# Application of directed enzyme evolution in synthesis

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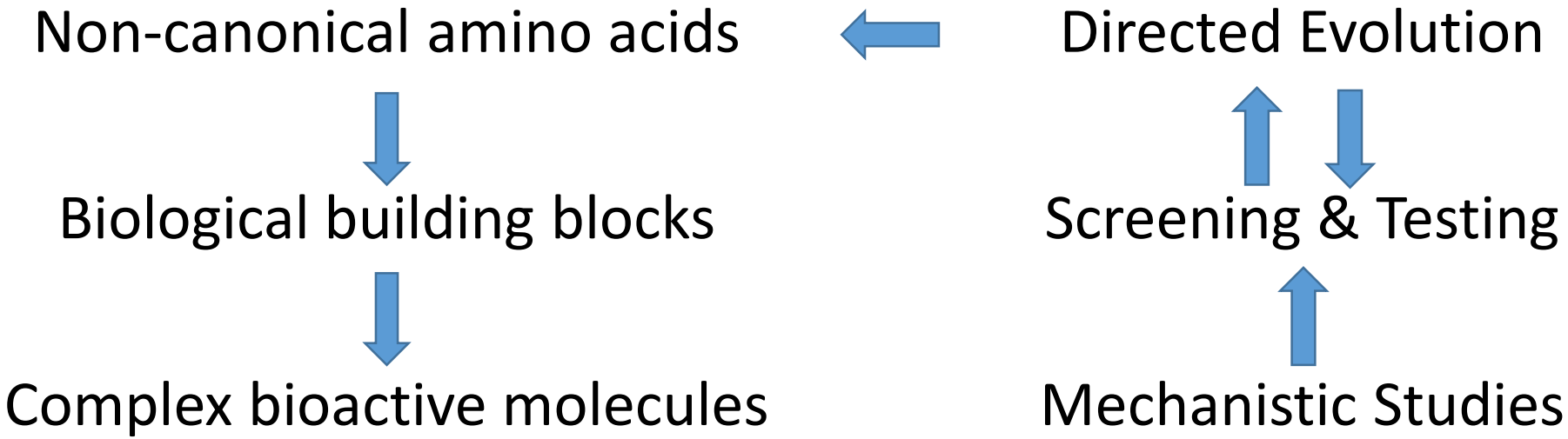
# Agenda

- 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.”

Hammer, S. C., Knight, A. M., Arnold, F. H, *Current Opinion in Green and Sustainable Chemistry*. 2017, 7:23–30

# Background

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# Directed Evolution

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

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

- computational design
- laboratory probe
- laboratory evolution

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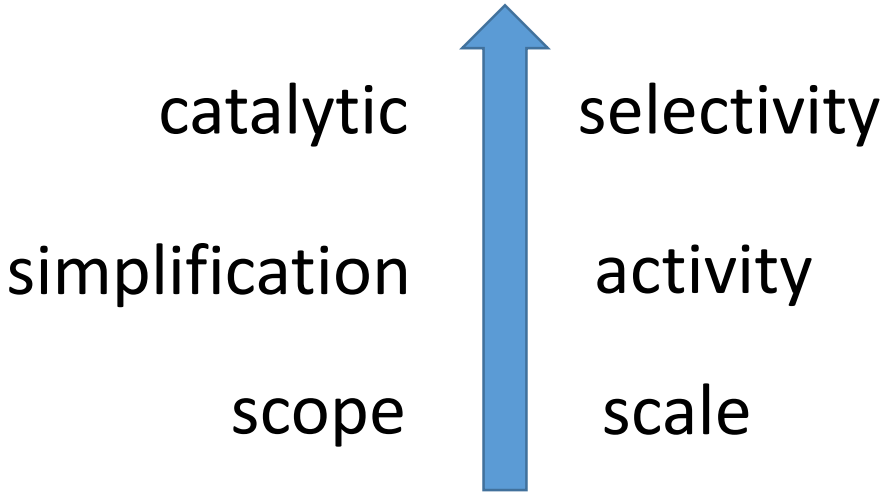
Zeymer, C., Zschoche, R., Hilvert, D. *J. Am. Chem. Soc*. **2017**, *139*, 12541-12549.

# Significance

Improvement of current chemical synthesis




Abiological reactions using engineered enzymes

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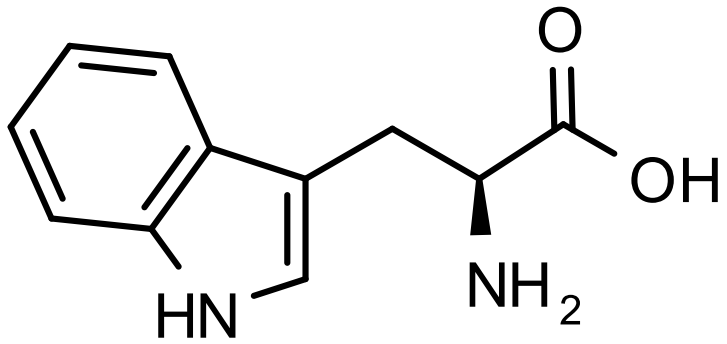
# Target – Tryptophan Analogues

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

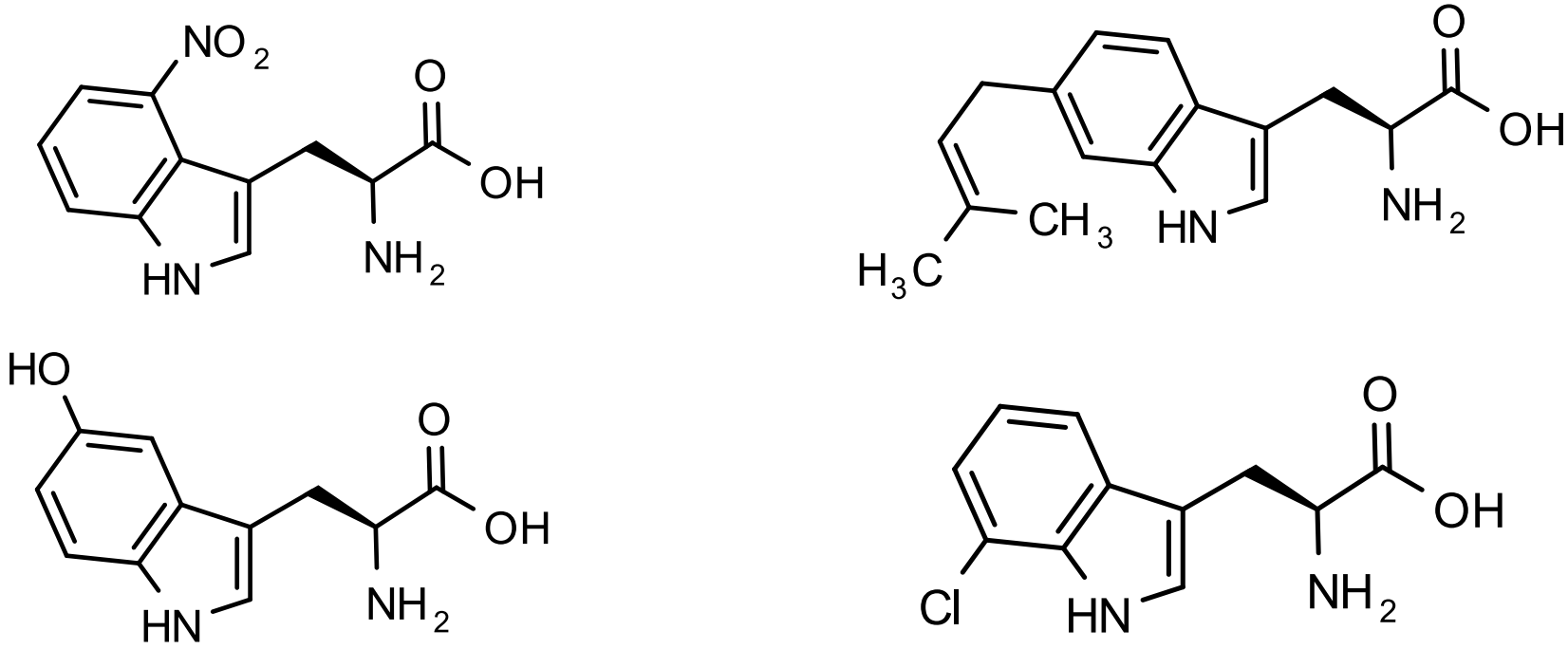



L-Tryptophan

Jordan, P. A.; Moore, B. S. Cell Chem. Bio. 2016, 23, 1504−1514.
Alkhalaf, L. M.; Ryan, K. S. Chem. Biol. 2015, 22, 317−328.

# Current Limitations

- natural Trp derivatives: each requires a different enzyme




Alkhalaf, L. M.; Ryan, K. S. Chem. Biol. 2015, 22, 317−328.

# 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 sterics 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

Ley, S. V.; Priour, A.; Heusser, C. Org. Lett. 2002, 4, 711−714.
Blaser, G.; Sanderson, J. M.; Batsanov, A. S.; Howard, J. Tetrahedron Lett. 2008, 49, 2795−2798.
Baldwin, J. E.; Dyer, R. L.; Ng, S. C.; Pratt, A. J.; Russell, M. A. Tetrahedron Lett. 1987, 28, 3745−3746.

# 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

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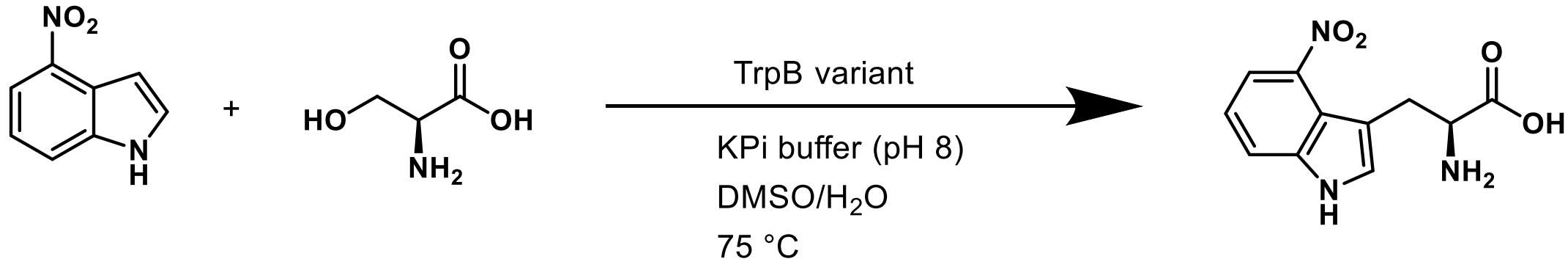
# Starting Point – 4-nitroindole

- Steric hindrance
- Electron withdrawing (deactivating)




4-nitroindole

4-nitroTrp

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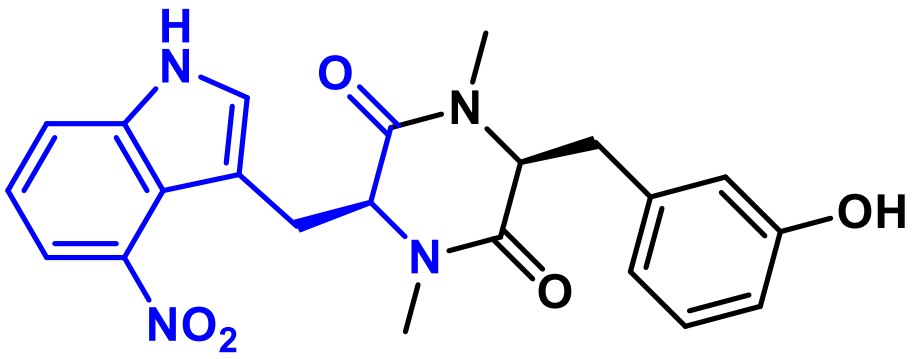
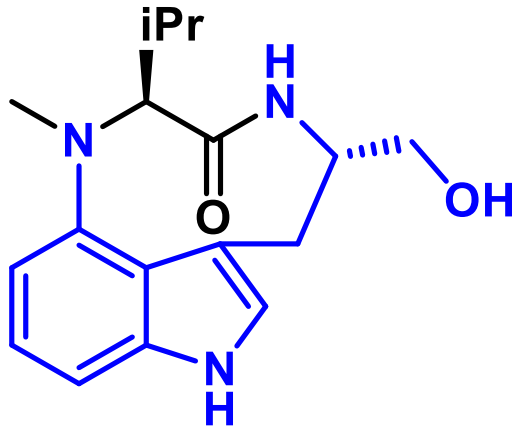
# Starting Point – 4-nitroTrp

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



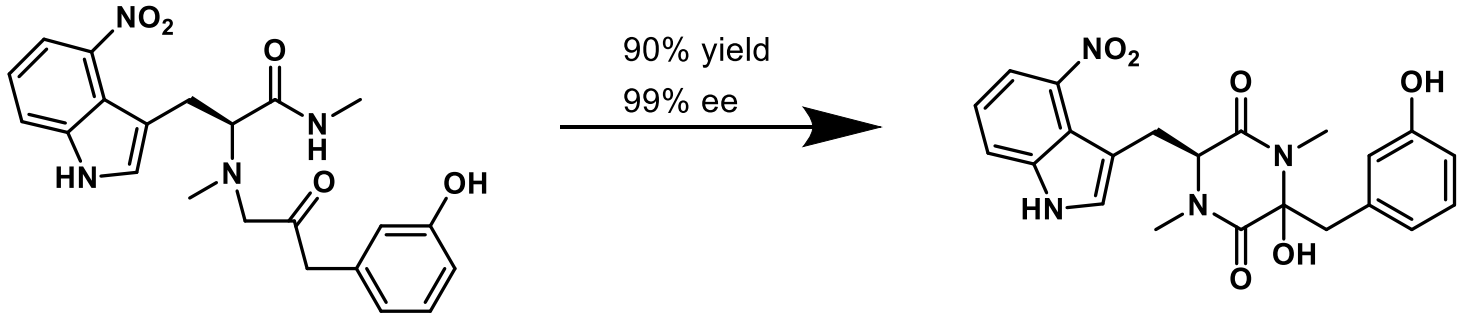

thaxtomin A

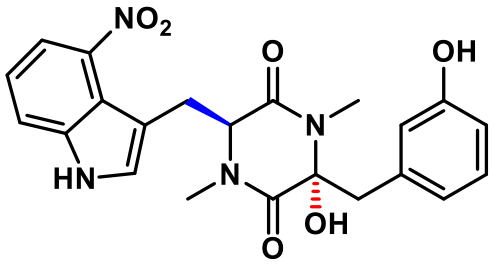



indolactam V

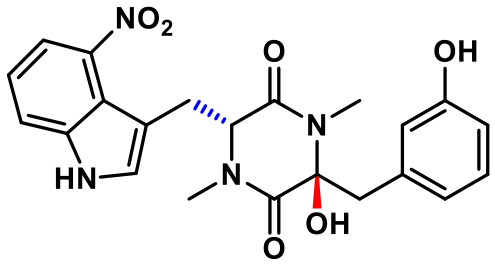
Zhang, H.; Ning, X.; Hang, H.; Ru, X.; Li, H.; Li, Y.; Wang, L.; Zhang, X.; Yu, S.; Qiao, Y.; Wang, X.; Wang, P. G. Org. Lett. 2013, 15, 5670−5673.
Barry, S. M.; Kers, J. A.; Johnson, E. G.; Song, L.; Aston, P. R.; Patel, B.; Krasnoff, S. B.; Crane, B. R.; Gibson, D. M.; Loria, R.; Challis, G. L. Nat. Chem. Biol. 2012, 8, 814−816.
Xu, Z.; Zhang, F.; Zhang, L.; Jia, Y. Org. Biomol. Chem. 2011, 9, 2512−2517.

# Synthesis of Thaxtomin A

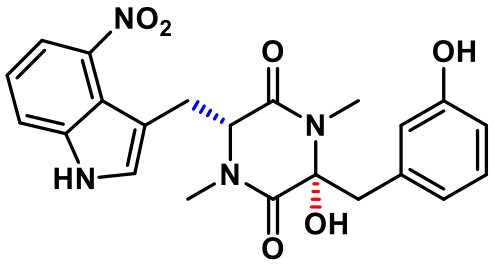


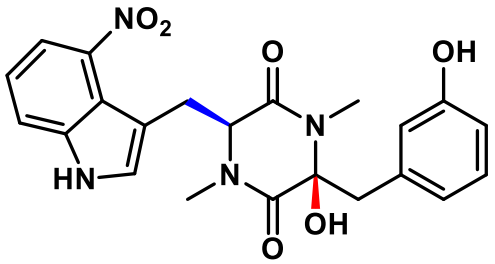

Thaxtomin A
herbicide




mirror-Thaxtomin A
fungicide



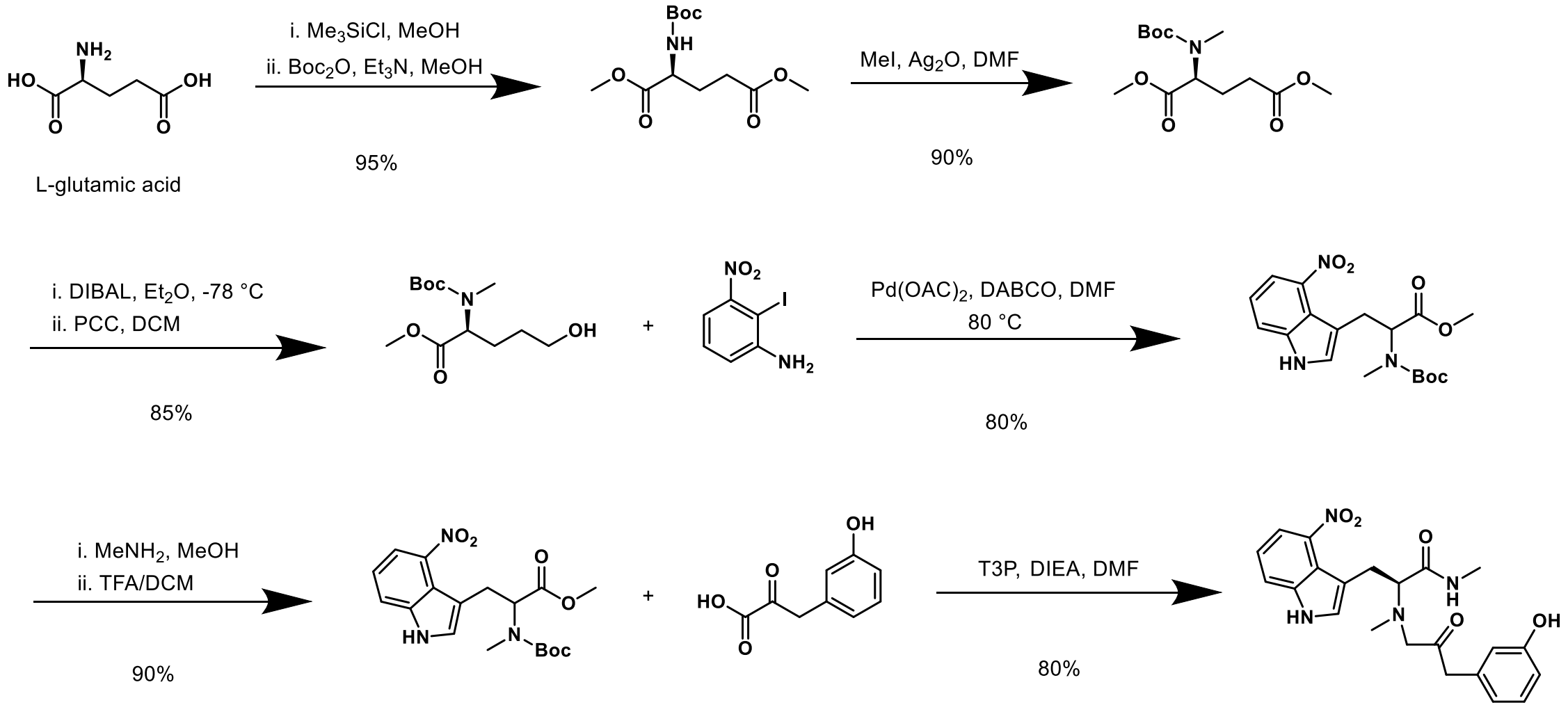

mirror-iso-Thaxtomin A
antiviral




iso-Thaxtomin A
antiviral

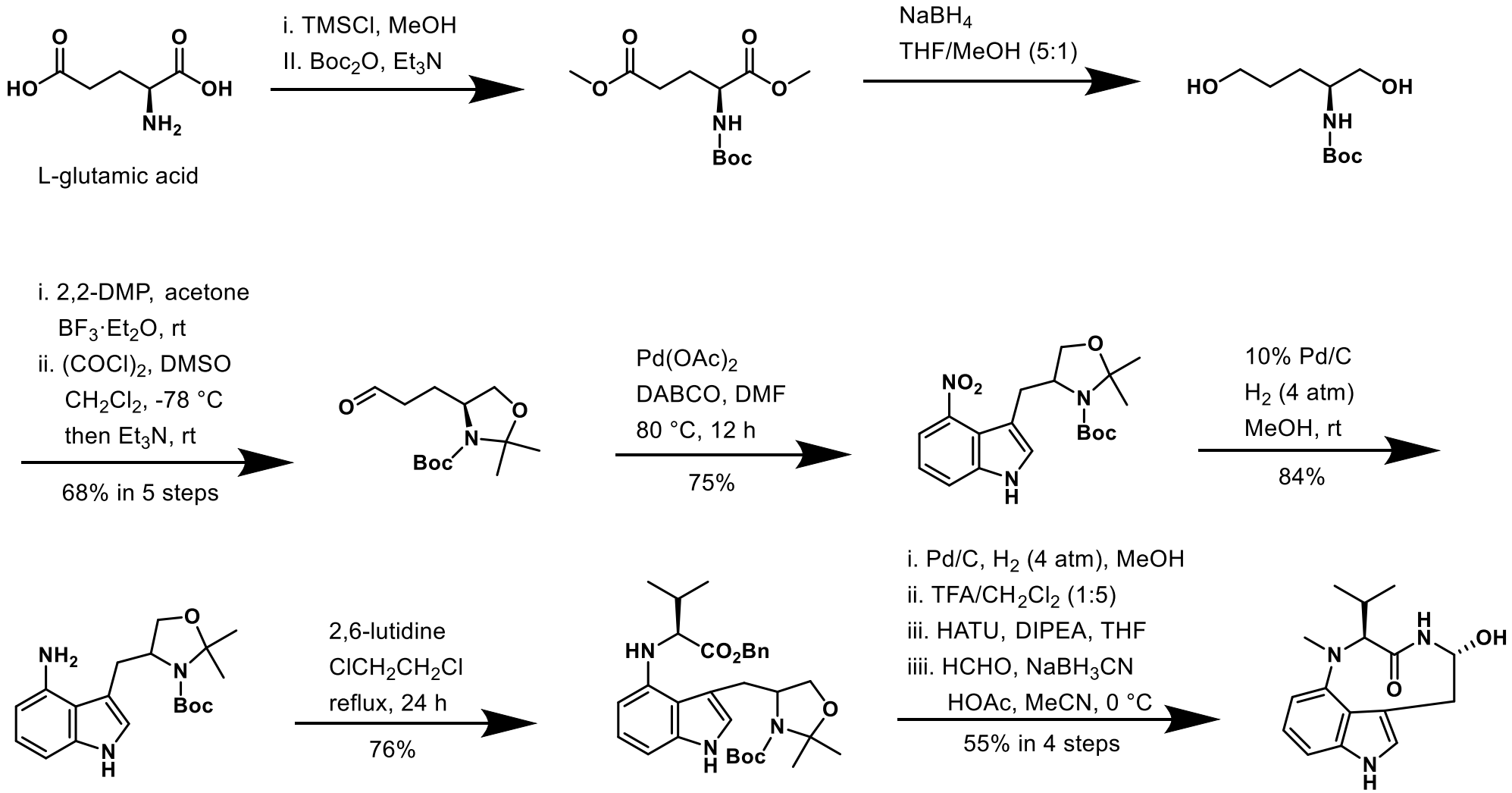
Zhang, H.; Ning, X.; Hang, H.; Ru, X.; Li, H.; Li, Y.; Wang, L.; Zhang, X.; Yu, S.; Qiao, Y.; Wang, X.; Wang, P. G. Org. Lett. 2013, 15, 5670−5673.

# Synthesis of Thaxtomin A Cont.




Zhang, H.; Ning, X.; Hang, H.; Ru, X.; Li, H.; Li, Y.; Wang, L.; Zhang, X.; Yu, S.; Qiao, Y.; Wang, X.; Wang, P. G. Org. Lett. 2013, 15, 5670−5673.

# Synthesis of Indolactam V




Xu, Z.; Zhang, F.; Zhang, L.; Jia, Y. Org. Biomol. Chem. 2011, 9, 2512–2517.

Screening

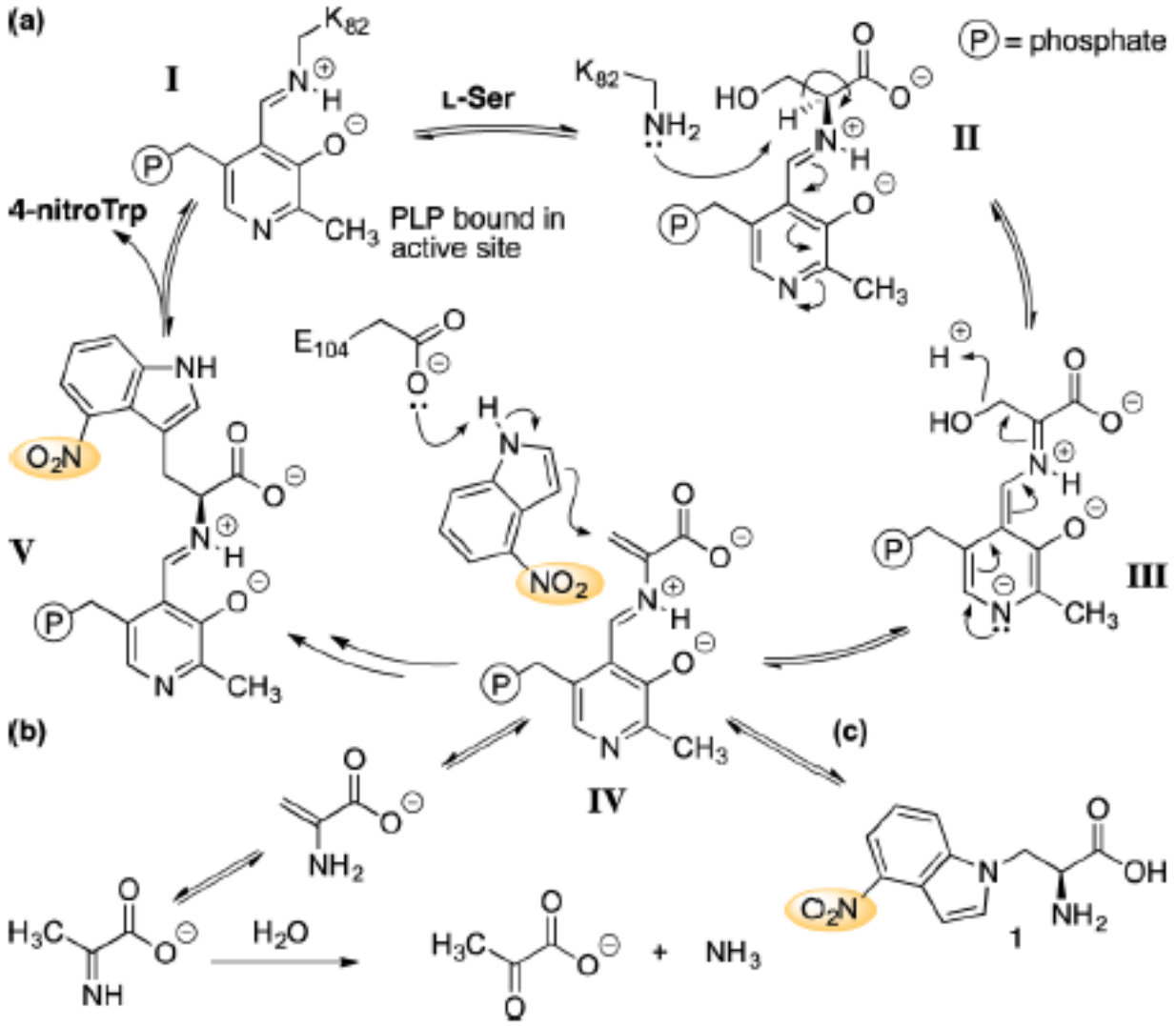



Figure 2. Putative pathways for reaction with 4-nitroindole. (a) Catalytic cycle for formation of 4-nitroTrp. (b) Enzymatic decomposition of Ser. (c) Competitive formation of isotryptophan 1.

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

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# Active Site Mutagenesis- Sterics

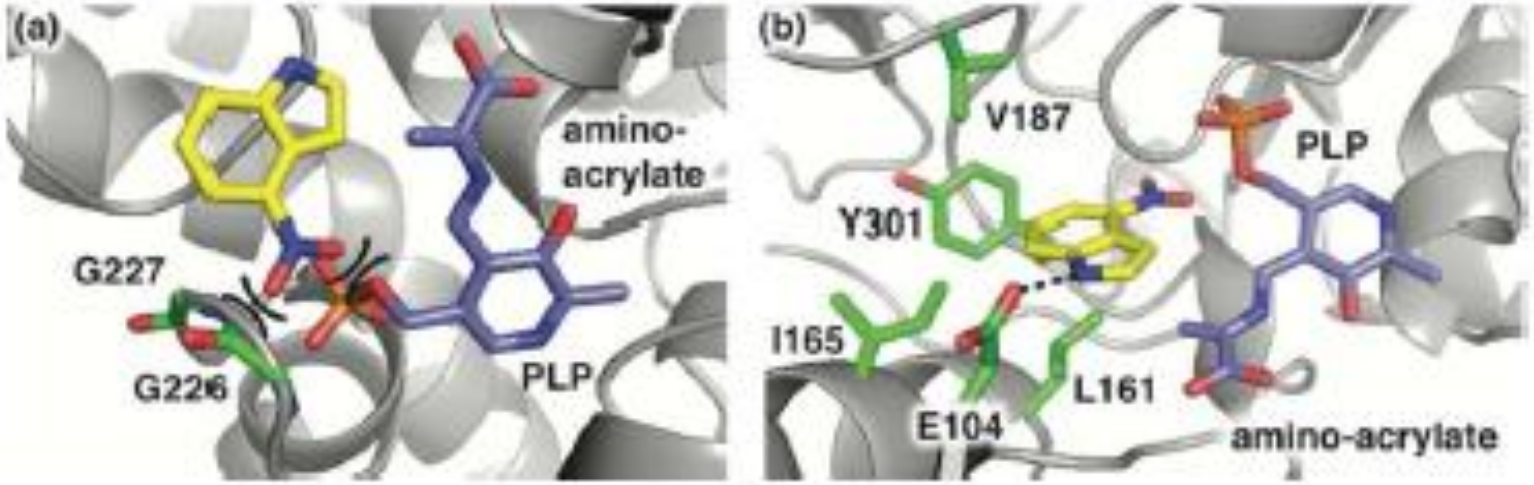


**Figure 3.** Model of 4-nitroindole (yellow) and the amino-acrylate in the active site of *Pf*TrpB (see Section 8.13 of Supporting Information). (a) Nitro group clashes with the protein backbone (green) and the PLP cofactor (purple). (b) Alternative view showing side-chains extending in to the active site and hydrogen bond with E104.

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

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# Random Mutagenesis

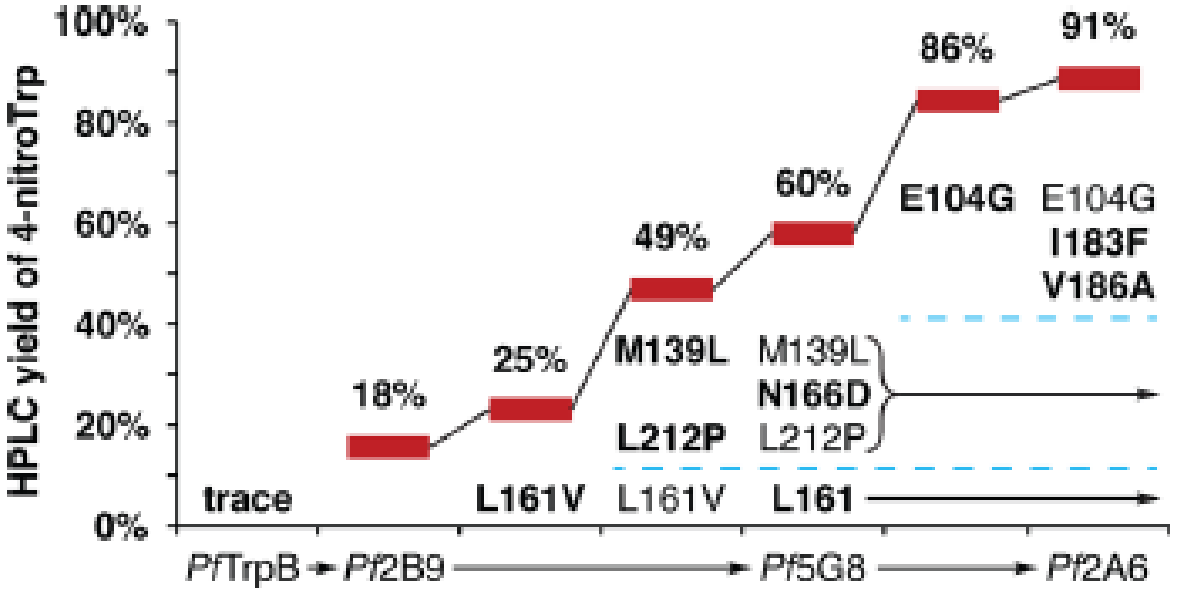


**Figure 4.** Evolutionary progression in production of 4-nitroTrp. Mutations in bold were added in the corresponding round of mutagenesis and screening. Dashed lines denote a new round of random mutagenesis. The horizontal axis indicates catalyst designations. See Section 8.7 of the Supporting Information for experimental details.

- 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)

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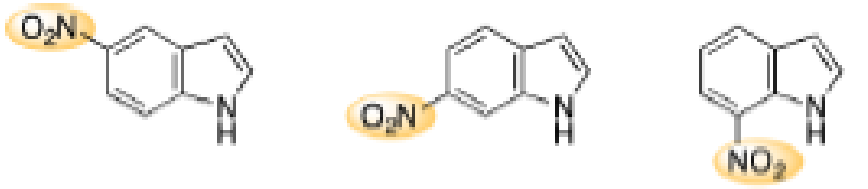
# Optimization towards Nitroindoles

**Chart 2. Nitro Substitution at Other Positions**




5-nitroindole 6-nitroindole 7-nitroindole

**Table 1. Optimizing Catalysts for Other Nitroindole Isomers**

| | | HPLC yield of nitroTrp (%)[b] | | | |
|---|---|---|---|---|---|
| entry | catalyst[a] | 4-nitro | 5-nitro | 6-nitro | 7-nitro |
| 1 | *Pf*2B9 | 18 | 8 | 17 | 29 |
| 2 | *Pf*5G8 | 60 | 64 | 64 | 64 |
| 3 | *Pf*2A6 | 91 | 5 | 26 | >99 |
| 4 | *Tm*2F3 | | 76 | | |
| 5 | *Tm*2F3 I184F | | 86 | | |
| 6 | *Pf*2B9 I165F Y301H | | | 66 | |
| 7 | *Pf*0A9 | | | 86 | |
| 8 | *Pf*0A9 E104G | | | 91 | |

[a]See Table 2 for catalyst designations. [b]Reactions used equimolar amounts of nitroindole and Ser. See Section 8.7 of the Supporting Information for experimental details.

- 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

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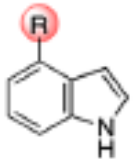
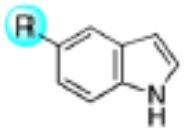
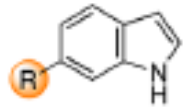
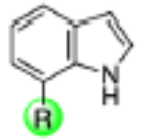
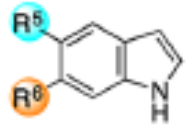
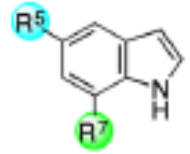
# Scope of Catalyst Substrate

**Table 3. Tryptophan Analogues Produced by Catalyst Panel[a]**

| Entry | Substrate | R | Catalyst | Isolated yield (%) |
|---|---|---|---|---|
| 1 |  | $NO_2$ | *Pf*2A6 | 95[b] |
| 2 | | F | *Tm*2F3 | 97 |
| 3 | | Br | *Tm*2F3 | 72 |
| 4 | | CN | *Tm*2F3 I184F | 41[b] |
| 5 | | $B(OH)_2$ | | ND |
| 6 |  | $NO_2$ | *Tm*2F3 I184F | 88[b] |
| 7 | | CN | *Tm*2F3 | 79 |
| 8 | | $CONH_2$ | *Tm*2F3 | 77 |
| 9 | | $B(OH)_2$ | *Pf*0A9 | 37 |
| 10 | | I | *Pf*0A9 | 74[b] |
| 11 | | $CF_3$ | *Pf*2A6 | 19[b,c] |
| 12 |  | $NO_2$ | *Pf*0A9 E104G | 91 |
| 13 | | Cl | *Pf*0A9 | 98 |
| 14 | | Br | *Pf*0A9 | 97 |
| 15 | | CN | *Pf*0A9 | 99 |
| 16 | | $B(OH)_2$ | *Pf*0A9 | 49 |
| 17 |  | $NO_2$ | *Pf*2A6 | 98 |
| 18 | | CN | *Pf*2A6 | 98 |
| 19 | | Cl | *Pf*0A9 | 99 |
| 20 | | I | *Pf*0A9 | 91 |
| 21 | | Br | *Pf*5G8 | 53 |
| 22 | | $B(OH)_2$ | | ND |
| 23 |  | 5,6-$Cl_2$ | *Pf*5G8 | 87 |
| 24 |  | 5-Br-7-F | *Tm*2F3 I184F | 56 |
| 25 | | 5-Cl-7-I | *Tm*2F3 | 10 |

[a]Reactions used 0.02 mol % catalyst loading (maximum 5000 turnovers) and 1.1 equiv Ser relative to indole substrate. [b]Catalyst loading was 0.1 mol % (maximum 1000 turnovers). [c]Reaction gives alkylation at nitrogen. ND, not detected.

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# Kinetics of the Mutations

**Table 4. Initial Rates Throughout Evolution**[a]

| | | initial turnover frequency ($min^{-1}$) | | |
|---|---|---|---|---|
| entry | catalyst | to 4-nitroTrp | to pyruvate | to Trp |
| 1 | *Pf*TrpB | – | 25.0 ± 0.3 | 19 ± 1.2 |
| 2 | *Pf*2B9 | 1.25 ± 0.07 | 12.2 ± 0.5 | 60.9 ± 0.16 |
| 3 | *Pf*5G8 | 1.8 ± 0.12 | 2.0 ± 0.2 | 9.9 ± 0.5 |
| 4 | *Pf*5G8 E104G | 3.5 ± 0.2 | 0.9 ± 0.12 | 7.03 ± 0.07 |
| 5 | *Pf*2A6 | 7.0 ± 0.3 | 1.4 ± 0.10 | 17.6 ± 0.3 |

[a]See Sections 8.10, 8.11, and 8.12 of the Supporting Information.

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

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

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# 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

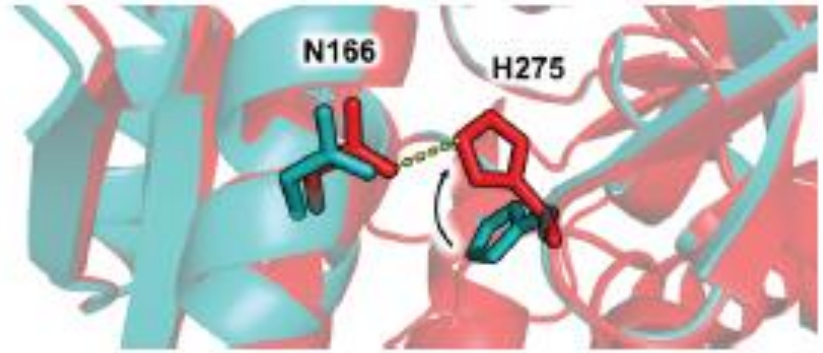


**Figure 5.** Overlaid crystal structures of *Pf*TrpB (PDB ID: 5DVZ) in the open state (cyan) and partially closed state (PDB ID: 5DW0, red) showing the side-chain motion of N166, and H275.

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# 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

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# 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.

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# 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
  - starightforward synthesis
  - easy purification
    - precipitation
    - Amino-acrylate @ AS only
  - high expression level
  - thermostability

mediates 4-nitroindole

- Electronically deactivated
- Sterically hindered
- Poorly soluble

*Expandable Scope*

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# Will engineered biocatalysts open a door to new syntheses?