Tailoring Tryptophan Synthase TrpB for Selective Quaternary Carbon Bond Formation

Markus Dick,[1] Nicholas S. Sarai,[1] Michael W. Martynowycz,[2] Tamir Gonen,[2] and Frances H. Arnold[1,*]

[1]Division of Chemistry and Chemical Engineering 210-41, California Institute of Technology, 1200 East California Boulevard, Pasadena, California 91125, United States

[2]Howard Hughes Medical Institute, David Geffen School of Medicine, Departments of Biological Chemistry and Physiology, University of California, Los Angeles, Los Angeles, California 90095, United States

Supporting Information

Contents

1 Activity of TrpB towards N-alkylation product of 3-methyloxindole 1

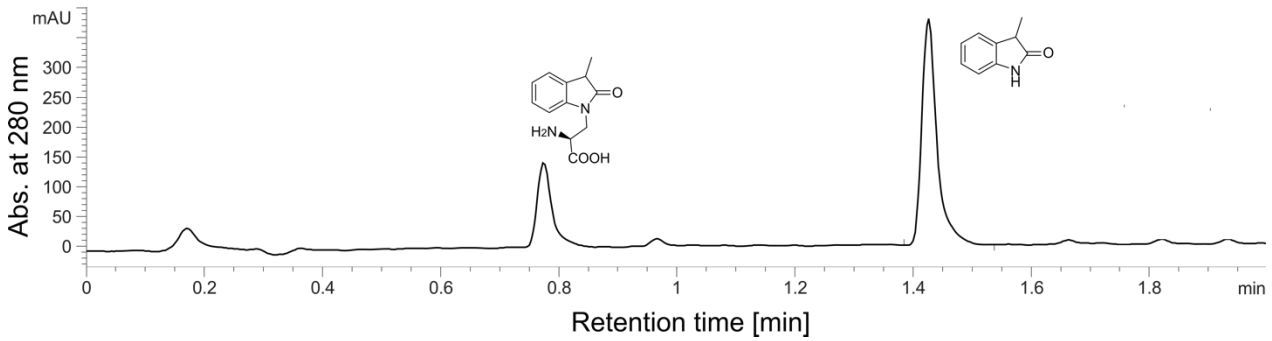


Figure S1: HPLC chromatogram for the TrpB catalyzed reaction of 3-methyloxindole 1 and serine 2. In this specific case, *Tm*TrpB9D8*-E103S[1] (20 µM) was used with 20 mM 3-methyloxindole **1** and 1 eq. serine **2** at 55 °C overnight, giving roughly 28% HPLC yield. A 2 min gradient from 5-50% acetonitrile in water (including 0.1 acetic acid) was used for reverse phase HPLC. The connectivity of the product was confirmed by NMR of the isolated product.

2 N- and C-alkylation products

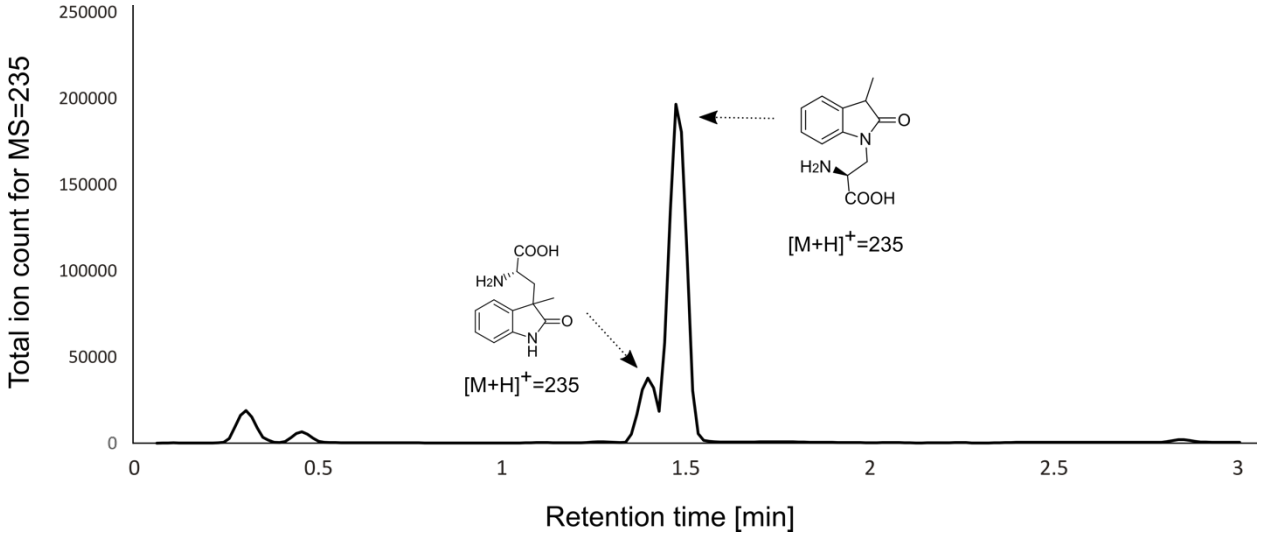


Figure S2: LC-MS chromatogram of the *Pf*0A9 E104G catalyzed reaction of 3-methyloxindole 1 and serine 2. The chromatogram only shows signals for compounds with a mass of 235 g/mol, which corresponds to the protonated products shown. For the reaction, 20 mM of 3-methyloxindole **1**, 1 eq. of serine **2** and 25% v/v clear cell lysate (corresponding to roughly 25 µM final concentration of *Pf*0A9 E104G[2]) were used. For separation, a 3 min gradient from 5-95% acetonitrile on reverse-phase LC-MS was used. The product yield (both N- and C-alkylation) was less than 0.1%. An authentic standard was used to assign the retention times to the corresponding products.

3 NMR of 3-methyloxindole in chloroform and water

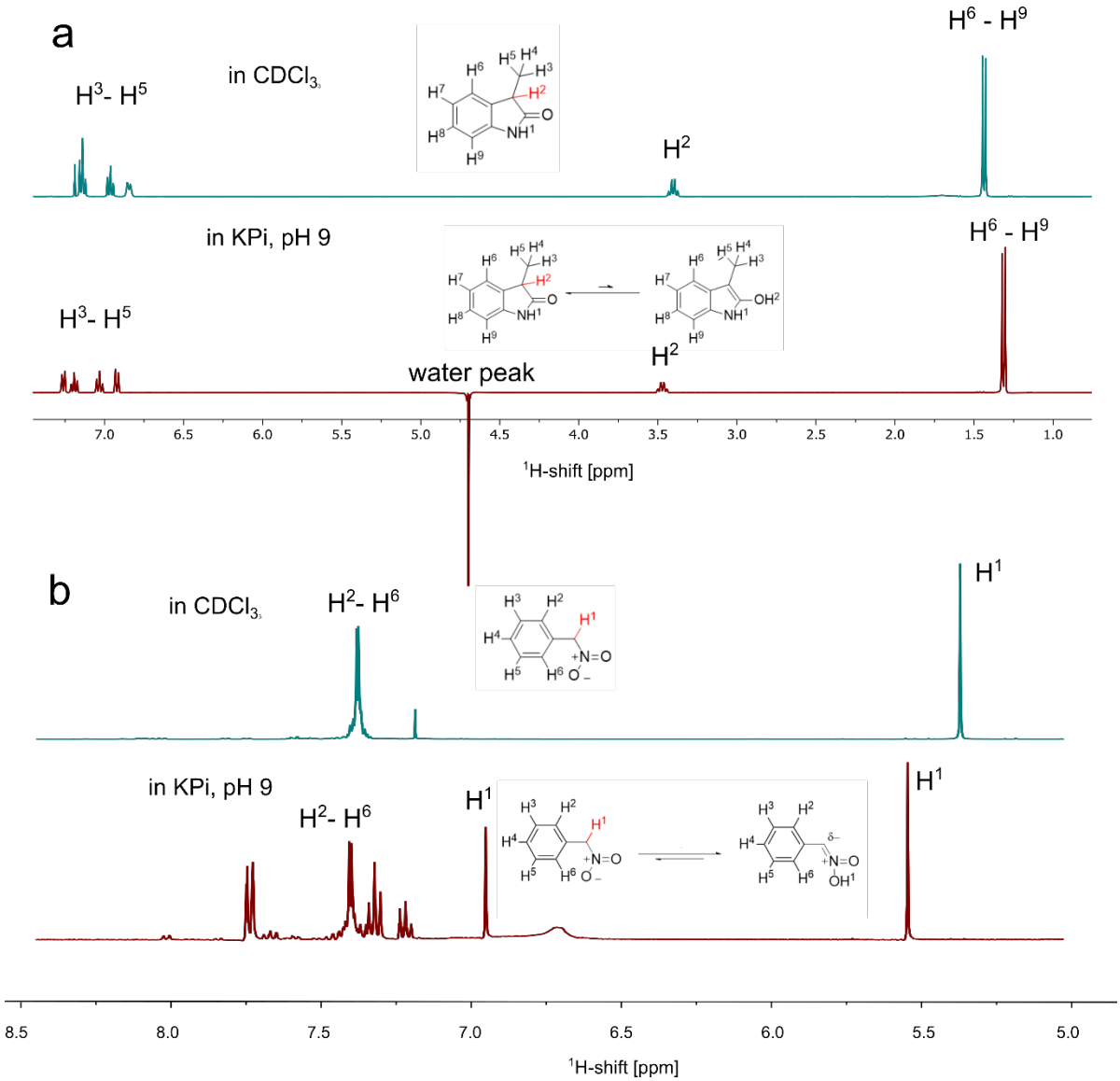


Figure S3: Comparison of NMR spectra of a) 3-methyloxindole and b) nitromethylbenzene in chloroform (green) and standard reaction conditions for TrpB (50 mM potassium phosphate buffer (KP_i), pH 9) (red). Water suppression was used for the NMR spectra in aqueous solution. In the case of the oxindole, both NMR spectra are very similar and show only the keto-form of oxindole. For the nitroalkane, an equilibrium of around 1:1 could be detected between the keto- and enolate-forms.

4 First round of random mutagenesis

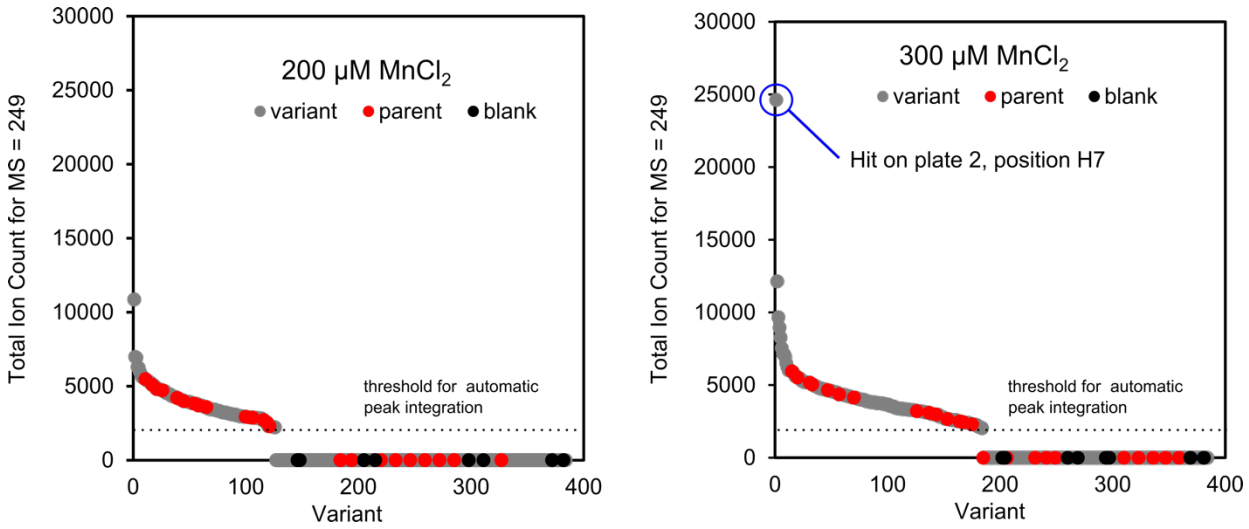


Figure S4: Global, random mutagenesis of *Pf*0A9 E104G using two different $MnCl_2$ concentrations for introducing random mutations. For screening, 20 mM of 1,3-dimethyloxindole **4**, 1 eq. serine **2** and 50 µL clarified cell lysate (see 8.4) were used. Product formation was measured by LC-MS (see 8.4). Because the parental activity for the substrate was very low, automatic peak integration failed for certain parent enzymes.

5 Cooperative effect of W172R and S185T on activity of *Pf*0A9 E104G

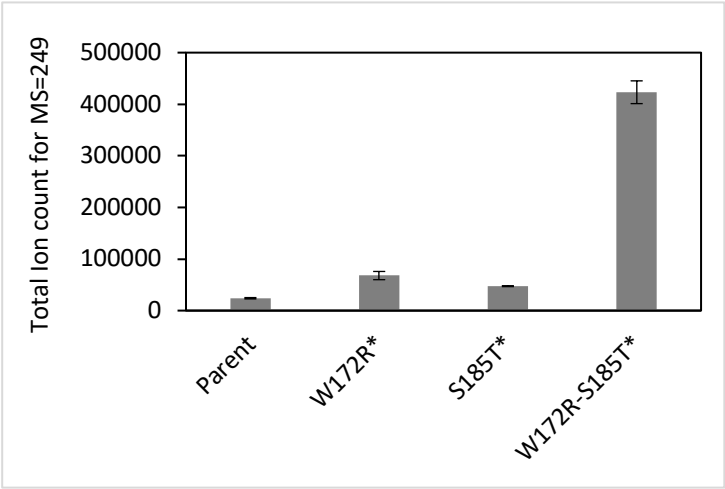


Figure S5: Influence of W172R and S185T and their combination on activity of *Pf*0A9 E104G (parent) for 1,3-dimethyloxindole alkylation activity.

*These variants carry an additional mutation (Q217R), which was randomly inserted in the first round of directed evolution. Because an activity assay showed that this mutation had no influence on either the parental activity or on the activity of W172R-S185T, this mutation was not removed and was kept as a neutral mutation for further rounds of directed evolution.

6 Active-site residues targeted in site-saturation mutagenesis libraries

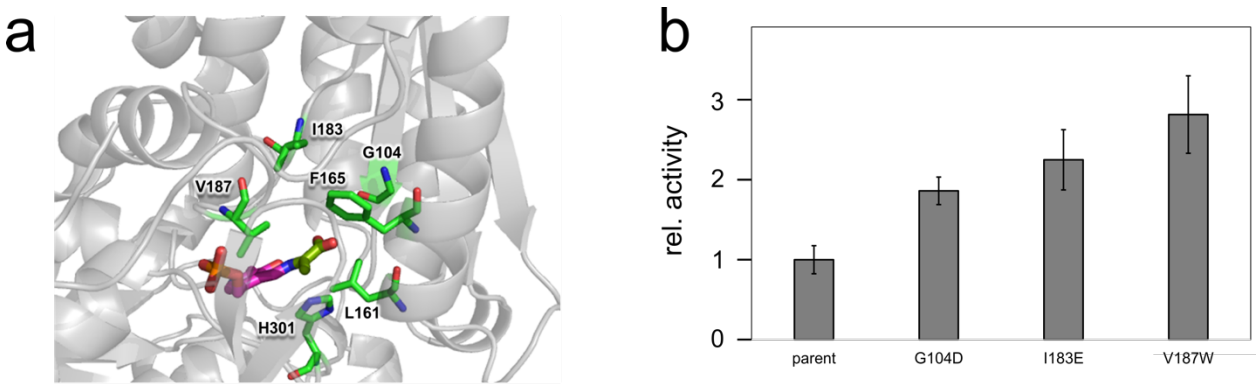


Figure S6: **a) Active site of *Pf*(β-Me) with the PLP-bound amino-acrylate (PDB ID: 5vm5).** All six residues were targeted for site-saturation libraries (see 8.2). **b) Best hits of six site-saturation libraries.** Shown is the relative activity compared to parent (*Pf*0A9 E104G after two rounds, containing additional mutations W172R-S185T and R170C-V221A).

7 List of substrates not accepted by Pf_{quat} for C–C bond formation

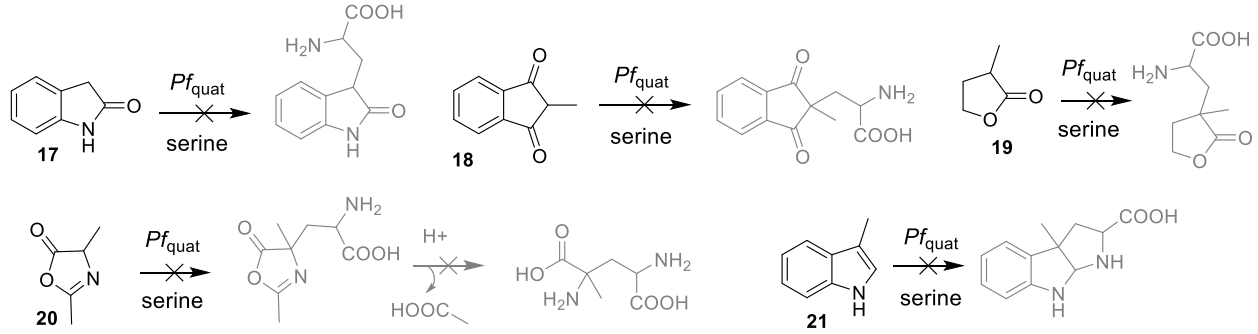


Figure S7: **No C–C bond formation was detected when 20 mM of these nucleophiles and 1 eq. serine were incubated with 40 µM Pf_{quat}. Hypothetical products are drawn in grey.** For substrate **21**, a product with the correct mass was detected. However, this was the N-alkylation product.

8 Procedures for protein expression and characterization

8.1 Cloning, expression, and purification of TrpB variants

The genes encoding *Pf*0A9 E104G and *Tm*TrpB 9D8star E103S (UNIPROT ID P50909 for wt TrpBs) were previously cloned into pET-22b(+) with a *C*-terminal His-tag. Protein expression of the variants was carried out in *Escherichia coli* BL21 *E. cloni* Express cells (Lucigen) by inoculating 5 mL of Lysogeny Broth containing 100 µg/mL carbenicillin (LB_{carb}) with a single colony and incubating the pre-culture overnight at 37 °C and 220 rpm. This overnight culture was used to inoculate 900 mL of Terrific Broth containing 100 µg/mL carbenicillin (TB_{carb}). The expression cultures were shaken at 37 °C and 220 rpm for ~2 h, at which point the OD_{600} was 0.5–0.7. The cultures were then cooled on ice for around 30 min and induced with by the addition of isopropyl β-D-thiogalactopyranoside (IPTG, final concentration of 1 mM). The culture was shaken at 220 rpm and 22 °C for another 20 h and then harvested by centrifugation at 5,000 × g for 5 min. The cell pellets were stored at –20 °C until further use.

Cells were resuspended in 5 volumes of potassium phosphate buffer (KP_i, 50 mM, pH 8) containing 0.2 mM PLP, disrupted *via* sonication, and after centrifugation (15 min at 5,000 × g) the supernatant was either directly used for reaction as cell lysate or subjected to purification. For protein purification, the lysate was loaded onto a NiNTA-column. After washing with KP_i-buffer containing 30 mM imidazole the enzyme was eluted with 250 mM imidazole. PD-10 desalting columns were used to buffer exchange the solution to 50 mM KP_i. The protein concentration was determined *via* the Bradford assay (Bio-Rad).

8.2 Construction of site-saturation mutagenesis libraries

PCR was done using Phusion polymerase (New England Biolabs) according to the standard protocol. For the construction of site-saturation libraries, the 22-codon trick first described by Kille et al. was used.[3] Three primers were designed containing codons NDT (encoding Ile, Asn, Ser, Gly, Asp, Val, Arg, His, Leu, Phe, Tyr, and Cys), VHG (encoding Met, Thr, Lys, Glu, Ala, Val, Gln, Pro, and Leu), and TGG (encoding Trp). These three primers were mixed 12:9:1. Then, the mutations were introduced with QuikChange PCR using a plasmid that contained the parent gene in the pET-22b(+) vector as template. The linear plasmid was digested with DpnI, purified with a preparative agarose gel, cyclized *via* the Gibson method, purified again, and transformed into BL21 *E. cloni* Express cells using electroporation.

8.3 Construction of random mutagenesis libraries

Random mutations were inserted into the TrpB gene with error-prone PCR using Taq polymerase (New England Biolabs) according to the following scheme.

	added volume [µL]
Taq buffer (10×)	10
dNTP mix (200 µM)	2
forward primer (10 µM)[a]	2
reverse primer (10 µM)[b]	2
template DNA (50 ng/µl)	1
$MnCl_2$ (1 mM)	20/30
Taq DNA Polymerase	0.5
Water	add to 100

Thermocycler program		
95 °C	40 s	
95 °C	30 s	30x
55 °C	30 s	
68 °C	80 s	
68 °C	5 min	

[a]GAAATAATTTTGTTTAACTTTAAG, corresponding to the NdeI restriction site of the pET-22b(+) vector.
[b]GCCGGATCTCAGTGGTGGTGGT, corresponding to the XhoI restriction site of the pET-22b(+) vector.

The PCR product was purified using a preparative agarose gel, and the gene was cloned into an empty pET-22b(+) vector between restriction sites NdeI and XhoI using the Gibson method, purified again and transformed into BL21 *E. cloni* Express cells using electroporation. Libraries with 200 and 300 µM $MnCl_2$ were screened to validate which library gave the optimal balance of inactivation rate and diversity.

8.4 Library expression and screening

BL21 *E. cloni* Express cells carrying the gene for TrpB variants were grown overnight in 96-well deep-well plates (300 µL/well LB_{carb}) at 37 °C, 250 rpm according to the following scheme.

	1	2	3	4	5	6	7	8	9	10	11	12
A	P											
B		P										
C			P									
D				P								
E					P							
F						P						
G							B					
H								B				

P = parent enzyme; B = blank (sterile control)

1 mL of TB_{carb} medium in each well of a new 96-well plate was inoculated with 20 µL of the overnight cultures, incubated for 2 h at 200 rpm, 37 °C and then chilled on ice for 30 min. Gene expression was induced by the addition of IPTG (1 mM final concentration). The cultures were shaken at 250 rpm and 22 °C. After 20 h, the cultures were centrifuged at 5,000 × g for 10 min and frozen at –20 °C. For screening, cells were thawed and lysed by the addition of 300 µL/well of KP_i (50 mM, pH 8.0), with 1 mg/mL lysozyme, 0.1 mg/mL DNase I, 200 µM PLP, and 2 mM $MgCl_2$. The plates were incubated for 30 min at 37 °C, then transferred to a water bath equilibrated to 75 °C. After 30 min, the plates were chilled in ice, subjected to centrifugation at 5,000 × g and 4 °C for 10 min. 20-50 µL of the clear lysate was added to a reaction mixture containing 20 mM serine (0.4 M stock solution in water) and 20 mM 3-methyloxindole (0.4 M stock solution in DMSO) in 50 mM KP_i buffer (pH 9) such that the total volume of the wells in the 96-well plate was 200 µL. The plates were incubated for 14 h at 55 °C, chilled on ice and 800 µL of acetonitrile / 1 M HCl (ratio 1:1) was added. The plates were subjected to centrifugation (5,000 × g, 5 min) and 200 µL of the supernatant was transferred to an assay plate. Each well was analyzed with a C-18 silica column (4.6 × 50 mm) using acetonitrile/water (0.1% acetic acid by volume): 5% to 70% acetonitrile over 1 min, 70% for 0.5 min; 1.5 mL/min. For validation, a gradient of 5% to 95% acetonitrile over 2 min followed by 0.5 min at 95% was used.

8.5 Small- and large-scale reactions with TrpB

Small-scale reactions to measure the activity of variants in terms of total turnover numbers (TTN) and to evaluate the substrate scope were performed in a similar way to the plate screening reactions (see 8.4). For these reactions, 20-50 µM of purified enzyme and 50-100 mM substrate were used in 200 µL total volume (the exact concentrations are shown in Figure 4 and 5). The reaction to product **12** was run under anaerobic conditions because the substrate can easily oxidize. For NMR and high-resolution mass spectrometry, 50 mM of the nucleophile, 2 eq. of serine and 0.2 mol% of purified enzyme were used and the total volume was increased to 30 mL. A preparative-scale reaction using 1 mmol nucleophile (50 mM concentration) and cell lysate from 100 mL expression culture (resuspended in 5 mL of 50 mM KP_i buffer, pH 9) was run for 3-methyloxindole **1** with Pf_{quat}. In order to increase the overall yield, 5 eq. of serine were added while the reaction time was extended to 24 h.

All reactions were performed overnight at 55 °C in either plastic Eppendorf tubes, glass vials, or in 100 mL flasks. For non-preparative scale reactions, the work-up is described in 8.4. Preparative-scale reactions were frozen, lyophilized and the residual solid was washed twice with toluene to remove residual DMSO. The solid was then dissolved in 2-5 mL of 1 M HCl. The product was then subjected to purification by preparative reverse-phase chromatography performed on a Biotage Isolera One purification system; C-18 silica was used as the stationary phase, with methanol as the strong solvent and water as the weak solvent. The product was eluted with a gradient from 1% to 100% methanol over 10 CV. The fractions containing product were combined and methanol was removed *in vacuo*. Then the fractions were frozen and lyophilized to remove water. The products were obtained as the hydrochloride salts.

8.6 Separation of diastereomers

For separation of the major diastereomers of the 3-methyloxindole product **3b** for analysis *via* micro-crystal electron diffraction (Section 9), 1-5 mg of the lyophilized substance (see 8.5) was dissolved in 100 µL of water. 20 µL of the solution was loaded on an Eclipse XDB-C8, 4.6 x 150mm column. A 4.5 min isocratic method with a ratio of 9:1 (water : acetonitrile) including 0.1% acetic acid with a flowrate of 1 mL/min on a reverse-phase HPLC was used. The major diastereomer eluted at 3.48 min and the minor at 3.83 min.

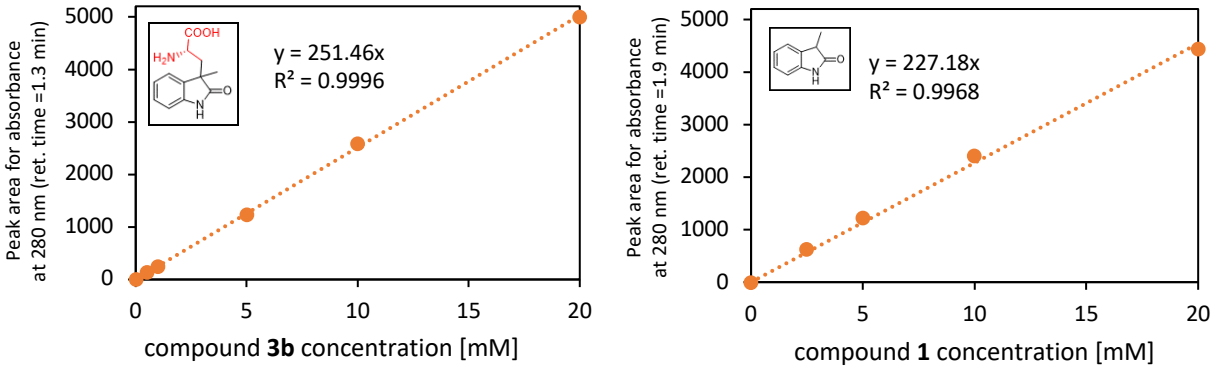
8.7 Determination of optical purity

FDNP-alaninamide was used as a solution in acetone (33 mM). In a 2-mL vial, the amino acid (0.50 µmol) was dissolved in 1 M aq. $NaHCO_3$ (100 µL). FDNP- alaninamide (10 µL, 0.33 µmol) was added, then the vial was placed in an incubator at 37 °C and shaken at 230 rpm. After 4 h, the reaction mixture was allowed to cool to room temperature, then diluted with 1:1 CH_3CN/H_2O (500 µL). The resulting solution was analyzed directly by LCMS with a

20-30 min isocratic method of 20% acetonitrile plus 0.1% acetic acid in H_2O. The total ion count filtered for the expected mass was used to identify LC peaks for the derivatization products. Each amino acid was derivatized with both racemic and enantiopure FDNP-alaninamide for comparison. Since the diastereomers for most of the products were not separated, both diastereomers were analyzed simultaneously. As such, four independent peaks could be observed for the control sample derivatized with racemic FDNP-alaninamide while two were observed for the product derivatized with enantiopure FDNP-alaninamide. This procedure was adapted from Marfey *et al.*[4]

8.8 Calibration curve for measuring HPLC yields

To determine the HPLC yield of the C-alkylation product **3b** of 3-methyloxindole **1** and serine **2,** different concentrations of the isolated product (see 8.5) in acetonitrile/1 M HCl (1:1) were prepared and analyzed by HPLC using the 2.5 min method described in 8.4. For comparison, the same calibration curve was measured for substrate **1**. The ratio of **1** divided by **3b** was 0.91, which was used calculate product formation based on an authentic standard with a defined substrate concentration.



9 Microcrystal electron diffraction (MicroED)

9.1 Sample preparation

MicroED sample preparation was conducted as described.[5-6] Briefly, samples were received as lyophilized powders taken directly after purification without crystallization attempts. Oxindole **3b** was found to quickly change from a dry powder to a hard, resin-like material, whereas ketone **14** remained a dry powder while stored. Pre-clipped Quantifoil R2/2 Cu300 grids were dropped directly into the vials of purified ketone **14** and mechanically agitated for 15 s. The oxindole **3b** resin-like material was dissolved in 20 µL of ethanol; 1 µL of this was applied to the carbon side of the pre-clipped EM grid and allowed to dry under vacuum for 15 min. The grids were then plunged into liquid nitrogen, and cryo-transferred into the transmission electron microscope for investigation.

9.2 Data collection

Data were collected on a Thermo-Fisher Talos Arctica transmission electron microscope operating at an acceleration voltage of 200kV and cooled to liquid nitrogen temperatures as described.[7] Crystals were identified by low-magnification imaging. MicroED data collection was conducted on select crystals as previously described.[7-8] Crystals were continuously rotated in a parallel electron beam while MicroED data were collected by a bottom-mount Thermo Fisher CetaD CMOS 4k x 4k detector operating in rolling shutter mode. Images were binned by 2. Typical data collection was performed using a continuous rotation rate of 0.5°/s with frames being read out every 1 s over an angular wedge ~60°. The exposure rate was calibrated to 0.01$e^{-}Å^{-2}s^{-1}$. Individual crystals were isolated using a select area aperture to reduce noise.

Movies were initially saved as MRC files and converted to SMV format using the MicroED tools software developed in-house and freely available at: https://cryoem.ucla.edu/pages/MicroED.[9]
MicroED data were indexed and integrated in XDS.[10] Integrated datasets were merged and scaled in XSCALE.[10] The structures were solved by *ab initio* direct methods in SHELXT,[11] and refined in SHELXL.[12]

9.3 Structural data and statistics

Individual integration and refinement statistics for these two structures can be found below with their corresponding densities.

Oxindole (3b)	
Temperature [K]	80
Space group	P $2_1/c$
Unit cell (a,b,c) [Å] (α,β,ɣ) (°)	12.15, 8.98, 10.28 90, 99.41, 90
Reflections [#]	2524
$R_{observed}$	10.1
$CC_{1/2}$	98.8
Resolution range [Å]	7.19 – 0.9
Completeness [%]	65.1
Exposure [$e^{-}Å^{-2}s^{-1}$]	<1
R / wR2 / GooF	20 / 41.9 / 2.3

Ketone (14)	
Temperature [K]	80
Space group	P $2_12_12_1$
Unit cell (a,b,c) [Å] (α,β,ɣ) (°)	6.66, 9.10, 20.26 90, 90, 90
Reflections [#]	3055
$R_{observed}$	19.6
$CC_{1/2}$	99.4
Resolution range [Å]	6.76 – 0.9
Completeness [%]	83.0
Exposure [$e^{-}Å^{-2}s^{-1}$]	<1
R / wR2 / GooF	28.5 / 53.9 / 1.59

10 Synthesis and characterization of oxindole derivatives and related substrates

10.1 General information

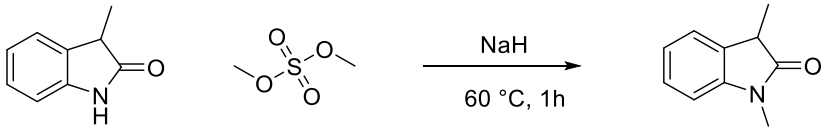
Carbon and proton NMR spectra were recorded on a Bruker 400 MHz (100 MHz) spectrometer equipped with a cryogenic probe. Proton chemical shifts are reported in ppm (δ) relative to tetramethylsilane and calibrated using the solvent resonance of $CDCl_3$ (δ 7.18 ppm), methanol-d_4 (δ 3.31 ppm), or D_2O (δ 4.79 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), doublet of doublets (dd), doublet of doublets of doublets (ddd), triplet (t), triplet of doublets (td), multiplet (m)], coupling constants [Hz], integration). Carbon NMR spectra were recorded with complete proton decoupling. Carbon chemical shifts are reported in ppm relative to tetramethylsilane and calibrated using the residual solvent proton resonance as an absolute reference. All NMR spectra were recorded at around 25 °C.

Normal phase purification was performed on a Biotage Isolera One purification system, using silica as the stationary phase, with ethyl acetate as the strong solvent and hexane as the weak solvent.

High-resolution mass spectrometry (HRMS) data were acquired using an Agilent 6200 TOF equipped with electrospray ionization (ESI).

10.2 Synthesis of starting material for biocatalytic reactions

10.2.1 Methylation of 3-methyl-2-oxindole

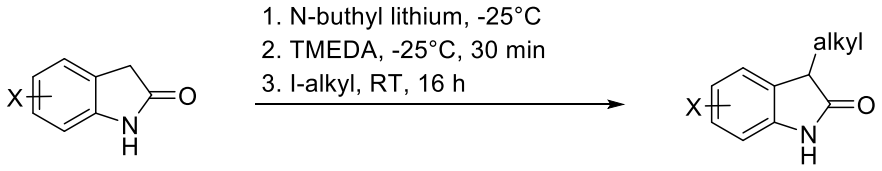


1 **4**

Methylation of oxindoles at the nitrogen was performed according to Liegault *et al.* with small adaptations.[13] 4 mmol 3-methyl-2-oxindole **1** was dissolved in 100 mL anhydrous toluene, 1.05 eq. of NaH were added and the reaction mixture was stirred for 20 min at 60 °C. 1.2 eq. of dimethyl sulfate were added dropwise. After 2 h at 60 °C and 1 h at 90 °C, the reaction mixture was cooled to room temperature and quenched with 100 mL brine. The organic layer was extracted using 2 x 40 mL EtOAc, dried with $MgSO_4$, concentrated under reduced pressure, and purified by silica gel chromatography (gradient: hexane : ethyl acetate 10:1 → 2:1) to afford **4** with 64% yield.

^{1}H NMR (300 MHz, chloroform-*d*) δ 7.40 – 7.16 (m, 2H), 7.05 (td, *J* = 7.5, 1.1 Hz, 1H), 6.82 (ddt, *J* = 7.8, 1.0, 0.5 Hz, 1H), 3.20 (s, 2H), 1.47 (d, *J* = 7.6 Hz, 3H).

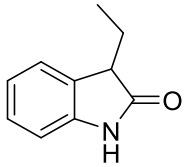
10.2.2 Alkylation of 2-oxindole at C_3



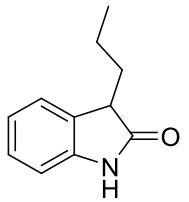
X = H, Cl, F

A solution of oxindole (3 mmol) in dry THF (4 mL) under argon was cooled to -25 °C using dry ice in acetone and treated drop wise with *n*-butyl lithium (2 eq.). To the resulting solution was added 2 eq. of N,N,N',N'-tetramethylethylenediamine (TMEDA). After 30 min., 6 mmol of iodoalkane were added and the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was poured into aqueous

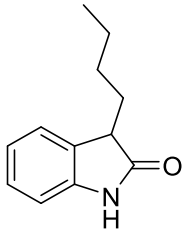
NH_4Cl solution and extracted with EtOAc (2x), the combined organic layers were washed with dil. HCl, water, brine, dried ($MgSO_4$) and concentrated under reduced pressure. The crude extract was purified by silica gel chromatography (gradient: hexane : ethyl acetate 10:1 → 2:1) to afford the desired product with 50-70% yield (the desired C-alkylation product eluted later than the less polar N-alkylation site product (around 20-30%).



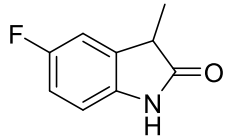
^{1}H NMR (400 MHz, chloroform-*d*) δ 7.47 (s, 1H), 7.20 – 7.10 (m, 2H), 6.97 (td, *J* = 7.5, 1.0 Hz, 1H), 6.79 (ddt, *J* = 7.7, 1.1, 0.6 Hz, 1H), 3.38 (t, *J* = 5.7 Hz, 1H), 2.04 – 1.89 (m, 2H), 0.85 (t, *J* = 7.4 Hz, 3H).



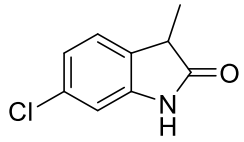
^{1}H NMR (400 MHz, chloroform-*d*) δ 7.66 (s, 1H), 7.20 – 7.09 (m, 2H), 6.97 (td, *J* = 7.5, 1.0 Hz, 1H), 6.80 (ddt, *J* = 7.7, 1.1, 0.6 Hz, 1H), 3.4 (t, *J* = 5.7 Hz, 1H), 2.04 – 1.89 (m, 2H), 0.85 (t, *J* = 7.4 Hz, 3H).



^{1}H NMR (400 MHz, chloroform-*d*) δ 7.18 – 7.10 (m, 2H), 6.96 (td, *J* = 7.6, 1.1 Hz, 1H), 6.80 (ddd, *J* = 7.7, 1.1, 0.6 Hz, 1H), 3.43 (t, *J* = 5.7 Hz, 1H), 2.00 – 1.73 (m, 2H), 1.40 – 1.17 (m, 4H), 0.81 (t, *J* = 6.9 Hz, 3H).

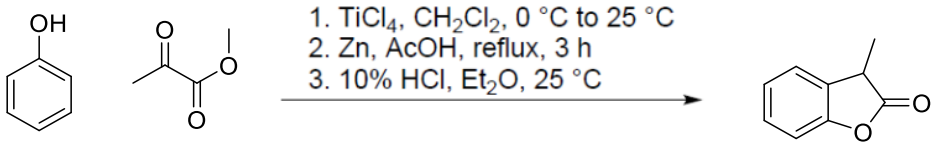


^{1}H NMR (400 MHz, chloroform-*d*) δ 7.81 (s, 1H), 6.90 (dddt, *J* = 8.0, 2.5, 1.2, 0.5 Hz, 1H), 6.85 (dddd, *J* = 9.3, 8.4, 2.6, 0.8 Hz, 1H), 6.73 (dd, *J* = 8.5, 4.3 Hz, 1H), 3.45 – 3.35 (m, 1H), 1.43 (d, *J* = 7.7 Hz, 3H).



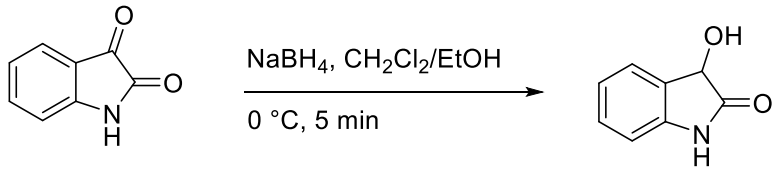
^{1}H NMR (400 MHz, chloroform-*d*) δ 7.59 (s, 1H), 7.06 (ddt, *J* = 8.0, 1.1, 0.5 Hz, 1H), 6.95 (dd, *J* = 7.9, 1.9 Hz, 1H), 6.82 (d, *J* = 1.8 Hz, 1H), 3.36 (qd, *J* = 7.6, 1.1 Hz, 1H), 1.41 (d, *J* = 7.7 Hz, 3H).

10.2.3 Synthesis of the lactone



Synthesis of the lactone was performed according to Wang et al. with small adaptations.[14] In a dry 50 mL two-necked flask, to a solution of phenol (10 mmol) in DCM (20 mL) at 25 °C was added 12 mmol $TiCl_4$ dropwise. The reaction mixture was cooled to 0 °C and methyl pyruvate (12 mmol) slowly added, then stirred at 25 °C for 2 h. AcOH (6 mL) and Zn (10 mmol) was then added slowly and the reaction mixture was heated at reflux for 3 h, filtered and concentrated under vacuum. To the residue was added 10% HCl (6 mL) and Et_2O (6 mL); the mixture was stirred at 25 °C for 3 h. After the reaction was completed, it was extracted with EtOAc (10 mL × 3) and the combined organic phases were dried over Na_2SO_4. The residue was concentrated under reduced pressure and purified by column chromatography (gradient: hexane : ethyl acetate 10:1 → 1:2) to afford the desired product with 30% yield.
^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.27 – 7.18 (m, 1H), 7.22 – 7.15 (m, 1H), 7.13 – 7.00 (m, 2H), 3.66 (q, 7.6 Hz, 1H), 1.51 (d, *J* = 7.6 Hz, 3H).

10.2.4 Synthesis of 3-hydroxyl oxindole

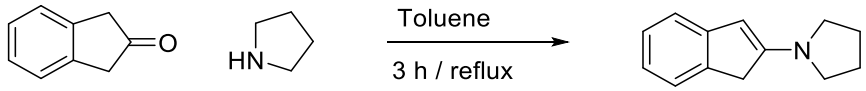


Sodium borohydride (7.5 mmol, 1.5 eq.) was added in small portions to a stirred suspension of isatin (5 mmol) in 30 mL of a 1:1 dichloromethane/ethanol mixture at 0 °C. The mixture was stirred at 0 °C until the suspension became colorless (about 5 min). Then water (5 mL) was added and the reaction mixture was stirred until bubbling stopped. The mixture was extracted with dichloromethane (3 x 20 mL). The combined organic extracts were dried ($MgSO_4$) and the solvent evaporated under reduced pressure (yield: 85%). The procedure was performed according to Wu et al.[15]
^{1}H NMR (400 MHz, deuterium oxide) δ 7.44 (ddq, *J* = 7.4, 1.2, 0.6 Hz, 1H), 7.35 (dddd, *J* = 8.4, 7.7, 1.7, 1.0 Hz, 1H), 7.15 (tt, *J* = 7.7, 0.7 Hz, 1H), 6.99 (dq, *J* = 7.8, 0.5 Hz, 1H), 5.11 (s, 1H).

10.2.5 Synthesis of 1-methyl-2,3-dihydro-1H-inden-2-one

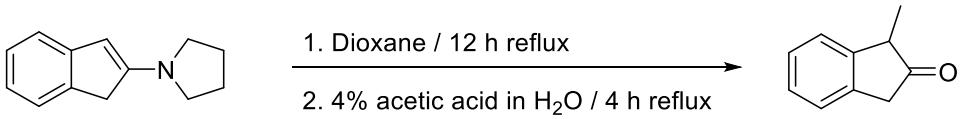
Methylation of the ketone at the 1-position was performed according to Koppes et al.[16]

<u>Step 1: Synthesis of 1-(1H-inden-2-yl)pyrrolidine</u>



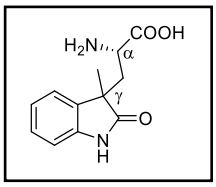
A mixture of 2-indanone (3.0 g, 22.65 mmol, 1 eq.) and pyrrolidine (2.4 mL, 29.45 mmol, 1.3 eq.) in anhydrous toluene was refluxed under nitrogen with azeotropic removal of water for 3 h. The mixture was then cooled to room temperature and concentrated to dryness *in vacuo*.

<u>Step 2: Synthesis of 1-methyl-2,3-dihydro-1H-inden-2-one</u>

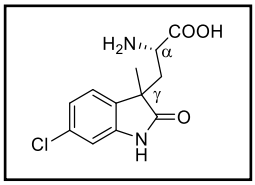


4 g of 1-(1H-inden-2-yl)pyrrolidine (1 eq., 22 mmol) was dissolved in 50 mL of dioxane and 41 mL (66 mmol, 3 eq.) methyl iodide was added. After 12 h of reflux, 10 mL of water and 0.4 mL of acetic acid was added to the reaction mixture. After a further 4 h of reflux, the solution was quenched by pouring it into a saturated $NaHCO_3$ solution and the resulting mixture was extracted with ether. The combined ether extracts were washed with water and brine, dried over $MgSO_4$, and concentrated to dryness *in vacuo*. The crude product was purified by column chromatography (gradient: hexane : ethyl acetate 10:1 → 1:2) to afford the desired product with 10% yield.
^{1}H NMR (400 MHz, chloroform-*d*) δ 7.29 – 7.15 (m, 4H), 3.55 – 3.37 (m, 3H), 1.35 (d, *J* = 7.5 Hz, 3H).

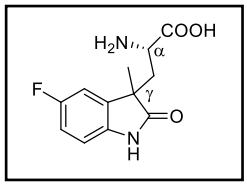
10.3 Analytics of TrpB catalyzed product



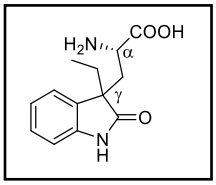
Compound 3b (HCl salt). The product was obtained as a 5:1 mixture of two diastereomers, which were separated using preparative HPLC (see 8.6) to determine the relative configuration using MicroED (see section 9).
Major diastereomer (*S*, *S*) – (α, γ): **^{1}H NMR** (400 MHz, deuterium oxide) δ 7.38 – 7.29 (m, 2H), 7.15 (t, *J* = 7.8 Hz, 1H), 7.05 (d, *J* = 7.6 Hz, 1H), 3.62 (dd, *J* = 8.8, 3.8 Hz, 1H), 2.68 (dd, *J* = 14.6, 8.8 Hz, 1H), 2.41 (dd, *J* = 14.6, 4.9 Hz, 1H), 1.42 (s, 3H). **^{13}C NMR** (101 MHz, deuterium oxide) δ 183.3, 171.0, 140.4, 132.0, 128.9, 123.4, 123.2, 110.8, 50.6, 47.3, 36.5, 24.8.
Minor Diastereomer (*S*, *R*) – (α, γ): **^{1}H NMR** (400 MHz, deuterium oxide) δ 7.43 – 7.29 (m, 2H), 7.20 (td, *J* = 7.5, 1.0 Hz, 1H), 7.09 (d, *J* = 7.7 Hz, 1H), 3.73 (dd, *J* = 7.5, 6.4 Hz, 1H), 2.61 (dd, *J* = 15.0, 7.5 Hz, 1H), 2.34 (dd, *J* = 15.0, 6.5 Hz, 1H), 1.45 (s, 3H). **^{13}C NMR** (101 MHz, deuterium oxide) δ 183.9, 171.1, 140.2, 132.4, 128.9, 123.3, 123.0, 111.0, 50.4, 47.3, 35.8, 24.7.
HRMS (ESI) (*m*/*z*) for $[M+H]^+$ ($C_{12}H_{14}ClN_2O_3$) calculated 235.1077, observed 235.108.
Enantiopurity (both diastereomers): >99%.



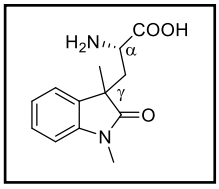
Compound 5 (HCl salt). The product was obtained as a 5:1 mixture of two diastereomers. Due to signal overlap, all NMR signals could only be assigned for the major diastereomer.
Major diastereomer (*S*, *S*) – (α, γ): **^{1}H NMR** ^{1}H NMR (400 MHz, deuterium oxideδ 7.26 (d, *J* = 8.0 Hz, 1H), 7.13 (d, *J* = 8.1 Hz, 1H), 7.08 (s, 1H), 3.59 (dd, *J* = 9.2, 4.3 Hz, 1H), 2.63 (dd, *J* = 14.6, 9.2 Hz, 1H), 2.39 (dd, *J* = 14.5, 4.8 Hz, 1H), 1.41 (s, 3H). **^{13}C NMR** (101 MHz, deuterium oxide) δ 183.31, 171.15, 141.7, 133.7, 130.6, 124.5, 122.9, 111.1, 50.7, 47.1, 36.5, 24.8.
HRMS (ESI) (*m*/*z*) for $[M+H]^+$ ($C_{12}H_{15}N_2O_3$) calculated 269.0687, observed 269.0689.
Enantiopurity (both diastereomers): >99%.



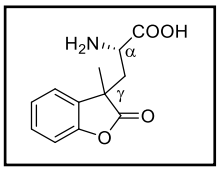
Compound 6 (HCl salt). The product was obtained as a 7:2 mixture of two diastereomers. Due to signal overlap, all NMR signals could only be assigned for the major diastereomer.
Major diastereomer (*S*, *S*) – (α, γ): **^{1}H NMR** (400 MHz, Deuterium Oxide) δ 7.04 (dd, *J* = 8.4, 2.6 Hz, 1H), 6.98 – 6.93 (m, 1H), 6.89 (dd, *J* = 8.6, 4.4 Hz, 1H), 3.55 (dd, *J* = 9.2, 4.7 Hz, 1H), 2.54 (dd, *J* = 15.2, 9.3 Hz, 1H), 2.31 (dd, *J* = 14.9, 4.8 Hz, 1H), 1.32 (s, 3H).
^{13}C NMR (101 MHz, Deuterium Oxide) δ 183.31, 170.9, 160.4, 158.6, 136.4, 133.7, 133.6, 115.3, 50.4, 47.9, 36.5, 24.8.
HRMS (ESI) (*m*/*z*) for $[M+H]^+$ ($C_{12}H_{14}FN_2O_3$) calculated 253.0983, observed 253.0984.
Enantiopurity (both diastereomers): >99%.



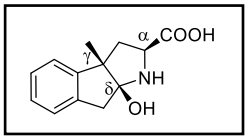
Compound 9 (HCl salt). The product was obtained as a 2:1 mixture of two diastereomers. Due to signal overlap, all NMR signals could only be assigned for the major diastereomer.
Major diastereomer (*S*, *S*) – (α, γ): **^{1}H NMR (**400 MHz, deuterium oxide) δ 7.37 – 7.29 (m, 2H), 7.17 (td, *J* = 7.6, 1.0 Hz, 1H), 7.05 (dd, *J* = 7.9, 0.9 Hz, 1H), 3.57 (dd, *J* = 8.6, 5.0 Hz, 1H), 2.68 (dd, *J* = 14.5, 8.5 Hz, 1H), 2.38 (dd, *J* = 14.6, 5.5 Hz, 1H), 2.03 – 1.78 (m, 2H), 0.55 (t, *J* = 7.5 Hz, 3H).
^{13}C NMR (101 MHz, deuterium oxide) δ 182.76, 171.42, 141.39, 129.97, 128.92, 123.83, 123.19, 110.55, 50.85, 38.68, 36.55, 31.94, 6.97.
HRMS (ESI) (*m*/*z*) for $[M+H]^+$ ($C_{13}H_{17}N_2O_3$) calculated 249.1234, observed 249.124.
Enantiopurity >99%. Due to signal overlap after derivatization and LCMS separation (see 8.7), an *ee*>99% could only be confirmed for one diastereomer.



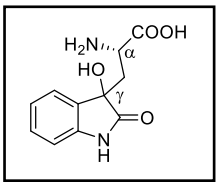
Compound 13 (HCl salt). The product was obtained as a 5:4 mixture of two diastereomers.
Major diastereomer (*S*, *S*) – (α, γ): **^{1}H NMR (**400 MHz, deuterium oxide) δ 7.48 – 7.41 (m, 2H), 7.20 (td, *J* = 7.6, 1.0 Hz, 1H), (dd, *J* = 7.9, 0.8 Hz, 1H), 3.58 (dd, *J* = 10.2, 5.4 Hz, 1H), 3.27 (s, 3H), 2.65 (dd, *J* = 15.4, 8.8 Hz, 1H), 2.44 (dd, *J* = 14.5, 4.9 Hz, 1H), 1.41 (s, 3H).
^{13}C NMR (101 MHz, deuterium oxide) δ 181.23, 171.19, 142.75, , 131.49, 128.97, 123.60, 122.97, 109.60, 50.81, 46.96, 36.60, 26.29, 24.86.
Minor diastereomer (*S*, *R*) – (α, γ): **^{1}H NMR (**400 MHz, deuterium oxide) δ 7.42 – 7.35 (m, 2H), 7.26 (td, *J* = 7.6, 1.0 Hz, 1H), 7.17 (dd, *J* = 7.8, 1.3 Hz, 1H), 3.57 (dd, *J* = 11.0, 5.1 Hz, 2H), 3.22 (s, 3H), 2.63 (dd, *J* = 15.4, 8.7 Hz, 1H), 3.57 (dd, *J* = 11.0, 5.1 Hz, 1H), 1.42 (s, 3H).
^{13}C NMR (101 MHz, deuterium oxide) δ 181.71, 171.41, 142.64, 131.71, 128.97, 123.73, 122.69, 109.79, 50.59, 46.90, 36.35, 26.20, 24.58.
HRMS (ESI) (*m*/*z*) for $[M+H]^+$ ($C_{13}H_{17}N_2O_3$) calculated 249.1234, observed 249.1236.
Enantiopurity (both diastereomers): >99%.



Compound 7 (HCl salt). The product was obtained as a mixture of >98:2 of two diastereomers (according to HPLC-MS analysis). The minor diastereomer was not detectable in NMR.
Major diastereomer (*S*, *S*) – (α, γ): **^{1}H NMR** (400 MHz, deuterium oxide) δ 7.31 (dd, *J* = 7.8, 1.7 Hz, 1H), 7.21 (dt, *J* = 7.8, 1.6 Hz, 1H), 6.92 (dt, *J* = 7.6, 1.3 Hz, 1H), 6.86 (dd, *J* = 8.0, 1.2 Hz, 1H), 4.46 (dd, *J* = 11.0, 3.2 Hz, 1H), 2.97 – 2.85 (m, 1H), 2.16 (dd, *J* = 13.6, 3.2 Hz, 1H), 1.52 (s, 3H).
^{13}C NMR (101 MHz, deuterium oxide) δ 185.23, 177.11, 153.76, 129.06, 127.70, 120.12, 116.08, 53.21, 46.11, 38.82, 22.94.
HRMS (ESI) (*m*/*z*) for $[M+H]^+$ ($C_{12}H_{14}NO_4$) calculated 236.0917, observed 236.0913.
Enantiopurity: >99%.



Compound 8 (HCl salt). The product was obtained as a mixture of >99:1 of two diastereomers (according to HPLC-MS analysis). The minor diastereomer was not detectable in NMR.
Major diastereomer (*S*, *S, S*) – (α, γ, δ): **^{1}H NMR** (400 MHz, methanol-d_4) δ 7.46 – 7.22 (m, 4H), 3.90 (dd, *J* = 10.8, 7.2 Hz, 1H), 3.56 (dd, *J* = 31.6, 17.3 Hz, 2H), 2.84 (dd, *J* = 13.4, 7.3 Hz, 1H), 2.42 (dd, *J* = 13.4, 10.7 Hz, 1H), 1.34 (s, 3H). **^{13}C NMR** (101 MHz, methanol-d_4) δ 169.12, 144.95, 135.72, 128.54, 128.54, 125.12, 122.96, 109.26, 58.14, 57.29, 39.59, 37.23, 20.54.
HRMS* (ESI) (*m*/*z*) for $[M+H-H_2O]^+$ ($C_{12}H_{12}NO_3$) calculated 216.1019, observed 216.1025.
Enantiopurity: >99%.
**Protonation of the hydroxyl group at C_δ leads to the formation of the imine via water elimination.*



Compound 12 (HCl salt). The product was obtained as a 5:4 mixture of two diastereomers, which could be separated using a C-18 silica column on a Biotage Isolera One purification system (see 8.5).

Major diastereomer (*S*, *S*) – (α, γ): **^{1}H NMR (**400 MHz, deuterium oxide) δ 7.49 (d, *J* = 7.8 Hz, 1H), 7.41 (d, *J* =8.1 Hz, 1H), 7.20 (d, *J* = 10.8 Hz, 1H), 7.04 (d, *J* = 7.5 Hz, 1H), 4.47 (dd, *J* = 9.1, 3.8 Hz, 1H), 2.59 (dd, *J* = 15.5, 3.8 Hz, 1H), 2.36 (dd, *J* = 15.5, 9.1 Hz, 1H). **^{13}C NMR** (101 MHz, deuterium oxide) δ 180.62, 171.54, 140.22, 130.65, 129.61, 123.90, 123.67, 111.40, 74.90, 49.48, 35.97.

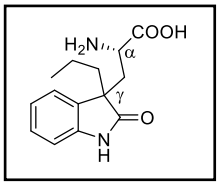
Minor diastereomer (*S*, *R*) – (α, γ): **^{1}H NMR (**400 MHz, deuterium oxide) δ 7.45 (d, *J* = 7.6 Hz, 1H), 7.38 (t, *J* = 7.7 Hz, 1H), 7.18 (t, *J* = 7.7 Hz, 1H), 7.03 (d, *J* = 7.8, 0.8 Hz, 1H), 4.64 (dd, *J* = 9.6, 3.7 Hz, 1H), 2.59 (dd, *J* = 15.6, 9.4 Hz, 1H), 2.43 (dd, *J* = 15.8, 3.7 Hz, 1H). **^{13}C NMR** (101 MHz, deuterium oxide) δ 180.09, 171.58, 139.88, 130.77, 130.24, 123.72, 123.60, 111.12, 74.45, 49.15, 36.21.

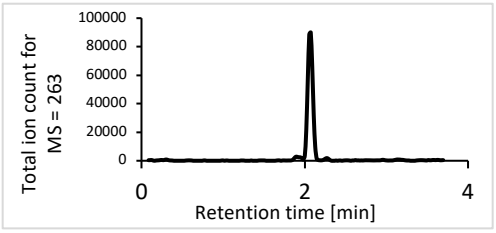
HRMS (ESI) (*m*/*z*) for $[M+H]^+$ ($C_{11}H_{13}N_2O_4$) calculated 237.087, observed 237.0866.

Enantiopurity >99%. Due to signal overlap after derivatization and LCMS separation (see 8.7), an *ee*>99% could only be confirmed for one diastereomer.

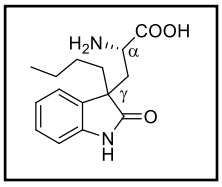
For the following two products, detailed analytics (NMR, HRMS and optical purity) could not be accomplished as the overall yield was not sufficient to obtain >1 mg of isolated product.

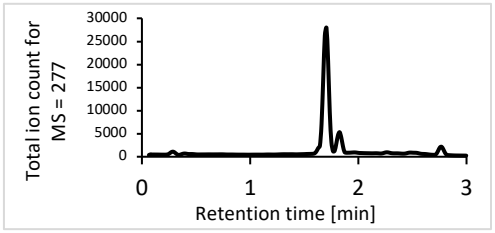
Compound **10**: LC-MS chromatogram for expected mass $[M+H]^+$=263





Compound **11**: LC-MS chromatogram for expected mass $[M+H]^+$=277





11 DNA and Protein sequences

Parent Protein *Pf*0A9 E104G[2]

Nucleotide sequence:

ATGTGGTTCGGTGAATTTGGTGGTCAGTACGTGCCAGAAACGCTGGTTGGACCCCTGAAAGAGCTGGAAAAAGCTTACAAACG
TTTCAAAGATGACGAAGAATTCAATCGTCAGCTGAATTACTACCTGAAAACCTGGGCAGGTCGTCCAACCCCACTGTACTACGC
AAAACGCCTGACTGAAAAAATCGGTGGTGCTAAAGTCTACCTGAAACGTGAAGACCTGGTTCACGGTGGTGCACACAAGACCA
ACAACGCCATCGGTCAGGCACTGCTGGCAAAGCTCATGGGTAAAACTCGTCTGATCGCTGGGACCGGTGCTGGTCAGCACGGC
GTAGCGACTGCAATGGCTGGTGCACTGCTGGGCATGAAAGTGGACATTTACATGGGTGCTGAGGACGTAGAACGTCAGAAAC
TGAACGTATTCCGTATGAAGCTGCTGGGTGCAAACGTAATTCCAGTTAACTCCGGTTCTCGCACCCTGAAAGACGCATTTGACG

AGGCTCTGCGTGATTGGGTGGCTACTTTTGAATACACCCACTACCTAATCGGTTCCGTGGTCGGTCCACATCCGTATCCGACCAT
CGTTCGTGATTTTCAGTCTGTTATCGGTCGTGAGGCTAAAGCGCAGATCCTGGAGGCTGAGGGTCAGCTGCCAGATGTAATCG
TTGCTTGTGTTGGTGGTGGCTCTAACGCGATGGGTATCTTTTACCCGTTCGTGAACGACAAAAAAGTTAAGCTGGTTGGCGTTG
AGGCTGGTGGTAAAGGCCTGGAATCTGGTAAGCATTCCGCTAGCCTGAACGCAGGTCAGGTTGGTGTGTCCCATGGCATGCTG
TCCTACTTTCTGCAGGACGAAGAAGGTCAGATCAAACCAAGCCACTCCATCGCACCAGGTCTGGATCATCCAGGTGTTGGTCCA
GAACACGCTTACCTGAAAAAAATTCAGCGTGCTGAATACGTGGCTGTAACCGATGAAGAAGCACTGAAAGCGTTCCATGAACT
GAGCCGTACCGAAGGTATCATCCCAGCTCTGGAATCTGCGCATGCTGTGGCTTACGCTATGAAACTGGCTAAGGAAATGTCTCG
TGATGAGATCATCATCGTAAACCTGTCTGGTCGTGGTGACAAAGACCTGGATATTGTCCTGAAAGCGTCTGGCAACGTGCTCGA
GCACCACCACCACCACCACTGAG

Protein sequence:
MWFGEFGGQYVPETLVGPLKELEKAYKRFKDDEEFNRQLNYYLKTWAGRPTPLYYAKRLTEKIGGAKVYLKREDLVHGGAHKTNNAI
GQALLAKLMGKTRLIAGTGAGQHGVATAMAGALLGMKVDIYMGAEDVERQKLNVFRMKLLGANVIPVNSGSRTLKDAFDEALRD
WVATFEYTHYLIGSVVGPHPYPTIVRDFQSVIGREAKAQILEAEGQLPDVIVACVGGGSNAMGIFYPFVNDKKVKLVGVEAGGKGLES
GKHSASLNAGQVGVSHGMLSYFLQDEEGQIKPSHSIAPGLDHPGVGPEHAYLKKIQRAEYVAVTDEEALKAFHELSRTEGIIPALESAH
AVAYAMKLAKEMSRDEIIIVNLSGRGDKDLDIVLKASGNVLEHHHHHH

Final variant *Pf*$_{quat}$

Nucleotide sequence:
ATGTGGTTCGGTGAATTTGGTGGTCAGTACGTGCCAGAAACGCTGGTTGGACCCCTGAAAGAGCTGGAAAAAGCTTACAAACG
TTTCAAAGACGACGAAGAATTCAATCGTCAGCTGAATTACTACCTGAAAACCTGGGCAGGTCGTCCAACTCCACTGTACTACGC
AAAACGCCTGACTGAAAAAATCGGTGGTGCTAAAGTCTACCTGAAACGTGAAGACCTGGTTCACGGTGGTGCACACAAGACCA
ATAACGCCATCGGTCAGGCACTGCTGGCAAAGCTCATGGGTAAAACTCGTCTGATCGCTGGGACCGGTGCTGGTCAGCACGGC
GTAGCGACTGCAATGGCTGGTGCACTGCTGGGCATGAAAGTGGACATTTACATGGGTGCTGAGGACGTAGAACGTCAGAAAC
TGAACGTATACCGTATGAAGCTGCTGGGTGCAAACGTAATTCCAGTTAACTCCGGTTCTCGCACCGTGAAAGACGCATTTGACG
AGGCTCTGTGTGATCGGGTGGCTACTTTTGAATACACCCACTACCTAATCGGTACCGTGTGGGGTCCACATCCGTATCCGACCA
TCGTTCGTGATTTTCAGACTGTTATCGGTCGTGAGGCTAAAGCGCAGATCCTGGAGGCTGAAGGTCGGCTGCCAGATGCAATC
GTTGCTTGTGTTGGTGGTGGCTCTAACGCGATGGGTATCTTTTACCCGTTCGTGAACGACAAAAAAGTTAAGCTGGTTGGCGTT
GAGGCTGGTGGTAAAGGCCTGGAATCTGGTAAGCATTCCGCTAGCCTGAACGCAGGTCAGGTTGGTGTGTCCCATGGCATGCT
GTCCTACTTTCTGCAGGACGAAGAAGGTCAGATCAAACCAAGCCACTCCATCGCACCAGGTCTGGATCATCCAGGTGTTGGTCC
AGAACACGCTTACCTGAAAAAAATTCAGCGTGCTGAATACGTGGCTGTAACCGATGAAGAAGCACTGAAAGCGTTCCATGAAC
TGAGCCGTACCGAAGGTATCATCCCAGCTCTTGAATCTGCGCATGCTGTGGCTTACGCTATGAAACTGGCTAAGGAAATGTCTC
GTGATGAGATCATCATCGTAAACCTGTCTGGTCGTGGTGACAAAGACCTGGATATTGTCCTGAAAGCGTCTGGCAACGTGCTC
GAGCACCACCACCACCACCACTGA

Protein sequence (The 9 beneficial mutations are highlighted in red):
MWFGEFGGQYVPETLVGPLKELEKAYKRFKDDEEFNRQLNYYLKTWAGRPTPLYYAKRLTEKIGGAKVYLKREDLVHGGAHKTNNAI
GQALLAKLMGKTRLIAGTGAGQHGVATAMAGALLGMKVDIYMGAEDVERQKLNVYRMKLLGANVIPVNSGSRTVKDAFDEALCD
RVATFEYTHYLIGTVWGPHPYPTIVRDFQTVIGREAKAQILEAEGRLPDAIVACVGGGSNAMGIFYPFVNDKKVKLVGVEAGGKGLES
GKHSASLNAGQVGVSHGMLSYFLQDEEGQIKPSHSIAPGLDHPGVGPEHAYLKKIQRAEYVAVTDEEALKAFHELSRTEGIIPALESAH
AVAYAMKLAKEMSRDEIIIVNLSGRGDKDLDIVLKASGNVLEHHHHHH

12 References

1. Boville, C. E.; Romney, D. K.; Almhjell, P. J.; Sieben, M.; Arnold, F. H., Improved Synthesis of 4-Cyanotryptophan and Other Tryptophan Analogues in Aqueous Solvent Using Variants of TrpB from Thermotoga maritima. *J Org Chem* **2018,** *83* (14), 7447-7452.

2. Romney, D. K.; Murciano-Calles, J.; Wehrmuller, J. E.; Arnold, F. H., Unlocking Reactivity of TrpB: A General Biocatalytic Platform for Synthesis of Tryptophan Analogues. *J Am Chem Soc* **2017,** *139* (31), 10769-10776.
3. Kille, S.; Acevedo-Rocha, C. G.; Parra, L. P.; Zhang, Z. G.; Opperman, D. J.; Reetz, M. T.; Acevedo, J. P., Reducing codon redundancy and screening effort of combinatorial protein libraries created by saturation mutagenesis. *ACS Synth Biol* **2013,** *2* (2), 83-92.
4. Marfey, P., Determination ofD-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Research Communications* **1984,** *49* (6), 591-596.
5. Gallagher-Jones, M.; Glynn, C.; Boyer, D. R.; Martynowycz, M. W.; Hernandez, E.; Miao, J.; Zee, C. T.; Novikova, I. V.; Goldschmidt, L.; McFarlane, H. T.; Helguera, G. F.; Evans, J. E.; Sawaya, M. R.; Cascio, D.; Eisenberg, D. S.; Gonen, T.; Rodriguez, J. A., Sub-angstrom cryo-EM structure of a prion protofibril reveals a polar clasp. *Nat Struct Mol Biol* **2018,** *25* (2), 131-134.
6. Jones, C. G.; Martynowycz, M. W.; Hattne, J.; Fulton, T. J.; Stoltz, B. M.; Rodriguez, J. A.; Nelson, H. M.; Gonen, T., The CryoEM Method MicroED as a Powerful Tool for Small Molecule Structure Determination. *ACS Cent Sci* **2018,** *4* (11), 1587-1592.
7. Martynowycz, M. W.; Zhao, W.; Hattne, J.; Jensen, G. J.; Gonen, T., Collection of Continuous Rotation MicroED Data from Ion Beam-Milled Crystals of Any Size. *Structure* **2019,** *27* (3), 545-548 e2.
8. Nannenga, B. L.; Shi, D.; Leslie, A. G. W.; Gonen, T., High-resolution structure determination by continuous-rotation data collection in MicroED. *Nat Methods* **2014,** *11* (9), 927-930.
9. Hattne, J.; Martynowycz, M. W.; Penczek, P. A.; Gonen, T., MicroED with the Falcon III direct electron detector. *IUCrJ* **2019,** *6* (5), 921-926.
10. Kabsch, W., XDS. *Acta Crystallogr D Biol Crystallogr* **2010,** *66* (Pt 2), 125-132.
11. Sheldrick, G. M., SHELXT - integrated space-group and crystal-structure determination. *Acta Crystallogr A Found Adv* **2015,** *71* (Pt 1), 3-8.
12. Sheldrick, G. M., Crystal structure refinement with SHELXL. *Acta Crystallogr C Struct Chem* **2015,** *71* (Pt 1), 3-8.
13. Liegault, B.; Petrov, I.; Gorelsky, S. I.; Fagnou, K., Modulating reactivity and diverting selectivity in palladium-catalyzed heteroaromatic direct arylation through the use of a chloride activating/blocking group. *J Org Chem* **2010,** *75* (4), 1047-60.
14. Wang, M.; Zhang, X.; Ling, Z.; Zhang, Z.; Zhang, W., Direct enantioselective C-acylation for the construction of a quaternary stereocenter catalyzed by a chiral bicyclic imidazole. *Chem Commun (Camb)* **2017,** *53* (8), 1381-1384.
15. He, R.; Wu, S.; Tang, H.; Huo, X.; Sun, Z.; Zhang, W., Iridium-Catalyzed Enantioselective and Diastereoselective Allylation of Dioxindoles: A One-Step Synthesis of 3-Allyl-3-hydroxyoxindoles. *Org Lett* **2018,** *20* (19), 6183-6187.
16. Koppes, M. J. C. M.; Cerfontain, H., Photochemical reactivities of cyclic α-phenyl-β,γ-enones. Singlet 1,3-acyl shift, decarbonylation and unquenchable oxa-di-π-methane reactions upon direct irradiation. *Recueil des Travaux Chimiques des Pays-Bas* **1988,** *107* (9), 549-562.