Application of directed enzyme evolution in synthesis

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10/25/2018
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Agenda

• Background and Significance

• Directed Evolution

• **Synthesis Application-Tryptophan**
  - Tryptophan Analogs
  - Natural Products
  - Directed evolution of TrpB
“Enzymes are used in biocatalytic processes for the efficient and sustainable production of pharmaceuticals, fragrances, fine chemicals, and other products. Most bioprocesses exploit chemistry found in nature, but we are now entering a realm of biocatalysis that goes well beyond. Enzymes have been engineered to catalyze reactions previously only accessible with synthetic catalysts. Because they can be tuned by directed evolution, many of these new biocatalysts have been shown to perform abiological reactions with high activity and selectivity.”
Background

Non-canonical amino acids

Directed Evolution

Biological building blocks

Screening & Testing

Complex bioactive molecules

Mechanistic Studies

Directed Evolution

- protein engineering technique
- mutagenesis
- mimics natural selection
- specific goals

- KIE
- isotope trajectory
- $R_{\text{turnover}}$
- site-specific mutagenesis
- surrogate study

- computational design
- laboratory probe
- laboratory evolution

Significance

Improvement of current chemical synthesis

catalytic  selectivity
simplification  activity
scope  scale

Abiological reactions using engineered enzymes

Target – Tryptophan Analogues

- biosynthetic precursors for a wide range of bioactivities
  - anticancer
  - antibiotic
  - antifungal
  - immunosuppressant
  - phytotoxic
  - chemical synthesis

L-Tryptophan

Current Limitations

• natural Trp derivatives: each requires a different enzyme

Current Limitations Cont.

• acylases and transaminates: only useful in setting the final stereoselectivity

• esterases: kinetic resolution (50% yield max)

• tryptophan synthase (TrpS): sensitive to the electronics and stericstics of the substrates
  • complex: α-subunit (TrpA) + β-subunit (TrpB)
  • TrpA is a necessary allosteric actuator
  • TrpB is directly involved in synthesis of tryptophan analogues

Starting Point

• recent development of TrpB variants: full activity without TrpA

• substrate: 4- substituted indoles
  • common in natural products
  • low reactivity with natural TrpB
  • 4-nitroindole

Starting Point – 4-nitroindole

- Steric hindrance
- Electron withdrawing (deactivating)

Starting Point – 4-nitroTrp

- 4-nitrotryptophan (4-nitroTrp) as a chemical precursor
  - complex enantiopure synthesis
  - limited scale using the natural enzyme


Synthesis of Thaxtomin A

90% yield
99% ee

Thaxtomin A herbicide

mirror-Thaxtomin A fungicide

mirror-iso-Thaxtomin A antiviral

iso-Thaxtomin A antiviral

Synthesis of Thaxtomin A

L-glutamic acid

1. Me$_3$SiCl, MeOH
2. Boc$_2$O, Et$_3$N, MeOH

95%

Mel, Ag$_2$O, DMF

90%

i. DIBAL, Et$_2$O, -78 °C
ii. PCC, DCM

85%

Pd(OAC)$_2$, DABCO, DMF

80%

NO$_2$


90%

T3P, DIEA, DMF

80%
Synthesis of Indolactam V

L-glutamic acid

i. TMSCI, MeOH
II. Boc₂O, Et₃N

NaBH₄
THF/MeOH (5:1)

i. 2,2-DMP, acetone
 BF₃·Et₂O, rt
ii. (COCl)₂, DMSO
 CH₂Cl₂, -78 °C
then Et₃N, rt
68% in 5 steps

Pd(OAc)₂
DABCO, DMF
80 °C, 12 h
75%

10% Pd/C
H₂ (4 atm)
MeOH, rt
84%

i. Pd/C, H₂ (4 atm), MeOH
ii. TFA/CH₂Cl₂ (1:5)
iii. HATU, DIPEA, THF
iii. HCHO, NaBH₃CN
HOAc, MeCN, 0 °C
55% in 4 steps

• PfTrpB & TmTrpB
  • trace activity
• variants of 4-nitroTrp
  • Pf2B9: 18% conversion
• RDS: binding Ser
• reversible formation of side products is undesired

Active Site Mutagenesis- Sterics

- E104 – not modified
  - binds to indole through NH
  - Promotes the attack from C3
- L161V: 25%, no side product
- Not a steric problem

Random Mutagenesis

- Pf5G8 (leucine)
  - M139, L212,N166D: 60%, equimolar 4-nitroindole and Ser

- Pf2A6
  - E104G mutation: 86%
  - disapproves the supposition that H-bonding interaction with 4-nitroindole promotes the rxn
  - V186A, I183F & precedent: 91%
  - glycine is optimal (site-saturation screening)

Optimization towards Nitroindoles

- Tm2F3 1184F: 86% @5
  - Transferred mutations from PfTrpB to the corresponding positions in TmTrpB
- Pf0A9 E104G: 91% @6
  - 6-nitroindole has a different reactivity from 4-nitroindole

### Table 3. Tryptophan Analogues Produced by Catalyst Panel

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>R</th>
<th>Catalyst</th>
<th>Isolated yield (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>NO₂</td>
<td>P₂A6</td>
<td>95&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>2</td>
<td>F</td>
<td>T₂F₃</td>
<td>97</td>
<td></td>
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<tr>
<td>3</td>
<td>Br</td>
<td>T₂F₃</td>
<td>72</td>
<td></td>
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<tr>
<td>4</td>
<td>CN</td>
<td>T₂F₃ I₁₈₄F</td>
<td>41&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>5</td>
<td>B(OH)_₂</td>
<td>ND</td>
<td></td>
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<tr>
<td>6</td>
<td>NO₂</td>
<td>T₂F₃ I₁₈₄F</td>
<td>88&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>7</td>
<td>CN</td>
<td>T₂F₃</td>
<td>79</td>
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<td>8</td>
<td>CONH₂</td>
<td>T₂F₃</td>
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<td>9</td>
<td>B(OH)_₂</td>
<td>P₀A₉</td>
<td>37</td>
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</tr>
<tr>
<td>10</td>
<td>I</td>
<td>P₀A₉</td>
<td>74&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>11</td>
<td>CF₃</td>
<td>P₂A6</td>
<td>19&lt;sup&gt;b, c&lt;/sup&gt;</td>
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<tr>
<td>12</td>
<td>NO₂</td>
<td>P₀A₉ E₁₀₄G</td>
<td>91</td>
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<tr>
<td>13</td>
<td>Cl</td>
<td>P₀A₉</td>
<td>98</td>
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<tr>
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<td>Br</td>
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<td>97</td>
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<td>CN</td>
<td>P₀A₉</td>
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<td>16</td>
<td>B(OH)_₂</td>
<td>P₀A₉</td>
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<tr>
<td>17</td>
<td>NO₂</td>
<td>P₂A6</td>
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<td>18</td>
<td>CN</td>
<td>P₂A₆</td>
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</tr>
<tr>
<td>19</td>
<td>Cl</td>
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<tr>
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<td>I</td>
<td>P₀A₉</td>
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<tr>
<td>21</td>
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<td>P₅G₈</td>
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<td>22</td>
<td>B(OH)_₂</td>
<td>ND</td>
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<td>23</td>
<td>5,6-Cl₂</td>
<td>P₅G₈</td>
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<td>24</td>
<td>5-Br-7-F</td>
<td>T₂F₃ I₁₈₄F</td>
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<td>5-Cl-7-I</td>
<td>T₂F₃</td>
<td>10</td>
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</tr>
</tbody>
</table>

<sup>a</sup>Reactions used 0.02 mol % catalyst loading (maximum 5000 turnovers) and 1.1 equiv Ser relative to indole substrate. <sup>b</sup>Catalyst loading was 0.1 mol % (maximum 1000 turnovers). <sup>c</sup>Reaction gives alkylation at nitrogen. ND, not detected.
Kinetics of the Mutations

The rate of Ser deamination: incubating the enzymes with Ser, excluding a Nu substrate and measuring the production of pyruvate.

<table>
<thead>
<tr>
<th>entry</th>
<th>catalyst</th>
<th>initial turnover frequency (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>to 4-nitroTrp</td>
</tr>
<tr>
<td>1</td>
<td>PfTrpB</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Pf2B9</td>
<td>1.25 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>PfSG8</td>
<td>1.8 ± 0.12</td>
</tr>
<tr>
<td>4</td>
<td>PfSG8 E104G</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>Pf2A6</td>
<td>7.0 ± 0.3</td>
</tr>
</tbody>
</table>

*Table 4. Initial Rates Throughout Evolution*

- competing hydrolysis of amino-acrylate IM
- accelerating Nu attack of the substrate
  - Binding at the active site
  - Increasing the persistence of amino-acrylate

The New Catalyst

• M139 & N166 in open/close conformational states of TrpB
  • fully open: inactive/substrate entry and product release
  • fully closed: amino-acrylate formation, Nu addition/blocking access
  • stabilization of closed state = decreased Ser amination
  • H-bond between N166 and H275
  • rotameric switch for closure
  • basic aspartate to stabilize H-bond

The New Catalyst Cont.

• Active site mutations
  • reshape to accommodate 4-nitroindole
  • bind in more reactive conformation
  • E104: modulates the transition to the closed state
  • ongoing study

The New Catalyst Cont.

• Directed evolution towards a challenging substrate unlocked the reactivity for many related substrates.

• The reaction was only limited by Ser hydrolysis, therefore, mutations to decrease Ser hydrolysis was the focus, and this focus was independent of substrates.

TrpB in Organic Synthesis

• challenge: Installing chiral amino acid moiety (α-C stereochemistry)
  • using existing amino acids: amine and carboxylate functional groups require protecting groups
• solution: TrpB
  • unprotected Ser
  • almost 100% enantioselective
  • straightforward synthesis
  • easy purification
    • precipitation
    • Amino-acrylate @ AS only
  • high expression level
  • thermostability
  
mediates 4-nitroindole
  - Electronically deactivated
  - Sterically hindered
  - Poorly soluble

Expandable Scope

Will engineered biocatalysts open a door to new syntheses?