A colorimetric competitive displacement assay for the evaluation of catalytic peptides


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General methods

Reagents were purchased from Sigma Aldrich or TCI unless otherwise noted. Amino acids were purchased from Novabiochem EMD Millipore. Fmoc-Leu-Wang resin was purchased from Advanced Chem Tech. Column chromatography was performed using a Teledyne CombiFlash Rf+ automatic chromatography system, using 4g silica columns. Aldol reactions were dried using a BioChromato Spiral Plug Smart Evaporator. Nuclear magnetic resonance (NMR) spectra were collected on a Bruker Avance III 400 MHz NMR spectrometer. UV-Vis spectra were collected on a Thermo Scientific Evolution 300 UV-Vis spectrophotometer. Mass spectra were collected on a Thermo Scientific LCQ Fleet Mass Spectrometer.

Abbreviations used:
DCM - Dichloromethane
MeOH - Methanol
MeCN - Acetonitrile
Fmoc - Fluorenylmethyloxycarbonyl
NMP - N-Methyl-2-pyrrolidone
DiPEA - N,N-Diisopropylethylamine
LCMS - Liquid chromatography mass spectrometry
HCTU - O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
TFA - Trifluoroacetic acid
TIPS - Triisopropylsilane
pTSA - p-Toluene sulfonic acid
Determination of dissociation constant between PV and R

A solution containing the receptor R (1.5 mM, with 100 µM PV, in a buffer containing 10 mM pTSA and 10 mM DiPEA in a 1:1 mixture of MeOH and DCM) was titrated into a solution of PV (100 µM, in a buffer containing 10 mM pTSA, and 10 mM DiPEA in a 1:1 mixture of MeOH and DCM), and the absorbance was measured after each addition (Figure S1). The absorbance change at 520 nm was monitored, and the data were analyzed in GraphPad Prism 7 using the “Receptor Binding: Saturation Binding -- One site specific binding” (Figure S2), which calculated $K_D = 112 \pm 8.6 \mu M$, $R^2 = 0.993$.

![Figure S1 Titration between receptor R and PV.](image1)

![Figure S2 Change in absorbance at 520 nm during the titration between R and PV.](image2)
Determination of dissociation constant between \( R \) and \( 3 \)

A solution containing purified diol product \( 3 \) (30 mM, with 250 \( \mu \)M \( R \) and 100 \( \mu \)M \( PV \) in a buffer containing 10 mM pTSA and 10 mM DiPEA in 1:1 mixture of MeOH and DCM) was titrated into a solution of \( R \) and \( PV \) (250 \( \mu \)M \( R \) and 100 \( \mu \)M \( PV \) in a buffer containing 10 mM pTSA and 10 mM DiPEA in 1:1 mixture of MeOH and DCM), and the absorbance was measured after each addition (Figure S3). The absorbance change at 520 nm was monitored, and the data were analyzed in GraphPad Prism 7 using the “Receptor Binding: Competitive Binding -- One site - Fit \( K_i \)” (Figure 2 in main text), which calculated \( K_i = 1.83 \pm 0.065 \) mM, \( R^2 = 0.999 \).

![Figure S3](image.png)
Control titrations

A solution containing aldol starting material 1 or 2 (30 mM, with 250 µM R and 100 µM PV in a buffer containing 10 mM pTSA and 10 mM DiPEA in 1:1 mixture of MeOH and DCM) was titrated into a solution of R and PV (250 µM R and 100 µM PV in a buffer containing 10 mM pTSA and 10 mM DiPEA in 1:1 mixture of MeOH and DCM), and the absorbance was measured after each addition (Figures S4 and S5).

Figure S4 Titration of starting material 1 into a solution containing R and PV.

Figure S5 Titration of starting material 2 into a solution containing R and PV.
Peptide synthesis and characterization

**Experimental Determination of Resin Loading:**
Fmoc-Leu-Wang resin was purchased from AdvancedChemTech with a loading of 0.3-1.5 mmol/g. The specific loading level on the batch purchased was determined using the following protocol. Two to six mg of dried resin was weighed into a small glass vial, and 3 mL of a 20% by volume piperidine in NMP solution was added. After 5 minutes of rotation on a Labquake Rotisserie Shaker, the absorbance of the solution at 290 nm was measured using 20% by volume piperidine in NMP as a blank. The loading in mmol/g was calculated by dividing the absorbance at 290 nm by the mass of the resin times 1.65.

**Fmoc deprotection:**
The Fmoc group was removed by incubating the resin in a 20% by volume piperidine in NMP solution for 20 minutes with the solution refreshed halfway through. For every 100 mg of resin, 2 mL of the deprotection solution was used. The resin was then rinsed with NMP 5 times.

**Amino Acid Coupling:**
Double couplings were performed to produce high purity peptides that could be used as catalysts on the solid phase without purification. For the first coupling, six equivalents of Fmoc amino acid were weighed into a glass scintillation vial, to which six equivalents of HCTU were added. The reagents were dissolved in NMP (2 mL per 100 mg resin used) and 12 equivalents of DiPEA were added. The amino acid solution was added to the resin in a polypropylene syringe outfitted with a porous polypropylene disc at the bottom (Torvq, USA) and allowed to rotate on a Labquake Rotisserie Shaker. After 1 h, the solution was drained and the coupling was repeated with three equivalents of Fmoc amino acid, three equivalents of HCTU, and six equivalents of DiPEA for 1 h. After the second coupling the resin was washed five times with NMP.

**LCMS analysis:**
For analysis of peptide purity, peptides were cleaved from the resin by incubating for two hours in a cleavage cocktail consisting of 95% TFA, 2.5% H$_2$O, and 2.5% TIPS (1 mL per 10 mg resin used). The solution was then collected and the solvent was evaporated under inert gas. The peptide was then dissolved in MeCN and water for LCMS analysis. LCMS data was obtained on a Thermo Scientific LCQ Fleet Mass Spectrometer.

**Characterization data:**

<table>
<thead>
<tr>
<th>Catalytic Peptide</th>
<th>Sequence</th>
<th>Calculated [M+H]$^+$ (m/z)</th>
<th>Found [M+H]$^+$ (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>VdPGL</td>
<td>385.24</td>
<td>385.11</td>
</tr>
<tr>
<td>4b</td>
<td>VPGL</td>
<td>385.24</td>
<td>385.19</td>
</tr>
<tr>
<td>4c</td>
<td>VdPPPL</td>
<td>425.27</td>
<td>425.21</td>
</tr>
<tr>
<td>4d</td>
<td>VAAL</td>
<td>373.24</td>
<td>373.08</td>
</tr>
</tbody>
</table>
Figure S6 LCMS and $^1$H NMR characterization of catalytic peptide 4a, VbPGL, [M+H]$^+$ calculated $m/z = 385.24$, found $m/z = 385.11$. 
Figure S7 LCMS and $^1$H NMR characterization of catalytic peptide 4b, VPGL, [M+H]$^+$ calculated $m/z = 385.24$, found $m/z = 385.19$. 

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Figure S8 LCMS and $^1$H NMR characterization of catalytic peptide 4c, VdPPL, [M+H]$^+$ calculated $m/z = 425.27$, found $m/z = 425.21$. 
Figure S9 LCMS and $^1$H NMR characterization of catalytic peptide 4d, VAAL, [M+H]$^+$ calculated $m/z$ = 373.24, found $m/z$ = 373.08.
Protocol for peptide catalyzed aldol reactions

\[
\begin{align*}
\text{CH(OH)COOH} & \quad + \quad \text{HOC}_{6}H_{4}NO_{2} \\
& \xrightarrow{20 \text{ mol } \% \text{ catalyst 4}} \quad \text{CH(OH)}\text{C}_{6}H_{4}NO_{2}
\end{align*}
\]

DCM / RT / 48 h

Approximately 18 mg of peptide resin (corresponding to 0.017 mmol of peptide, or 0.2 equivalents, based on an experimentally determined resin loading of 0.9 mmol/g), was weighed into a 3 mL polypropylene syringe outfitted with a porous polypropylene disc at the bottom (Torviq, USA). The resin was incubated with DCM for 20 min, and then washed with DCM three times to remove residual traces of NMP from the peptide synthesis. In a 20-mL scintillation vial, 12.5 mg of 4-nitrobenzaldehyde (0.083 mmol, 1 eq) and hydroxyacetone (17 µL, 0.25 mmol, 3 eq) were dissolved in 1 mL of DCM. The solution was then pulled into the fritted syringe containing the resin-bound catalyst and the reaction rotated on a Labquake Rotisserie Shaker for 48 h at room temperature. After 48 hours, the reaction solution was expelled into a graduated cylinder, and the resin washed three times with DCM, with each wash expelled into the same graduated cylinder. The total volume of solution after all three washes was recorded.
Protocol for and results of catalytic peptide evaluation

After the completion of a peptide-catalyzed aldol reaction, the extruded crude reaction solution was divided into two portions. One portion was used for crude $^1$H NMR analysis by evaporating the solvent with a BioChromato Spiral Plug Smart Evaporator and redissolving in CDCl$_3$ (see representative crude NMR spectrum in Figure S10). The NMR conversion was calculated by comparing the integration of aromatic peaks from nitrobenzaldehyde starting material 2 and diol product 3 (H$_i$ and H$_e$ respectively, Figure S11). The other portion of the crude reaction solution was combined with an equal volume of a solution of 500 µM R and 200 uM PV in a buffer containing 20 mM pTSA and 20 mM DiPEA in MeOH to create a ‘buffered reaction solution’. The absorbance of a ‘dye receptor solution’ (250 µM R and 100 µM PV in a 1:1 mixture of methanol and dichloromethane with 10 mM pTSA and 10 mM DiPEA) was measured. An equal volume of the buffered reaction solution was added to the dye receptor solution and the change of absorbance at 520 nm was recorded. The interpolation function on GraphPad Prism 7 was used with a titration of the purified diol product 3 as a standard curve to translate the change in absorbance at 520 nm into the concentration of product in the buffered reaction solution. This value, with the volume of the crude reaction solution, was then used to calculate the reaction conversion (Table S2).

Figure S10 Representative crude $^1$H NMR spectrum.
Figure S11 Peaks used to calculate reaction conversion by crude $^1$H NMR spectroscopy.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Sequence</th>
<th>$\Delta A_{520}$</th>
<th>Assay conversion</th>
<th>Crude NMR conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>VbPGL</td>
<td>-0.196</td>
<td>75%</td>
<td>73%</td>
</tr>
<tr>
<td>4b</td>
<td>VPGL</td>
<td>-0.256</td>
<td>112%</td>
<td>90%</td>
</tr>
<tr>
<td>4c</td>
<td>VbPPL</td>
<td>-0.213</td>
<td>80%</td>
<td>71%</td>
</tr>
<tr>
<td>4d</td>
<td>VAAL</td>
<td>-0.161</td>
<td>51%</td>
<td>35%</td>
</tr>
<tr>
<td>4e</td>
<td>Wang resin</td>
<td>-0.009</td>
<td>2%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Assay results with alternate substrates

The dye displacement assay was tested with several other aldol reaction aldehyde substrates. The assay-calculated reaction conversion was compared to the crude NMR reaction conversion to examine the validity of the assay for various substrates.

![Chemical structures and diagram](image)

**Figure S12** Results of the dye displacement assay with other aldehyde substrates.
Enantioselectivity of catalysts 4a and 4b

The enantioselectivity of catalysts 4a and 4b was studied using chiral HPLC. The diol product 3 was run on a CHIRALPAK IA-3 3 micron 4.6mm x 50mm column with a hexanes:isopropanol gradient. The peaks were assigned according to the literature (reference 10 in the main text). Catalyst 4a resulted in 6% ee of the anti RR isomer with 5.9:1 syn:anti dr. Catalyst 4b resulted in 16% ee of the anti SS isomer with 3.2:1 syn:anti dr.