicate, and all data were obtained based on the average values.

Growth Media. Terrific Broth media was prepared as follows. For 1 L Terrific Broth media, deionized H2O was added with 11.8 g of peptone 140 (pancreatic digest of casein), 23.6 g of yeast extract autolyzed low sodium, 9.4 g of dipotassium hydrogen phosphate, 2.2 g of potassium dihydrogen phosphate, 4 ml of glycerol and supplemented glucose (0.2 % w/v). Terrific Broth agar plates were prepared by adding 15 g agar to 1 L Terrific Broth media with ampicillin antibiotic selection and hemin (35 µg/mL). To media and plates was added ampicillin to a final concentration of 100 mg/L.

General Methods for Expression of Vitreoscilla Hemoglobin. The VHb gene and promoter region (promoter-VHb) were amplified by polymerase chain reaction (PCR) with Pfu polymerase and a pair of primers (5’-CCAAAGCTTACAGGACGCTGGGAAAGT-3’;
5′-CCGGAATTCTTAATGATGATGATGATGATGTTCAACCGCTTGAGCGTACAAATCT-3′). The promoter-VHb fragment was retrieved from plasmid DNA by using restriction enzymes EcoRI and HindIII. Finally, the promoter-VHb fragment was cloned into the pUC-19. Competent cells were transformed with the PUC-19 vector encoding for the appropriate VHb variant and selected on Terrific Broth agar plates containing ampicillin (100 mg/L). Single colonies were used to inoculate 5 mL of Terrific Broth media supplemented with ampicillin (100 mg/L), followed by incubation at 37°C with shaking (180 rpm) for 10 to 15 hours. For expression of VHb, the overnight cultures were transferred to 1 L Terrific Broth media containing ampicillin, followed by incubation at 37°C with shaking (180 rpm). At an OD₆₀₀ of 1.5, VHb gene was induced expression by its anaerobic promoter in an anaerobic environment and incubated at 25°C with shaking (110 rpm) for 30 hours. Cell cultures were harvested by centrifugation at 5000 rpm, 4°C. The overall pelleted bacteria were resuspended in 50 mL of 20 mM phosphate buffer (pH 7.4). After sonication (380W) for 30 min on ice, the cell lysates were centrifuged at 12,000 rpm, 4°C for 30 min.

**Protein Purification.** The clarified lysate was transferred to a Ni-NTA column equilibrated with Ni-NTA Lysis Buffer (20 mM phosphate buffer). The resin was washed with 50 mL of Ni-NTA Lysis Buffer and then 50 mL of Ni-NTA Wash Buffer (20 mM phosphate buffer, 20 mM imidazole, pH = 7.4). Proteins were eluted with Ni-NTA Elution Buffer (20 mM phosphate buffer, 250 mM imidazole, pH = 7.4). After elution from the Ni-NTA column, the protein was loaded into a 5ml PD-10 desalting column to remove imidazole. The TON for VHb was calculated based on purified protein concentration, which was measured by NanoDrop. VHb concentration was calculated using the extinction coefficient $\varepsilon_{419-436} \text{ nm} = 274 \text{ mM}^{-1} \text{ cm}^{-1}$, as previously described [1]

**Molecular Docking Analysis.** The initial structure of VHB was taken from PDB code of 2VHB. Olefin was docked into the active site of carbenoid intermediate and using AutoDock Vina tool.²³
Supporting Experimental Tables

Table S1. Optimize the feeding time of sodium nitrite.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Feeding time (min)</th>
<th>NaNO\textsubscript{2}</th>
<th>TON</th>
<th>%\textit{de}</th>
<th>%\textit{ee}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>1560</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1877</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>2105</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>2196</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>2213</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>2184</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>120</td>
<td>2208</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>8\textsuperscript{[a]}</td>
<td>30</td>
<td>2536</td>
<td>&gt;99.9</td>
<td>99.9</td>
<td></td>
</tr>
</tbody>
</table>

Reaction conditions: 5 mM 4-bromostyrene (1\textsuperscript{a}), 10 mM diazoacetonitrile (2\textsuperscript{a}, in situ generated from aminoacetonitrile hydrochloride (10 mM) and NaNO\textsubscript{2} (15 mM)), 5 mM sodium dithionite, promoting solvent: MeOH (50 µl), WT VHb (0.025% mol) at H\textsubscript{2}O (4 mL), RT, 20 h. \textsuperscript{[a]} reaction in 15 \textdegreeC.

Table S2. Optimized the reaction time, promotive solvent and reaction ratio of 4-bromostyrene with diazoacetonitrile.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time(h)</th>
<th>Promotive solvent</th>
<th>Equiv\textsuperscript{[a]}</th>
<th>TON</th>
<th>%\textit{de}</th>
<th>%\textit{ee}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>MeOH</td>
<td>2</td>
<td>1849</td>
<td>99.9</td>
<td>98.8</td>
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<tr>
<td>2</td>
<td>20</td>
<td>MeOH</td>
<td>2</td>
<td>2196</td>
<td>99.9</td>
<td>98.8</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>MeOH</td>
<td>2</td>
<td>2203</td>
<td>99.9</td>
<td>98.7</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>EtOH</td>
<td>2</td>
<td>2073</td>
<td>99.9</td>
<td>98.0</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>DMSO</td>
<td>2</td>
<td>1922</td>
<td>99.9</td>
<td>98.8</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>EA</td>
<td>2</td>
<td>2136</td>
<td>99.9</td>
<td>98.6</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>-</td>
<td>2</td>
<td>1697</td>
<td>99.9</td>
<td>98.8</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>MeOH</td>
<td>1</td>
<td>1339</td>
<td>99.9</td>
<td>98.8</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>MeOH</td>
<td>3</td>
<td>2193</td>
<td>99.9</td>
<td>98.7</td>
</tr>
</tbody>
</table>
Reactivity conditions: 5 mM 4-Bromostyrene (1a), diazaocetonitrile (2a, in situ generated from aminoacetonitrile hydrochloride (10 mM) and NaNO₂ (added in 30 min)), sodium dithionite (5 mM), WT VHb (0.025% mol) at H₂O (4 mL), promotive solvent (50 µl) room temperature.

[a] Relative to olefin.

**Table S3.** The effect of temperature on the cyclopropanation of diazoacetonitrile catalyzed by FeTPPCl.

![Chemical reaction diagram]

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Temperature(°C)</th>
<th>Yield</th>
<th>%de</th>
<th>%ee</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeTPPCl</td>
<td>5</td>
<td>58.7%</td>
<td>&gt;99.9</td>
<td>-</td>
</tr>
<tr>
<td>FeTPPCl</td>
<td>15</td>
<td>92.3%</td>
<td>&gt;99.9</td>
<td>-</td>
</tr>
<tr>
<td>FeTPPCl</td>
<td>25</td>
<td>76.8%</td>
<td>&gt;99.9</td>
<td>-</td>
</tr>
<tr>
<td>FeTPPCl</td>
<td>35</td>
<td>61.1%</td>
<td>&gt;99.9</td>
<td>-</td>
</tr>
<tr>
<td>FeTPPCl</td>
<td>45</td>
<td>37.4%</td>
<td>&gt;99.9</td>
<td>-</td>
</tr>
</tbody>
</table>

Reaction conditions: 20 mM 4-Bromostyrene (1a), 40 mM diazoacetonitrile (2a, in situ generated from aminoacetonitrile hydrochloride (40 mM) and NaNO₂ (60 mM, added in 30 min)), sodium dithionite (20 mM), DCM (100 µl), FeTPPCl (2% mol) at H₂O (1 mL), 20 h.
Table S4. The effect of temperature on the N-H insertion reaction catalyzed by VHb (as a carbene transferase).

\[
\text{NH}_2 + \text{EDA} \xrightarrow{\text{WT VHb}} \text{NH-COOEt}
\]

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Temperature(°C)</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT VHb</td>
<td>5</td>
<td>15.7%</td>
</tr>
<tr>
<td>WT VHb</td>
<td>15</td>
<td>47.8%</td>
</tr>
<tr>
<td>WT VHb</td>
<td>25</td>
<td>54.3%</td>
</tr>
<tr>
<td>WT VHb</td>
<td>35</td>
<td>67.7%</td>
</tr>
<tr>
<td>WT VHb</td>
<td>45</td>
<td>78.5%</td>
</tr>
</tbody>
</table>

Reaction conditions: 10 mM aniline, 10 mM EDA, sodium dithionite (10 mM), MeOH (20 µl), WT VHb (0.2% mol) at H_{2}O (1 mL), 8h.

Table S5. Validation of WT VHb docking results using a single mutation in alanine at the docking site.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>TON</th>
<th>%de</th>
<th>%ee</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2536</td>
<td>&gt;99.9</td>
<td>99.9</td>
</tr>
<tr>
<td>Y29A</td>
<td>3506</td>
<td>&gt;99.9</td>
<td>65.1</td>
</tr>
<tr>
<td>P54A</td>
<td>1944</td>
<td>&gt;99.9</td>
<td>80.2</td>
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<tr>
<td>L57A</td>
<td>1430</td>
<td>&gt;99.9</td>
<td>68.4</td>
</tr>
<tr>
<td>F43A</td>
<td>2047</td>
<td>&gt;99.9</td>
<td>78.4</td>
</tr>
</tbody>
</table>

Reaction conditions: 5 mM 4-Bromostyrene (1a), 10 mM diazonoacetonitrile (2a, in situ generated from aminoacetonitrile hydrochloride (10 mM) and NaNO₂ (15 mM, added in 30 min)), sodium dithionite (5 mM), MeOH (50 µl), purified protein (0.025% mol) at H_{2}O (4 mL), 15 °C, 20 h.
Figure S1. The time courses of cyclopropanation transformation. Reaction conditions: 5 mM 4-bromostyrene (1a), 10 mM diazoacetonitrile (2a, in situ generated from aminoacetonitrile hydrochloride (10 mM) and NaNO₂ (15 mM, added in 30 min)), 5 mM sodium dithionite, promoting solvent: MeOH (50 µl), WT VHB (0.025% mol) at H₂O (4 mL), 15°C.

Scheme S1. Docking results of hemoproteins (carbenoid intermediate) with 4-bromostyrene. 3D diagram (left), 2D diagram with type of interaction (right). Hb-Bovine, PDB ID: 6I1
Hb-human, PDB ID: 4N8T

Hb-Leporidae, PDB ID: 2RAO

Hb-porcine, PDB ID: 1QPW
**Mb-Horse heart, PDB ID: 5D5R**

![Mb-Horse heart, PDB ID: 5D5R](image1)

**Cytochrome C-Horse Heart, PDB ID: 6K9I**

![Cytochrome C-Horse Heart, PDB ID: 6K9I](image2)

**Table S6.** Optimized the substrate concentration of this reaction. Under the optimized conditions, increase substrate concentration by reducing solvents and promoting solvents.

<table>
<thead>
<tr>
<th>Substrate concentration (1a)</th>
<th>Yield</th>
<th>%(de)</th>
<th>%(ee)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5mM</td>
<td>0.69</td>
<td>&gt;99.9</td>
<td>99.9</td>
</tr>
<tr>
<td>25mM</td>
<td>0.75</td>
<td>&gt;99.9</td>
<td>99.9</td>
</tr>
<tr>
<td>33mM</td>
<td>0.77</td>
<td>&gt;99.9</td>
<td>99.9</td>
</tr>
<tr>
<td>50mM</td>
<td>0.86</td>
<td>&gt;99.9</td>
<td>99.9</td>
</tr>
</tbody>
</table>
Reaction conditions: 0.02 mmol 4-Bromostyrene (1a), 0.04 mmol diazonoacetonitrile (2a, in situ generated from aminoacetonitrile hydrochloride (0.04 mmol) and NaNO₂ (0.06 mmol, added in 30 min)), sodium dithionite (0.02 mmol), MeOH (1.25%), purified protein (0.025% mol), 15 °C, 20 h.

*Define the yield at 100 mM as 1.

**Scheme S2.** Green chemistry metric E-factor for this cyclopropanation reactions.

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>E-factor</th>
<th>de%</th>
<th>ee%</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT VHB</td>
<td>722.6</td>
<td>99.9</td>
<td>99.9</td>
</tr>
<tr>
<td>ruthenium porphyrin complexes[4]</td>
<td>1848.8</td>
<td>50.0</td>
<td>71.0</td>
</tr>
<tr>
<td>FeTPPCL[5]</td>
<td>558.5</td>
<td>71.4</td>
<td>0</td>
</tr>
<tr>
<td>Mb (H64V,V68A)-expressing cells[6]</td>
<td>985.7</td>
<td>99.9</td>
<td>96.0</td>
</tr>
</tbody>
</table>

When the Authors have not reported the amount of reagents used in the work-up and purification procedures procedure, we have accounted for the same amount we used (40 ml hexanes / ethyl acetate 8:1 and 20 mg MgSO₄)

**E-Factor for 3a using VHB (This Work):**

Total process used: 73.2 mg + 74 mg + 82 mg + 3.2 mg + 69.6 mg + 39.6 mg + 4000 mg + 15900 mg + 27400 mg + 4000 mg + 20 mg = 51661.6 mg

Amount of final product: 71.4 mg
Amount of waste: 51661.6 mg - 71.4 mg = 51590.2 mg

E-Factor = Amount of waste / Amount of product = 51590.2/71.4 = 722.6


\[
\begin{array}{c}
\text{Br} \\
\text{1a}
\end{array} \quad + \quad \begin{array}{c}
\text{NC} \quad \text{N}_2
\end{array} \quad \begin{array}{c}
\text{Ru} \quad 3.04 \text{ mg}
\end{array} \quad \text{DCM 1 ml}
\]
\[
\begin{array}{c}
\text{Br}
\end{array} \quad \begin{array}{c}
\text{CN}
\end{array} \quad \begin{array}{c}
\text{3a}
\end{array}
\]

1 mmol 183 mg 0.2 mmol 15.4 mg 40% 17.8 mg 71% ee

Total process used: 183 mg + 15.4 mg + 3.04 mg + 1325 mg + 31400 mg = 32926.4 mg
Amount of final product: 17.8 mg
Amount of waste: 32926.4 mg - 17.8 mg = 32908.6 mg
E-Factor = Amount of waste / Amount of product = 32908.6/17.8 = 1848.8

E-Factor for 3a using FeTPPCI (Koenigs’s work, Green Chem., 2017, 19, 2118-2122):

\[
\begin{array}{c}
\text{Br} \\
\text{1a}
\end{array} \quad + \quad \begin{array}{c}
\text{NC} \quad \text{N}_2
\end{array} \quad \begin{array}{c}
\text{FeTPPCI} \quad 2.82 \text{ mg}
\end{array} \quad \text{DCM 100 ul}
\]
\[
\begin{array}{c}
\text{Br}
\end{array} \quad \begin{array}{c}
\text{CN}
\end{array} \quad \begin{array}{c}
\text{3a}
\end{array}
\]

0.4 mmol 73.2 mg 84% 74.59 mg 0% ee

Total process used: 73.2 mg + 74 mg + 82 mg + 2.82 mg + 2000 mg + 132.5 mg + 7950 mg + 31400 mg + 20 mg = 41734.5 mg
Amount of final product: 74.59 mg
Amount of waste: 41734.5 mg - 74.59 mg = 41659.9 mg
E-Factor = Amount of waste / Amount of product = 41659.9/74.59 = 558.5
E-Factor for 3a using Mb (Fasan's work, Angew. Chem. Int. Ed, 2018, 57, 15852-15856):

Total process used: 109.8 mg + 231 mg + 0.016 mg + 21000 mg + 816 mg + 872mg + 42840 mg + 31400mg + 20mg = 97288.8 mg

Amount of final product: 98.6 mg

Amount of waste: 97288.8 mg - 98.6 mg = 97190.2 mg

E-Factor = Amount of waste/Amount of product = 97190.2/98.6 = 985.7

Beside the solvent used for the work-up and purification procedures, it can be concluded that in our case the most significant advantages are the environmentally friendly solvent and high efficiency of WT VHb. However, the lower substrate concentration limits the method from further reducing the E-factor.
HPLC analysis

The enantiomeric excess of the product was determined enantiomeric excess by HPLC analysis using Superchiral S-OD, serial: SOD541-10020903 5μm, column size: 4.6 mm I.D. × 150 mm L. The condition of HPLC was using a mobile phase of Hex:IPA=95:5, with a flow rate of 0.7 ml/minutes and a column temperature of 25 °C. The reference racemic samples were prepared as described in the experimental procedures.

Chiral HPLC analysis of racemic 3a (top) and enzymatically produced 3a product (bottom):
Chiral HPLC analysis of racemic 3b (top) and enzymatically produced 3b product (bottom):
Chiral HPLC analysis of racemic 3c (top) and enzymatically produced 3c product (bottom):
Chiral HPLC analysis of racemic 3d (top) and enzymatically produced 3d product (bottom):
Chiral HPLC analysis of racemic 3e (top) and enzymatically produced 3e product (bottom):
Chiral HPLC analysis of racemic 3f (top) and enzymatically produced 3f product (bottom):
Chiral HPLC analysis of racemic 3g (top) and enzymatically produced 3g product (bottom):
Chiral HPLC analysis of racemic 3h (top) and enzymatically produced 3h product (bottom):
Chiral HPLC analysis of racemic 3i (top) and enzymatically produced 3i product (bottom):
Chiral HPLC analysis of racemic 3j (top) and enzymatically produced 3j product (bottom):
Chiral HPLC analysis of racemic 3k (top) and enzymatically produced 3k product (bottom):
Chiral HPLC analysis of racemic 3l (top) and enzymatically produced 3l product (bottom):
**Synthetic Procedures**

General procedure for the biocatalytic cyclopropanation reactions using *in situ* generated diazoacetonitrile (Procedure A):

In a typical procedure, the olefin (5mM, 0.02 mmol in 50 uL methyl alcohol) was added to a 10 mL three necked flask containing 3mL water solutions of hemoproteins (0.025% mol) and aminoacetonitrile hydrochloride (10mM, 0.04mmol), equipped with a magnetic stir bar and sealed with a rubber septum. A solution of (15mM, 0.06 mmol) sodium nitrite and sodium dithionite (5mM, 0.02 mmol) in degassed deionized water (1 mL) was injected into the three necked flask slowly in 30 min at 15°C. The reaction mixture was stirred 20h at 15°C under nitrogen atmosphere. For product analysis, the reaction mixtures were extracted with dichloromethane (4 mL x 3) and the combined organic layers were dried over MgSO\textsubscript{4} and concentrated under reduced pressure. The crude product was purified by flash column chromatography using silica gel and ethyl acetate/petroleum ether as the eluent to isolate the cyclopropanation product. The purified product was characterized by NMR and chiral HPLC for stereoselectivity determination and they were used as authentic standards for the construction of calibration curves for determination of TON and yield values.

**Synthesis of racemic standards:**

General Procedure B:

FeTPPCI (3 mol%), amino acetonitrile hydrochloride (2 eq) and styrene derivative (0.4 mmol, 1 eq) were dissolved in 0.1 mL degassed dichloromethane and 1 mL degassed water under nitrogen atmosphere. NaNO\textsubscript{2} (2.5 eq) was dissolved in 1 mL degassed water, the solution was purged with nitrogen for a minute and was added via syringe pump in 30min. After complete addition the resulting reaction mixture was stirred for 20 h. The crude mixture was analyzed by NMR. The aqueous phase was extracted three times with dichloromethane; the combined organic layers were dried over MgSO\textsubscript{4} and concentrated under reduced pressure. The residue was purified on silica gel (hexanes / ethyl acetate 8:1), the product was dried by rotary evaporator below 25 °C to obtain the trans cyclopropanation product \[5\]. The isolated, racemic product was used for development of the HPLC analytical method for determination of enantiomeric excess. This procedure was applied for the preparation of racemic standards for 3a-3l.
Analytical data of products

2-(4-bromophenyl)cyclopropane-1-carbonitrile (3a). Following the procedure A, 3a was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.62), a brown solid, $^1$H NMR (400 MHz, Chloroform-d) δ 7.47 (d, $J = 8.4$ Hz, 2H), 7.02 (d, $J = 8.4$ Hz, 2H), 2.63 (ddd, $J = 9.2$, 6.4, 4.8 Hz, 1H), 1.68 (dt, $J = 9.2$, 5.6 Hz, 1H), 1.61 – 1.54 (m, 1H), 1.46 (ddd, $J = 8.8$, 6.8, 5.2 Hz, 1H).

2-(4-fluorophenyl)cyclopropane-1-carbonitrile (3b). Following the Procedure A, 3b was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.63), a white solid, $^1$H NMR (400 MHz, Chloroform-d) δ 7.12 (ddt, $J = 7.2$, 5.2, 2.0 Hz, 2H), 7.04 (td, $J = 8.4$, 1.8 Hz, 2H), 2.65 (dt, $J = 8.0$, 5.4 Hz, 1H), 1.66 (ddd, $J = 9.2$, 5.2, 3.6 Hz, 1H), 1.58 – 1.52 (m, 1H), 1.46 (ddt, $J = 9.2$, 6.8, 3.2 Hz, 1H).

2-(4-chlorophenyl)cyclopropane-1-carbonitrile (3c). Following the Procedure A, 3c was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.61), a yellow solid, $^1$H NMR (400 MHz, Chloroform-d) δ 7.32 (d, $J = 8.4$ Hz, 2H), 7.08 (d, $J = 8.4$ Hz, 2H), 2.64 (ddd, $J = 9.2$, 6.4, 4.8 Hz, 1H), 1.67 (dt, $J = 9.2$, 5.6 Hz, 1H), 1.57 (dt, $J = 10.0$, 5.2 Hz, 1H), 1.46 (ddd, $J = 8.8$, 6.8, 5.4 Hz, 1H).
2-(3-chlorophenyl)cyclopropane-1-carbonitrile (3d). Following the Procedure A, 3d was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.63), a yellow solid, \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 7.30 – 7.25 (m, 2H), 7.12 (d, \(J = 2.4\) Hz, 1H), 7.05 (dq, \(J = 5.6, 3.2, 2.0\) Hz, 1H), 2.64 (ddd, \(J = 9.2, 6.4, 4.8\) Hz, 1H), 1.68 (dt, \(J = 9.2, 5.6\) Hz, 1H), 1.60 (dt, \(J = 8.8, 5.2\) Hz, 1H), 1.49 (ddd, \(J = 8.7, 6.6, 5.3\) Hz, 1H).

2-(2-chlorophenyl)cyclopropane-1-carbonitrile (3e). Following the Procedure A, 3e was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.60), a yellow solid, \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 7.51 – 7.40 (m, 1H), 7.29 – 7.22 (m, 2H), 7.08 – 7.00 (m, 1H), 2.84 (ddd, \(J = 8.8, 6.6, 5.2\) Hz, 1H), 1.71 (dd, \(J = 9.2, 4.8\) Hz, 1H), 1.57 – 1.47 (m, 2H).

2-phenylcyclopropane-1-carbonitrile (3f). Following the Procedure A, 3f was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.66), a white solid, \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 7.38 – 7.28 (m, 3H), 7.15 (dd, \(J = 7.2, 2.0\) Hz, 2H), 2.68 (ddd, \(J = 9.2, 6.8, 4.8\) Hz, 1H), 1.69 – 1.64 (m, 1H), 1.60 (dt, \(J = 8.4, 5.2\) Hz, 1H), 1.50 (ddd, \(J = 8.4, 6.8, 5.2\) Hz, 1H).
2-(p-tolyl)cyclopropane-1-carbonitrile (3g). Following the Procedure A, 3g was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.53), a white solid, $^1\text{H NMR}$ (400 MHz, Chloroform-$d$) $\delta$ 7.16 (d, $J = 8.0$ Hz, 2H), 7.07 – 7.00 (m, 2H), 2.64 (ddd, $J = 9.2$, 6.8, 4.8 Hz, 1H), 2.37 (s, 3H), 1.65 – 1.60 (m, 1H), 1.54 (dt, $J = 10.0$, 5.2 Hz, 1H), 1.46 (ddd, $J = 8.8$, 6.8, 5.2 Hz, 1H).

2-(4-methoxyphenyl)cyclopropane-1-carbonitrile (3h). Following the Procedure A, 3h was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.57), a yellow solid, $^1\text{H NMR}$ (400 MHz, Chloroform-$d$) $\delta$ 7.11 – 7.05 (m, 2H), 6.88 (d, $J = 8.4$ Hz, 2H), 3.83 (s, 3H), 2.63 (ddd, $J = 9.2$, 6.4, 4.8 Hz, 1H), 1.61 (dt, $J = 9.6$, 5.2 Hz, 1H), 1.57 – 1.38 (m, 2H).

2-methyl-2-phenylcyclopropane-1-carbonitrile (3i). Following the Procedure A, 3i was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.62), a colorless liquid, $^1\text{H NMR}$ (400 MHz, Chloroform-$d$) $\delta$ 7.40 – 7.26 (m, 5H), 1.74 – 1.70 (m, 1H), 1.70 (s, 3H), 1.62 (t, $J = 4.4$ Hz, 1H), 1.35 (t, $J = 5.2$ Hz, 1H).
2-methyl-3-phenylcyclopropane-1-carbonitrile (3j). Following the Procedure A, 3j was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.62), a colorless liquid, $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.34 (dd, $J = 8.0, 6.4$ Hz, 2H), 7.28 (s, 1H), 7.11 (dd, $J = 7.2, 1.8$ Hz, 2H), 2.28 (t, $J = 5.6$ Hz, 1H), 1.78 – 1.70 (m, 2H), 1.49 (d, $J = 3.2$ Hz, 3H).

2-(naphthalen-2-yl)cyclopropane-1-carbonitrile (3k). Following the Procedure A, 3k was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.64), a yellow solid, $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.84 (td, $J = 9.2, 4.0$ Hz, 3H), 7.61 (d, $J = 1.6$ Hz, 1H), 7.52 (tt, $J = 7.2, 5.2$ Hz, 2H), 7.25 (dd, $J = 8.4, 1.6$ Hz, 1H), 2.84 (ddd, $J = 9.2, 6.4, 4.8$ Hz, 1H), 1.71 (ddd, $J = 18.4, 9.6, 4.8$ Hz, 2H), 1.65 – 1.60 (m, 1H).

4-(2-cyanocyclopropyl)butyl benzoate (3l). Following the Procedure A, 3l was purified by silicagel chromatography (petroleum ether:ethyl acetate=8:1, Rf=0.48), a yellow oil, $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.08 (dq, $J = 6.4, 2.4, 1.6$ Hz, 2H), 7.60 (dd, $J = 7.6, 2.8$ Hz, 1H), 7.53 – 7.45 (m, 2H), 4.38 (dt, $J = 10.4, 6.4$ Hz, 2H), 1.86 (dq, $J = 21.6, 6.8$ Hz, 2H), 1.66 (d, $J = 7.2$ Hz, 2H), 1.49 – 1.36 (m, 2H), 1.27 – 1.21 (m, 1H), 1.20 – 1.07 (m, 1H), 0.98 – 0.81 (m, 2H). $^{13}$C NMR (101 MHz, Chloroform-d) $\delta$ 166.71, 133.05, 132.98, 129.63, 128.48, 120.63, 64.62, 32.64, 30.42, 21.56, 18.54, 14.09, 2.81. MS (ESI): m/z = (M + H)$^+$ 243.23
NMR Spectra

3a-3k was full-scale reported by a published research.\[6\]

2-(4-bromophenyl)cyclopropane-1-carbonitrile (3a):

2-(4-fluorophenyl)cyclopropane-1-carbonitrile (3b):
2-(4-chlorophenyl)cyclopropane-1-carbonitrile (3c):
2-(3-chlorophenyl)cyclopropane-1-carbonitrile (3d):

2-(2-chlorophenyl)cyclopropane-1-carbonitrile (3e)

2-phenylcyclopropane-1-carbonitrile (3f):
2-(p-tolyl)cyclopropane-1-carbonitrile (3g):

2-(4-methoxyphenyl)cyclopropane-1-carbonitrile (3h):
2-methyl-2-phenylcyclopropane-1-carbonitrile (3i):

2-methyl-3-phenylcyclopropane-1-carbonitrile(3j)
2-(naphthalen-2-yl)cyclopropane-1-carbonitrile (3k):

4-(2-cyanocyclopropyl)butyl benzoate (3l):
Nucleotide and amino acid sequences of VHb variants

Nucleotide sequence of VHb (P54A)
ATGTTAGACCAAGCAACCATTAACATCATCAAAGCCACTGGTTCTGTATTGAAGGAGCACATGGCGTTACC
TACCACGACTTTTTTATAAACTGCTTTGCCAACACCCCTGAAAGTACGTCCTTTGGTATTGAGGTGCGCC
AGAATCTTTGGACCGAGCGAAGGCTTGGCCGATGACGTATTTGGCGCCAGGAAACCACATTTGAAAATTT
GCCAGCTATTTTTGCTGCGGTCAAAAAATTTGCACTACATGTGATTGTCGATGAAAGTATATTGCGCATGCGA
TATCCGATGGTCGCTAAAGAATTGTTGGGTGGATTAAAGAAGTATTGGCGATGCCGCAACCGATGACA
TTTTGGACCGCTGGGGCAAGGCATTATGGCGGTATTGCAGATGTGTTTATTCAAGTGGAAGCAGATTTGTACG
CTCAACGGCTTTGAACATCATCATCATCATCATATTAAA

Amino acid sequence of VHb (P54A)
MLDQQTINIIKATVPVLKEHGVTITTFFYKNLFAKHPEVRPLFDMGRQESLEQAKALAMTVLAAQNIENLPAIL
PAVKKIAVKHCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD
AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*.

Nucleotide sequence of VHb (Y29A)
ATGTTAGACCAAGCAACCATTAACATCATCAAAGCCACTGGTTCTGTATTGAAGGAGCACATGGCGTTACC
TACCACGACTTTTTGCGAAGGCTTGGCCGATGACGTATTTGGCGCCAGGAAACCACATTTGAAAATTT
GCCAGCTATTTTTGCTGCGGTCAAAAAATTTGCACTACATGTGATTGTCGATGAAAGTATATTGCGCATGCGA
TATCCGATGGTCGCTAAAGAATTGTTGGGTGGATTAAAGAAGTATTGGCGATGCCGCAACCGATGACA
TTTTGGACCGCTGGGGCAAGGCATTATGGCGGTATTGCAGATGTGTTTATTCAAGTGGAAGCAGATTTGTACG
CTCAACGGCTTTGAACATCATCATCATCATCATATTAAA

Amino acid sequence of VHb (Y29A)
MLDQQTINIIKATVPVLKEHGVTITTFFYKNLFAKHPEVRPLFDMGRQESLEQAKALAMTVLAAQNIENLPAIL
PAVKKIAVKHCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD
AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*.

Nucleotide sequence of VHb (F43A)
ATGTTAGACCAAGCAACCATTAACATCATCAAAGCCACTGGTTCTGTATTGAAGGAGCACATGGCGTTACC
TACCACGACTTTTTTATAAACTGCTTTGCCAACACCCCTGAAAGTACGTCCTTTGGTATTGAGGTGCGCC
AAGAATCTTTGGAGCAGTGTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTT
GCCAGCTATTTTGGCGCTGACCAAAAAAACATTGCAAGTAAATTTGGGCGAGATGGCGAGACCGCAGATGAC
TTTGGACGCGCTGGGCAAGGCGCTATGCGGATGATGGGTAAGATTTGGCAAGTGGATCTTCAGTGGAAACATTTGT

Amino acid sequence of VHb (F43A)
MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPENVPLADMGRQESLEHCKALAMTVLAAAQNIENLPAl
LPAVKKIAVHCAVAAAYIPIVGGQLLAIEVLEQLATADDILD
AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (L57A)
ATGTTAGACCCAAACATTCATACATCAAGGCGACTGTTCTGTATTTGAGAGCACTGGCTACCAT
TACCAGCCTTTTATAAATAACTGTTTGGGACCAAACACCCTGAAGTACGTCTTGTGGATATGGGTGCTGAC
AGAATCTTTTGGAGCAGTGTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTT
GCCAGCTATTTTGGCGCTGACCAAAAAAACATTGCAAGTAAATTTGGGCGAGATGGCGAGACCGCAGATGAC
TTTGGACGCGCTGGGCAAGGCGCTATGCGGATGATGGGTAAGATTTGGCAAGTGGATCTTCAGTGGAAACATTTGT

Amino acid sequence of VHb (L57A)
MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPENVPLADMGRQESLEHCKALAMTVLAAAQNIENLPAl
LPAVKKIAVHCAVAAAYIPIVGGQLLAIEVLEQLATADDILD
AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Reference