**SUPPORTING INFORMATION**

# Fragment-Based Drug Discovery of Inhibitors of Phosphopantetheine Adenylyltransferase from Gram-Negative Bacteria

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**Contents:**



## In vitro assays and methods

Biochemical assay: All enzymes were expressed with C-terminal His tags and purified in-house from *E. coli* using a nickel column. All biochemical reactions were carried out in a buffer consisting of 50 mM Tris pH 7.5, 50 mM KCl, 5 mM DTT, 1 mM $MgCl_2$, 0.01% (w/v) BSA, 0.01% (w/v) P20. 4'-Phosphopantetheine, the substrate for PPAT, was synthesized biosynthetically by incubating 12 μM *E. coli* PanK/CoaA enzyme with 10 mM ATP (V703, Promega) and 5 mM pantethine (P2125, Sigma) overnight at rt. This reaction produced two molecules of 4'-phosphopantetheine per molecule of pantethine and in the process consumed two molecules of ATP. The next morning, the ATP in the reaction was quantified to ensure the reaction had run to completion.

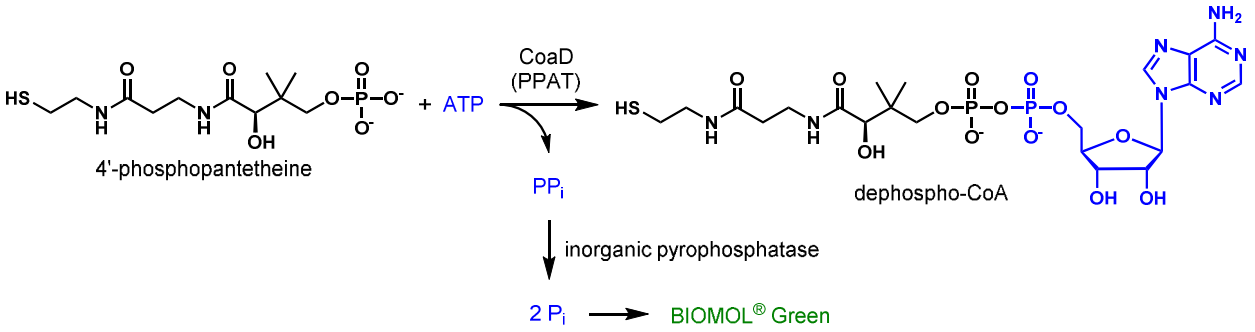



**Figure S1.** Assay format established for the characterization of specific PPAT activity. ATP components shown in blue.

PPAT activity was measured by coupling the production of pyrophosphate from the PPAT reaction to the production of inorganic phosphate by pyrophosphatase from *S. aureus* (Figure S1). Inorganic phosphate was then quantified using the BIOMOL® Green reagent (BML-AK111, Enzo Life Siences) and measuring the absorbance at 620 nm (Scheme S1). Reactions were assembled by incubating 24 nM *E. coli* PPAT (or 17.4 nM *P. aeruginosa* PPAT), 200 µM ATP, and compound together in assay buffer for five minutes, and then initiated by the addition of 20 µM 4'-phosphopantetheine (or 200 µM 4'-phosphopantetheine for the 10xPhP competition experiments) and 78 nM pyrophosphatase. Reactions were allowed to proceed for 30 minutes at rt before being stopped through addition of a volume of BIOMOL® Green reagent equal to the reaction volume. Compounds were delivered in sufficient concentrations to result in a final concentration of DMSO in each reaction of less than 5%.

$$P_i + (NH_4)_2MoO_4 \xrightarrow{H^+} H_3PMo_{12}O_{40}$$

$$H_3PMo_{12}O_{40} + HMG^{2+} \xrightarrow{H^+} (MG^+)(H_2PMo_{12}O_{40}^-) + 2H^+$$

(yellow) (malachite green) (green, $\lambda_{max}$ = 640 nm)

(yellow, $\lambda_{max}$ = 446 nm)

**Scheme S1.** Colorimetric detection of orthophosphate with the BIOMOL® Green reagent.

DSF assay: Differential scanning fluorimetry was carried out in the same buffer as the biochemical assay, lacking only BSA and P20 adjuvants. Enzyme (2.5 μM) was incubated with 2X SYPRO Orange (Invitrogen, diluted from a 5,000X stock), and data was collected from 25→75 °C at 0.5 °C intervals at a ramp rate of 1 °C/min in an Applied Biosystems ViiA 7 RTPCR instrument to generate thermal denaturation profiles of the protein in the presence and absence of a test compound. Data was analyzed using Applied Biosystems Thermal Shift software (v1.1) and the fluorescence intensity over the melting experiment was fit to the Boltzman equation. The melting point was calculated from the first derivative of the Boltzman fit. Across the series of experiments, a $T_m$ shift of >1 °C was deemed statistically significant evidence of ligand-induced stabilization.

SPR: Using a Biacore T200, Avidin-tagged PPAT was immobilized to a Biacore SA chip and any unbound streptavidin was blocked with biocytin (Sigma-Aldrich). Compounds were tested individually at varying concentrations in running buffer (50 mM HEPES pH 7.0, 150 mM KCl, 1 mM TCEP, 0.05% Tween 20, 2% DMSO) at 20°C. Sensorgrams were run in order from low to high concentration using a flow rate of 80 μL/min. All sensor chips were monitored for loss of activity with the injection of a control compound that retains >75% of the activity over the course of the run. Analysis of the binding curves and determination of the kinetic parameters were performed using evaluation software (Version 2.0, Biacore).

Susceptibility Testing: Susceptibility testing was performed using a broth microdilution assay following the recommended methodology of the Clinical and Laboratories Institute (CLSI) (REF: CLSI M7-A9). In brief, fresh bacterial overnight colony growth was resuspended in sterile saline, adjusted to a 0.5 McFarland turbidity standard and then diluted 1:200 into cation-adjusted Mueller-Hinton Broth II (CAMHB; Becton Dickinson, Franklin Lakes, NJ) to yield a

final target inoculum of $5x10^5$ colony-forming units (CFU)/mL. Two-fold serial dilutions of compounds were prepared in 100% DMSO at 100-fold the highest final assay concentration; the resulting dilution series of compounds were diluted 1:10 with sterile water. Assay microtiter plates, which contained 10 µl of 10-fold final concentration of compound per well, were inoculated with a volume of 90 µl of bacterial inoculum, sealed in a plastic bag to prevent moisture loss and incubated for 20 hours at 35°C in ambient air. Following incubation, assay plates were monitored for bacterial growth with a SPECTRAmax380 microtiter plate reader (Molecular Devices, Sunnyvale, CA) at 600 nm, as well as by visual observation with a reading mirror. The minimal inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic at which the visible growth of the organism is completely inhibited. Performance of the assay was monitored by testing gatifloxacin against laboratory quality control strains in accordance with guidelines of the CLSI (REF: CLSI M100-S22).

**Table S1.** MIC strain description.

| *E. coli* strain | Relevant characteristic | Source or reference |
|---|---|---|
| ATCC 25922 | CLSI Reference strain | ATCC |
| BW25113 | Parent of Keio collection, Δ(araD-araB)567, Δ*lacZ*4787(::*rrnB*-3) & lambda-, *rph*-1, Δ(*rhaD*-*rhaB*)568, *hsdR*514 | E coli Genetic Stock Center |
| Δ*tolC* | BW25113 Δ*tolC*732::kan | E coli Genetic Stock Center |

REF CLSI M7-A9: Clinical Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard - Ninth edition. CLSI document M07-A9. Wayne, PA; 2012.

REF CLSI M100-S22: Clinical Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. Wayne, PA; 2012.

**Single residue susceptibility testing**

Cloning, expression and purification of PPAT: The *coaD* genes from *E. coli* and *P. aeruginosa* were amplified from genomic DNA using the primers listed below. The resulting PCR fragments were digested with BamHI/SalI and ligated into a similarly digested pTrcHis2B vector and transformed into TOP10 cells. Each mutant was generated with site by overlap extension PCR using the indicated primers.

**Table S2.** *coaD* gene primers.

| *E. coli* Primers | | *P. aeruginosa* Primers | |
|---|---|---|---|
| WT 5' | aatcaatggatccgatgcaaaaacgggcgatttatc | WT 5' | aatcaatggatccgatgaaccgagtgctgtacccag |
| WT 3' | aatcaatgtcgaccgctaacttcgccatcagcgcc | WT 3' | aatcaatgtcgacgcgcttgaaacgttccgccagg |
| S41K 5' | ttctggcgattgccgccagccccaagaaaaaaccg | K40S 5' | ttctggcgattgccgccagccccaagaaaaaaccg |
| S41K 3' | ttccagggtaaacatcggttttttcttggggctggc | K40S 3' | ttccagggtaaacatcggttttttcttggggctggc |
| D72T 5' | aacgtggaagtggtcgggtttagtactttaatggcg | T71D 5' | aacgtggaagtggtcgggtttagtactttaatggcg |
| D72T 3' | ttacgggcgaagttcgccattaaagtactaaacccg | T71D 3' | ttacgggcgaagttcgccattaaagtactaaacccg |
| M74L 5' | gaagtggtcgggtttagtgatttactggcgaacttc | L73M 5' | gaagtggtcgggtttagtgatttactggcgaacttc |
| M74L 3' | tgttgattacgggcgaagttcgcagtaaatcacta | L73M 3' | tgttgattacgggcgaagttcgcagtaaatcacta |
| S130T 5' | aagagtggtcgtttatctcttcaactttggtgaaag | T129S 5' | aagagtggtcgtttatctcttcaactttggtgaaag |
| S130T 3' | gatggcgcgccacctctttcacaaagttgaagag | T129S 3' | gatggcgcgccacctctttcacaaagttgaagag |
| V135I 5' | atctcttcatcgttggtgaaagagattgcgcgccatc | I134V 5' | atctcttcatcgttggtgaaagagattgcgcgccatc |
| V135I 3' | ggtgacatcgccctgatggcgcgcaatctctttcac | I134V 3' | ggtgacatcgccctgatggcgcgcaatctctttcac |
| R137A 5' | tcatcgttggtgaaagaggtggcggcgcatcagggc | A136R 5' | tcatcgttggtgaaagaggtggcggcgcatcagggc |
| R137A 3' | gaaatgggtgacatcgccctgatgcgccgccacctc | A136R 3' | gaaatgggtgacatcgccctgatgcgccgccacctc |
| H138L 5’ | ttggtgaaagaggtggcgcgcctgcagggcgatgtc | L137H 5’ | ctggtccgggaaatcgccgctcatggcggggatatc |
| H138L 3’ | gaaatgggtgacatcgccctgcaggcgcgccacctc | L137H 3’ | gaacttgctgatatccccgccatgagcggcgatttc |

To express the *C*-terminal $His_6$-tagged protein, cultures growing in LB were induced at log phase with 1 mM IPTG and grown at 18 °C overnight. For purification, cultures were harvested by

centrifugation, lysed with B-PER, supplemented 1/1 (v/v) with a buffer of 50 mM Tris pH 8.0, 250 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole pH 8.2, and 1 mM TCEP, and centrifuged. Soluble protein in the supernatant was captured using TALON metal affinity resin, eluted with buffer containing 500 mM imidazole and then dialyzed overnight into a storage buffer containing 50 mM Tris, pH 8.0, 300 mM KCl, 1 mM $MgCl_2$, 10% glycerol, and 1 mM TCEP. Protein concentrations were determined at 595 nm using Bio-Rad protein reagent with bovine serum albumin (BSA) as the standard. Purified enzymes were stored in aliquots at -80 °C.

PPAT Enzymatic Assays: Initial rate kinetics were determined for each PPAT (WT and mutant) and the appropriate concentration of enzyme was then used to measure $IC_{50}$s. Dose-response reactions containing 100 mM Tris pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.01% P-20, 0.01% BSA, 5 mM DTT, PPAT, and varying concentrations of Fmoc-L-glutamyl-D-histidine isobutylamide (FGHI, **1** from main publication) that was serially diluted in DMSO were incubated for 20 min, initiated with 20 μM phosphopantetheine, 200 μM ATP and 4 U/mL pyrophosphatase coupling enzyme, and then quenched with a 3/2 (v/v) ratio of BIOMOL® Green reagent. After 30 min, absorbance was measured at 620 nm on a SpectraMax Plus384 absorbance microplate reader.

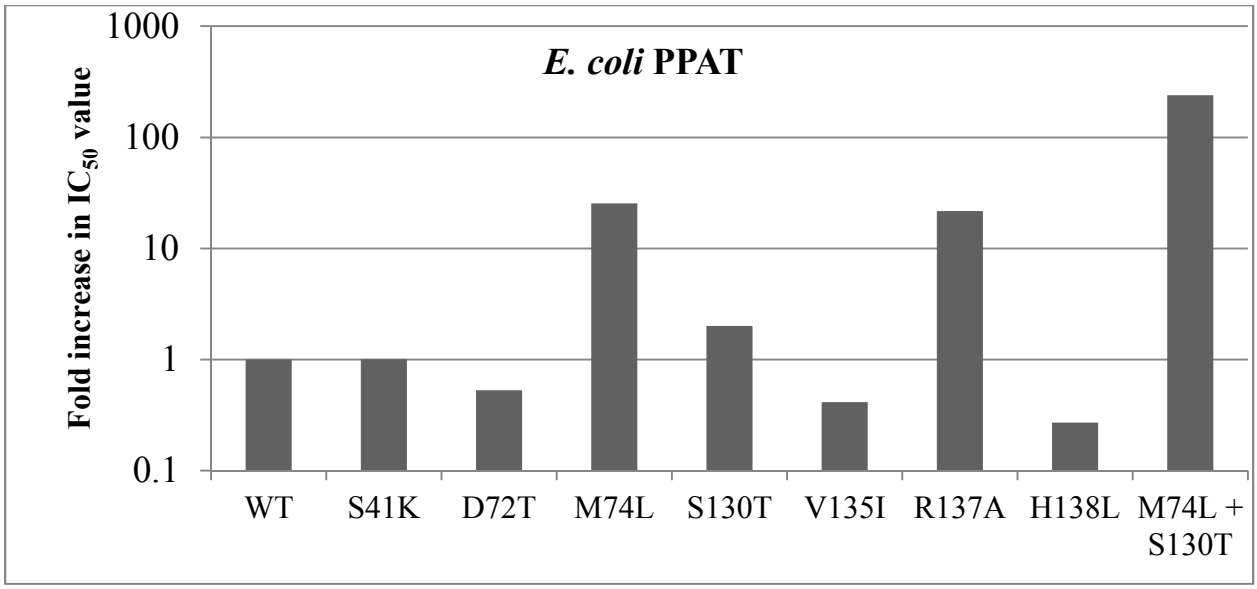



**Chart S1.** Relative differences in FGHI mutant *E. coli* PPAT activity. A fold increase >1 indicates reduced susceptibility to FGHI, while a fold increase <1 indicates enhanced susceptibility to FGHI.

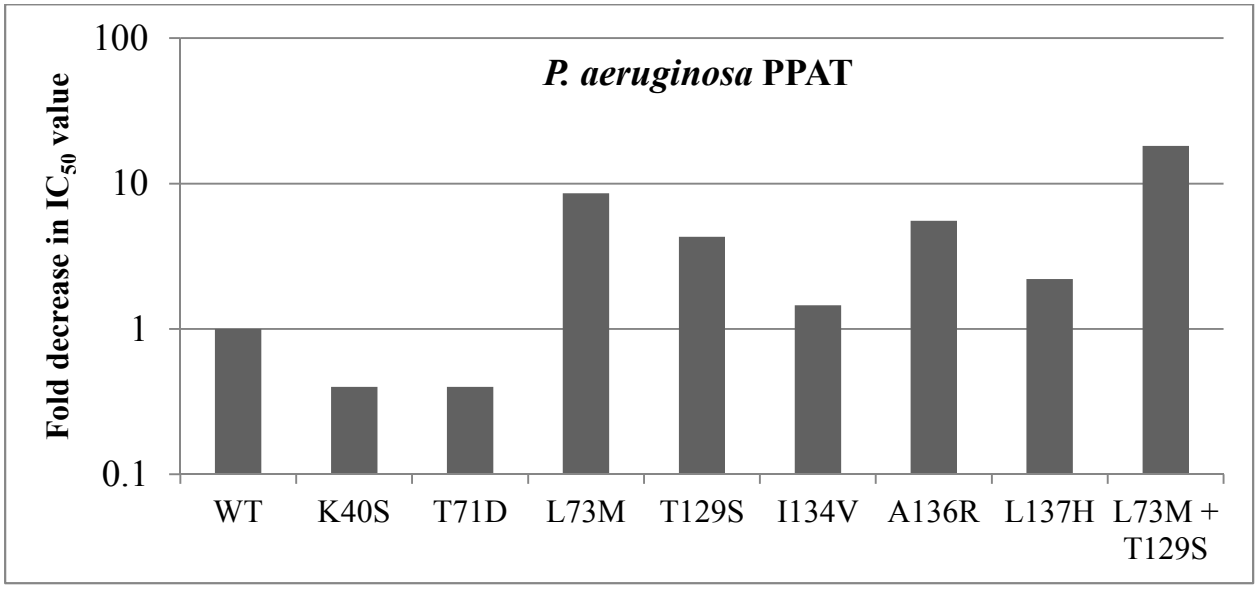



**Chart S2.** Relative differences in FGHI mutant *P. aeruginosa* PPAT activity. A fold decrease <1 indicates reduced susceptibility to FGHI, while a fold decrease >1 indicates enhanced susceptibility to FGHI.

**Computational methods**

All molecular electrostatic potential calculations were performed using the program Jaguar Version 8 (Schrodinger, LLC, New York, NY) and the visualizations were performed using Maestro (Schrodinger, LLC, New York, NY). The starting conformers of compounds **36**, **37**, **39**, **40** and **41** were derived from the binding conformation of compound **30** in the X-ray structure with *E. coli* PPAT (PDB code 6CCM). The molecular geometries were then optimized using the DFT/B3LYP level of theory and the 6-31G** basis set. The molecular electrostatic potential energetic values were computed at the B3LYP/6-31G** level of theory in kcal/mol.

**General experimental information**

If not indicated otherwise, the analytical HPLC conditions are as follows: the compounds and/or intermediates were characterized by HPLC using a UPLC Waters instrument (Milford, MA). HPLC solvent A was 100% water with 0.1% TFA and solvent B was 100% acetonitrile with 0.1% TFA from EMD Chemicals Inc. The instrument was a Waters ACQUITY UPLC system with 1.2 mL/min flow rate on a Kinetex-C18, 2.6 μm, 2.1 x 50 mm column from Phenomenex, column temperature: 50 °C; gradient: 2→88% solvent B over a 1.29 min or 9.79 min period. Compounds were detected by ultraviolet (UV) light absorption at either 220 or 254 nm. All final compounds possessed a purity of at least 95%.

LC-MS analysis was performed on Waters ACQUITY UPLC system and equipped with a ZQ 2000 or SQD MS system with 1.2 mL/min flow rate on a Kinetex-C18, 2.6 μm, 2.1 x 50 mm column by Phenomenex; column temperature: 50 °C; gradient: 2→88% (or 0→45%, or 65→95%) solvent B over a 1.29 min period. Compounds were detected by a Waters Photodiode

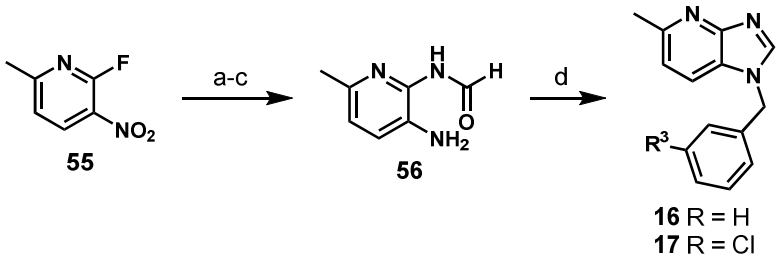
Array Detector. All masses were reported as those of the protonated parent ions, molecular weight range 150-850; cone voltage 20 V.

$^1$H NMR spectra were run on open access Varian 400 MHz or Bruker 500 MHz NMR spectrometers. Spectra were measured at 298K and were referenced using the solvent peak.

Specific optical rotations were measured on an Autopol IV Automatic Polarimeter (Rudolph Research Analytical) with a 100 mm path-length cylindrical glass cell at 20 °C. The wavelength of the light used was 589 nm (the sodium D line). Optical rotation of the same cell filled with solvent was subtracted as blank. The final result was the average of five measurements, each over 10 seconds. The solvent used was methanol.

Preparative separations were carried out using a Combiflash® Rf system (Teledyne Isco, Lincoln, NE) with RediSep silica gel cartridges (Teledyne Isco, Lincoln, NE) or SiliaSep silica gel cartridges (Silicycle Inc., Quebec City, Canada) or by flash column chromatography using silica gel (230–400 mesh) packing material, or by HPLC using a Waters 2767 Sample Manager, C18 reversed-phase Sunfire column, 30 x 50 mm, flow 75 mL/min. Solvents employed for the RP-HPLC were varying concentrations of acetonitrile and water with 0.1% trifluoroacetic acid, or varying concentrations of acetonitrile and 3.75 mM aqueous ammonium acetate.

Synthesis and characterization of compounds **16**–**52**:




**55** **56**

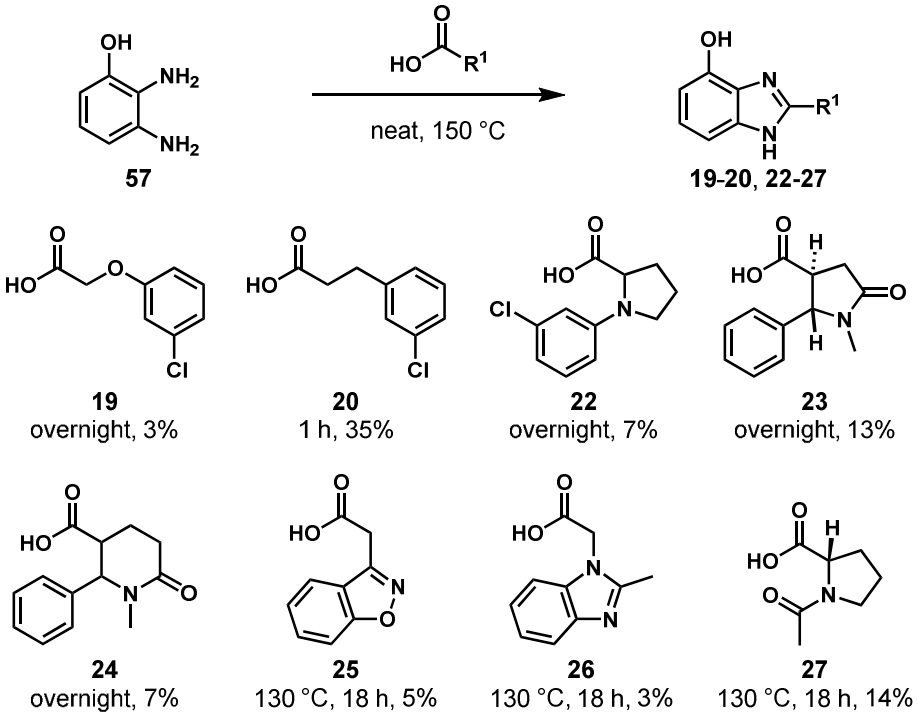
**16** R = H
**17** R = Cl

(a) ammonium hydroxide, MW, 130 °C, 30 min, 93%. (b) $Ac_2O$, HCOOH, 60 °C, overnight 100%. (c) $H_2$, Raney-Nickel, THF, MeOH, rt, overnight, 86%. (d) benzaldehyde or 3-chlorobenzaldehyde, $BH_3 \cdot Py$, 2 h; **16**: 28% and **17**: 34%.

***N*-(3-Amino-6-methylpyridin-2-yl)formamide (56)**. Step 1: 2-Fluoro-6-methyl-3-nitropyridine (**55**) (1.167 g, 7.48 mmol, 1.0 equiv.) was dispersed in ammonium hydroxide (28-30% in water, 2.91 mL, 74.8 mmol, 10 equiv.) and the mixture was heated at 130 °C in a microwave reactor for 30 min. The mixture was left to stand at rt, then the solid was collected by filtration, washed with water, and dried under vacuum to provide 1.059 g (93%) of 6-methyl-3-nitropyridin-2-amine. The same procedure was applied to 4 g scale and another 3.68 g of desired product were obtained. In total, 4.739 g of 6-methyl-3-nitropyridin-2-amine were obtained. MS (ESI): *m/z* 154.0 $[M+H]^+$. Step 2: A solution of acetic anhydride (64.2 mL, 681 mmol, 22 equiv.) and formic acid (43 mL, 1121 mmol, 22 equiv.) was heated at 60 °C for 3 h. The solution was cooled to rt and 6-methyl-3-nitropyridin-2-amine (4.738 g, 30.9 mmol, 1.0 equiv.) was added over 15 min. After stirring at rt overnight, the solvents were removed under reduced pressure at <45 °C to give 5.6 g (quant.) of *N*-(6-methyl-3-nitropyridin-2-yl)formamide. MS (ESI): *m/z* 182.1 $[M+H]^+$. Step 3: To a solution of *N*-(6-methyl-3-nitropyridin-2-yl)formamide (5.6 g, 30.9 mmol, 1.0 equiv.) in 160 mL dry THF in a round-bottomed flask was added a dispersion of Raney nickel (2.65 g, 30.9 mmol, 1.0 equiv.) in 6 mL MeOH. (Raney nickel in water was rinsed with MeOH several times, the supernatant was removed, and the black metal was dispersed in MeOH prior to use.) The reaction mixture was flushed with $N_2$ and $H_2$ several times, then maintained under 1 atm $H_2$ at rt for 4 h. The reaction was vented and purged with $N_2$. LCMS analysis showed that starting material remained, so the reaction mixture was flushed with $H_2$ and stirred under a balloon of $H_2$ overnight. The mixture was filtered through Celite®, the filtrate was concentrated and the crude product was purified by silica gel flash column chromatography (2% acetone/EtOAc) to give 4 g (86%) of **56**. MS (ESI): *m/z* 152.0 $[M+H]^+$.

**1-Benzyl-5-methyl-1*H*-imidazo[4,5-*b*]pyridine** (**16**). To a clear solution of **56** (200 mg, 1.323 mmol, 1.0 equiv.) and benzaldehyde (0.201 mL, 1.985 mmol, 1.5 equiv.) in 4 mL 1:1 $CH_2Cl_2$-AcOH at rt was added $BH_3 \cdot Py$ (0.134 mL, 1.323 mmol, 1.0 equiv.). After stirring for 2 h, the reaction mixture was neutralized to pH ≈ 7 with aqueous $NH_4OH$ and extracted with $CH_2Cl_2$. The organic layer was separated, dried over $Na_2SO_4$, and filtered. After concentration, the crude residue was purified by silica gel flash column chromatography [90→100% ($CH_2Cl_2$-MeOH-$NH_4OH$ 90:10:1)/$CH_2Cl_2$] to give 188 mg of impure material as a light yellow solid, which was further purified by RP-HPLC to give 83 mg (28%) of **16**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.73–8.67 (m, 1H), 7.94 (d, $J$ = 8.27 Hz, 1H), 7.37–7.25 (m, 5H), 7.17 (d, $J$ = 8.27 Hz, 1H), 5.52 (s, 2H), 2.55 (s, 3H). HRMS (ESI): *m/z* 224.1190 $[M+H]^+$.

**1-(3-Chlorobenzyl)-5-methyl-1*H*-imidazo[4,5-*b*]pyridine** (**17**). Prepared in a manner analogous to compound **16** to provide 118 mg (35%) of **17**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.57 (s, 1H), 7.86 (d, $J$ = 8.22 Hz, 1H), 7.41 (s, 1H), 7.37–7.34 (m, 2H), 7.26–7.22 (m, 1H), 7.11 (d, $J$ = 8.22 Hz, 1H), 5.50 (s, 2H), 2.52 (s, 3H). HRMS (ESI): *m/z* 258.0799 $[M+H]^+$.




**2,3-Diaminophenol** (**57**). A mixture of 2-amino-3-nitrophenol (500 mg, 3.24 mmol, 1.0 equiv.) and 10% Pd/C (173 mg, 0.162 mmol, 0.05 equiv.) in 15 mL 4:1 ethyl acetate-methanol was placed under a hydrogen atmosphere and allowed to stir at rt for 4 h. The reaction mixture was sparged with nitrogen, the catalyst removed by filtration over Celite® and the filter cake washed with ethyl acetate and methanol. The filtrate was evaporated to give 403 mg (quant.) of the desired product as a black solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.68 (s, 1H), 6.23 (t, $J$ = 7.8 Hz, 1H), 6.09–5.99 (m, 2H), 4.35 (s, 2H), 3.32 (s, 1H).

General procedure for the synthesis of 4-hydroxyazabenzimidazole analogs.

A microwave vial charged with a neat mixture of the appropriate carboxylic acid and **57** (1.0–1.1 equiv.) was flushed with nitrogen, sealed, then heated at 130 °C or 150 °C for the time indicated in the scheme above. After cooling to rt, the crude material was diluted with DMSO, filtered and

purified by preparative RP-HPLC to furnish the pure product. Compounds **25**–**27** were prepared by parallel synthesis.

**2-((3-Chlorophenoxy)methyl)-1*H*-benzo[*d*]imidazol-4-ol** (**19**). 5 mg (3%). $^1$H NMR (400 MHz, Methanol-$d_4$) δ 7.39 (d, $J$ = 8.3 Hz, 1H), 7.35 (d, $J$ = 8.4 Hz, 1H), 7.23–7.19 (m, 2H), 7.12–7.05 (m, 2H), 6.95–6.90 (m, 1H), 5.58 (s, 2H). MS (ESI): *m/z* 275.1 $[M+H]^+$.

**2-(3-Chlorophenethyl)-1*H*-benzo[*d*]imidazol-4-ol** (**20**). 55 mg (35%). HRMS (ESI): *m/z* 273.0801 $[M+H]^+$.

**2-(1-(3-Chlorophenyl)pyrrolidin-2-yl)-1*H*-benzo[*d*]imidazol-4-ol** (**22**). 3 mg (7%). $^1$H NMR (400 MHz, Methanol-$d_4$) δ 7.18 (t, $J$ = 8.02 Hz, 1H), 7.10–6.90 (m, 2H), 6.75 (d, $J$ = 7.83 Hz, 1H), 6.62 (d, $J$ = 7.83 Hz, 1H), 6.54 (s, 1H), 6.37 (dd, $J$ = 8.41, 2.15 Hz, 1H), 5.11–4.96 (m, 1H), 3.78 (t, $J$ = 7.04 Hz, 1H), 3.46–3.29 (m, 1H), 2.53–2.43 (m, 1H), 2.34–1.94 (m, 4H). MS (ESI): *m/z* 314.1 $[M+H]^+$.

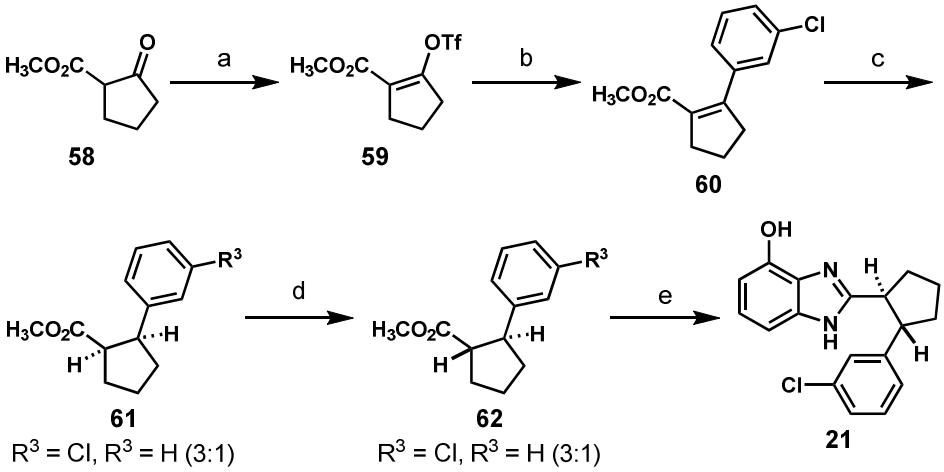
***trans*-4-(4-Hydroxy-1*H*-benzo[*d*]imidazol-2-yl)-1-methyl-5-phenylpyrrolidin-2-one** (**23**). 10 mg (13%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.53–7.17 (m, 1H), 7.07–6.74 (m, 1H), 6.61–6.37 (m, 1H), 5.08–4.63 (m, 1H), 3.70–3.46 (m, 2H), 3.03–2.84 (m, 1H), 2.83–2.63 (m, 2H), 2.60–2.52 (m, 6H). HRMS (ESI): *m/z* 308.1399 $[M+H]^+$.

**5-(4-Hydroxy-1*H*-benzo[*d*]imidazol-2-yl)-1-methyl-6-phenylpiperidin-2-one** (**24**). 5 mg (7%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.42–11.75 (m, 1H), 10.07–9.07 (m, 1H), 7.37–7.09 (m, 5H), 7.04–6.68 (m, 2H), 6.60–6.32 (m, 1H), 5.30–5.07 (m, 1H), 2.58 (s, 3H), 2.23–2.02 (m, 1H), 1.99–1.79 (m, 1H). HRMS (ESI): *m/z* 322.1553 $[M+H]^+$.

**2-(Benzo[*d*]isoxazol-3-ylmethyl)-1*H*-benzo[*d*]imidazol-4-ol** (**25**). 3 mg (5%). HRMS (ESI): *m/z* 266.0932 $[M+H]^+$.

**2-((2-Methyl-1*H*-benzo[*d*]imidazol-1-yl)methyl)-1*H*-benzo[*d*]imidazol-4-ol** (**26**). 2 mg (3%). MS (ESI): *m/z* 278.1 $[M+H]^+$.

**(*S*)-1-(2-(4-Hydroxy-1*H*-benzo[*d*]imidazol-2-yl)pyrrolidin-1-yl)ethan-1-one** (**27**). 8 mg (14%). HRMS (ESI): *m/z* 246.1241 $[M+H]^+$.




(a) NaH, $Tf_2O$, $Et_2O$, 0 °C, 1 h, 97% (b) $Pd(OAc)_2$, $PPh_3$, $Na_2CO_3$, EtOH-benzene (1:3), 140 °C, 30 min, MW, 86% (c) $PtO_2$, $H_2$ (1 atm), EtOH, 1.5 h, 72% (d) NaOMe, MeOH, reflux, 1 h. (e) **57**, neat, 150 °C, o/n, 18%.

**Methyl 2-(((trifluoromethyl)sulfonyl)oxy)cyclopent-1-ene-1-carboxylate** (**59**). To a solution of methyl 2-oxocyclopentane-1-carboxylate (**58**) (3 g, 21.10 mmol, 1.0 equiv.) in 20 mL ether at 0 °C was added NaH (60% in mineral oil, 1.182 g, 29.5 mmol, 1.4 equiv.) (gas evolution). The resulting suspension was allowed to stir at 0 °C for 10 min before $Tf_2O$ (4.28 mL, 7.15 g, 25.3 mmol, 1.2 equiv.) was added. The resulting suspension was stirred at 0 °C for 1 h, then the reaction was quenched with water and the product was extracted with EtOAc. The combined

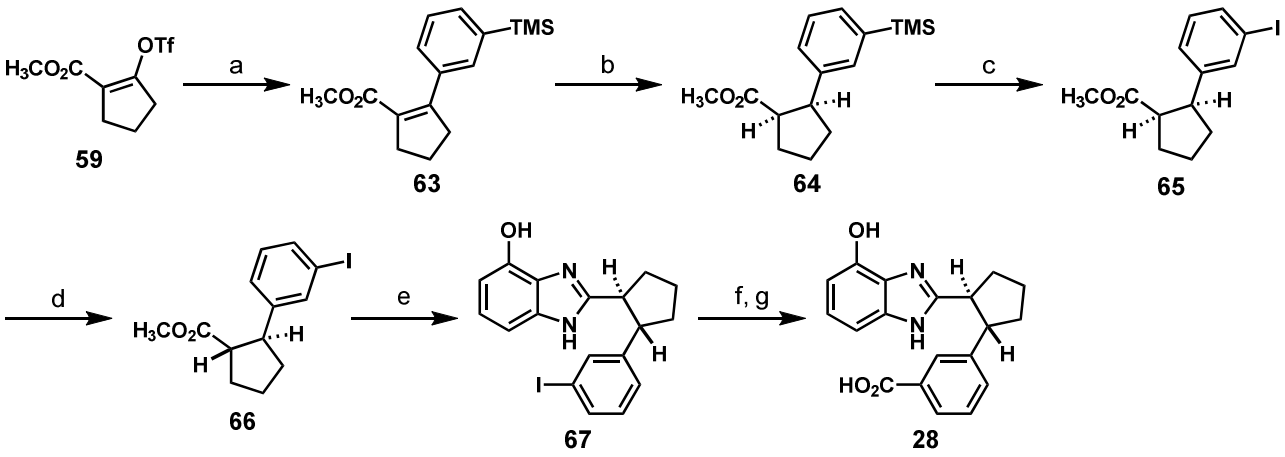
organic extracts were dried with $MgSO_4$, filtered and the solvent was evaporated to give 5.79 g (quant.) of crude **59**, which was taken on without further purification.

**Methyl 2-(3-chlorophenyl)cyclopent-1-ene-1-carboxylate** (**60**). A mixture of **59** (2.5 g, 9.12 mmol, 1.0 equiv.), $PPh_3$ (0.239 g, 0.912 mmol, 0.1 equiv.), $Na_2CO_3$ (1.933 g, 18.23 mmol, 2.0 equiv.) and $Pd(OAc)_2$ (0.102 g, 0.456 mmol, 0.05 equiv.) in 16 mL 3:1 benzene-ethanol was treated with (3-chlorophenyl)boronic acid (1.711 g, 10.94 mmol, 1.2 equiv.). The resulting mixture was sparged with nitrogen and subjected to microwave heating at 140°C for 30 min. The mixture was filtered through Celite®, concentrated, and re-suspended in EtOAc prior to a second filtration through Celite®. The solvent was evaporated and the residue was purified by silica gel flash column chromatography (EtOAc/heptane gradient) to provide 1.85 g (86%) of **60**. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.31–7.18 (m, 4H), 3.63 (s, 3H), 2.83 (t, $J$ = 7.6 Hz, 4H), 1.99 (p, $J$ = 7.6 Hz, 2H). MS (ESI): *m/z* 237.0 $[M+H]^+$.

***cis*-Methyl 2-(3-chlorophenyl)cyclopentane-1-carboxylate** (**61**). A mixture of **60** (275 mg, 1.162 mmol, 1.0 equiv.) and $PtO_2$ (80 mg, 0.352 mmol, 0.3 equiv.) in 10 mL EtOH was placed under a hydrogen atmosphere and allowed to stir well at rt for 1.5 h. The reaction was sparged with nitrogen, filtered through a pad of Celite®, and the solvent was evaporated to give 200 mg of crude **61**, which was contaminated with ~25% of the corresponding deschloro product. The mixture was taken on without further purification. MS (ESI): *m/z* 239.0 $[M+H]^+$.

***trans*-2-(2-(3-Chlorophenyl)cyclopentyl)-1*H*-benzo[d]imidazol-4-ol** (**21**). Step 1: A solution of **61** (58 mg, ~0.25 mmol, 1.0 equiv.) in 3 mL MeOH was treated with NaOMe (47 mg, 0.864 mmol, 3.5 equiv.) and the resulting solution was refluxed for 1 h. The solvent was evaporated to give 58 mg of crude **62**, which contained ~30% of the carboxylic acid, as well as the

corresponding mixture of deschloro products carried forward from the previous step. This mixture was used for the next reaction without further purification. MS (ESI): *m/z* 239.1 $[M+H]^+$. Step 2: A microwave vial containing a neat mixture of **62** (54.3 mg, ~0.24 mmol, 1.0 equiv.) and **57** (30 mg, 0.242 mmol, 1.0 equiv.) was flushed with nitrogen, sealed and heated at 150°C in an oil bath overnight. The reaction mixture was allowed to cool to rt, diluted with MeOH, filtered and purified by RP-HPLC to provide 14 mg (18%) of pure **21**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.31 (t, *J* = 1.8 Hz, 1H), 7.24 (dd, *J* = 8.7, 6.5 Hz, 1H), 7.21–7.15 (m, 2H), 6.97–6.74 (m, 2H), 6.52–6.38 (m, 1H), 3.66–3.48 (m, 1H), 3.41–3.34 (m, 1H), 2.30–2.15 (m, 2H), 2.02–1.87 (m, 3H), 1.83–1.70 (m, 1H). HRMS (ESI): *m/z* 313.1107 $[M+H]^+$.




(a) $Pd(OAc)_2$, $PPh_3$, $Na_2CO_3$, EtOH-benzene (1:3), 140 °C, 30 min, MW, quant. (b) $PtO_2$, $H_2$ (1 atm), EtOAc-MeOH, rt, o/n, quant. (c) ICl, 1 h, 0 °C (d) NaOMe, MeOH, reflux, 1 h (e) **57**, neat, 150 °C, o/n, 82% (f) $Mo(CO)_6$, $Pd(OAc)_2$, DPPF, *i*-$Pr_2$NEt, dioxane-DMF, 160 °C, 30 min, 79% (g) NaOH, THF-$H_2O$, rt, 1 h, 52%.

**Methyl 2-(3-(trimethylsilyl)phenyl)cyclopent-1-enecarboxylate** (**63**). A mixture of **59** (1.5 g, 5.47 mmol, 1.0 equiv.), $PPh_3$ (0.143 g, 0.547 mmol, 0.1 equiv.), $Na_2CO_3$ (1.160 g, 10.94 mmol, 2.0 equiv.) and $Pd(OAc)_2$ (0.061 g, 0.274 mmol, 0.05 equiv.) in 16 mL 3:1 benzene-ethanol was treated with (3-(trimethylsilyl)phenyl)boronic acid (1.274 g , 6.56 mmol, 1.2 equiv.). The resulting mixture was sparged with nitrogen and subjected to microwave heating at 140°C for 30

min. The mixture was filtered through Celite®, concentrated, and re-suspended in EtOAc prior to a second filtration through Celite®. The solvent was evaporated and the residue was purified by silica gel flash column chromatography (EtOAc/heptane gradient) to provide 1.5 g (quant.) of **63**. MS (ESI): *m/z* 275.1 $[M+H]^+$.

***cis*-Methyl 2-(3-(trimethylsilyl)phenyl)cyclopentanecarboxylate (64)**. A solution of **63** (1.6 g, 5.83 mmol, 1.0 equiv.) in 20 mL 3:1 EtOAc-MeOH was treated with 10% Pd/C (0.310 g, 0.292 mmol, 0.05 equiv.) and the resulting black mixture was placed under a hydrogen atmosphere and allowed to stir at rt overnight. No conversion of the starting material was observed, so the reaction was sparged with nitrogen and filtered through a pad of Celite®. The resulting solution was treated with $PtO_2$ (0.132 g, 0.583 mmol, 0.1 equiv.), placed under a hydrogen atmosphere and allowed to stir at rt overnight to achieve the desired olefin reduction. The reaction was sparged with nitrogen, filtered through a pad of Celite®, and the solvent was evaporated to give 1.612 g (quant.) of crude **64**, which was taken on without further purification. MS (ESI): *m/z* = 277.1 $[M+H]^+$.

***cis*-Methyl 2-(3-iodophenyl)cyclopentanecarboxylate (65)**. A solution of **64** (1.5 g, 5.43 mmol, 1.0 equiv.) in 20 mL $CH_2Cl_2$ was allowed to cool to 0°C, then iodine monochloride (1.0 M in $CH_2Cl_2$, 13.02 mL, 13.02 mmol, 2.4 equiv.) was added dropwise and the reaction was allowed to stir at 0 °C for 1 h. The reaction was quenched by the addition of 1 M sodium thiosulfate and the reaction stirred vigorously for 30 min. The organic phase was collected, dried over $MgSO_4$ and filtered through a plug of silica gel. After evaporation of the solvent, 1.791 g (quant.) of crude **65** was obtained and used for the next reaction. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.65–7.43 (m, 2H), 7.22–7.10 (m, 1H), 7.07–6.90 (m, 1H), 3.77–3.53 (m, 1H), 3.31 (s, 4H),

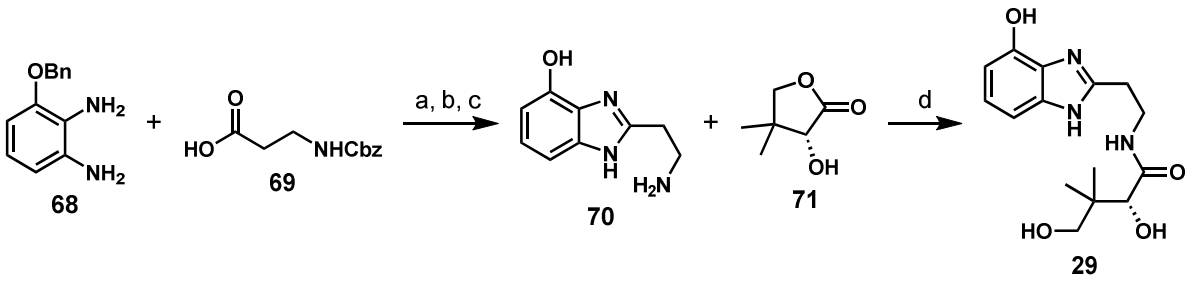
3.25–3.22 (m, 1H), 3.20–3.07 (m, 1H), 2.05 (br s, 6H), 1.82–1.61 (m, 1H), 1.60–1.44 (m, 1H). MS (ESI): *m/z* = 331.1 $[M+H]^+$.

***trans*-Methyl 2-(3-iodophenyl)cyclopentanecarboxylate (66).** A solution of **65** (1.6 g, 4.85 mmol, 1.0 equiv.) in 20 mL MeOH was treated with NaOMe (0.924 g, 17.11 mmol, 3.53 equiv.) and resulting solution was refluxed for 1 h. After the solution was allowed to cool to rt, it was diluted with EtOAc and washed with 1 N HCl and brine. The organic layer was dried with $MgSO_4$, filtered, and the solvent was evaporated to provide crude **66**, which contained ~30% of the corresponding carboxylic acid. The mixture was taken on without further purification.

***trans*-2-(2-(3-Iodophenyl)cyclopentyl)-1*H*-benzo[*d*]imidazol-4-ol (67).** A microwave vial containing a neat mixture of **66** (900 mg, ~2.73 mmol, 1.0 equiv) and **57** (508 mg, 4.09 mmol, 1.5 equiv.) was flushed with nitrogen, sealed and heated at 150°C in an oil bath overnight. The reaction mixture was allowed to cool to rt, diluted with MeOH, filtered and purified by RP-HPLC to give 900 mg (82%) of pure **67**. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.63–7.42 (m, 2H), 7.15–7.02 (m, 2H), 7.02–6.99 (m, 1H), 6.97–6.83 (m, 2H), 6.80–6.67 (m, 1H), 3.58–2.92 (m, 2H), 2.45–2.12 (m, 4H), 2.10–1.69 (m, 4H). MS (ESI): *m/z* = 405.0 $[M+H]^+$.

***trans*-3-(2-(4-Hydroxy-1*H*-benzo[*d*]imidazol-2-yl)cyclopentyl)benzoic acid (28).** Step 1: A mixture of **67** (60 mg, 0.148 mmol, 1.0 equiv.), $Mo(CO)_6$ (47.0 mg, 0.178 mmol, 1.2 equiv.), $Pd(OAc)_2$ (6.66 mg, 0.030 mmol, 0.2 equiv.), DPPF (8.23 mg, 0.015 mmol, 0.1 equiv.) and DIPEA (0.078 mL, 0.445 mmol, 3.0 equiv.) in a microwave vial was suspended in 1.5 mL dioxane. DMF (0.375 mL) was added and the resulting suspension was microwaved at 160°C for 30 min. The crude mixture was filtered through Celite® and the filter cake was rinsed with EtOAc. The filtrate was washed with brine, dried with $MgSO_4$, filtered and the solvents were

evaporated. The residue was purified by silica gel flash column chromatography (0→100% EtOAc/heptane, followed by 0→100% MeOH/$CH_2Cl_2$) to give 40 mg (79%) of methyl 3-(2-(4-hydroxy-1*H*-benzo[*d*]imidazol-2-yl)cyclopentyl)benzoate. MS (ESI): *m/z* = 337.2 $[M+H]^+$. Step 2: Half of the methyl ester from above (20 mg, 0.059 mmol, 1.0 equiv.) was dissolved in 0.5 mL THF, then 1 N NaOH (0.178 mL, 0.178 mmol, 3.0 equiv.) was added and the resulting suspension was allowed to stir at rt for 1 h. The reaction solution was neutralized with 2 N HCl and extracted with EtOAc. The combined organic extracts were washed with brine, dried with $MgSO_4$, and the solvents were evaporated. The residue was dissolved in MeOH-water, filtered and purified by RP-HPLC to provide 10 mg (52%) of pure **28**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.87 (s, 1H), 7.67 (dt, *J* = 7.7, 1.2 Hz, 1H), 7.31 (d, *J* = 7.6 Hz, 1H), 7.22 (t, *J* = 7.6 Hz, 1H), 6.87–6.78 (m, 2H), 6.46–6.39 (m, 1H), 3.71–3.60 (m, 1H), 3.50–3.41 (m, 1H), 2.31–2.19 (m, 2H), 2.02–1.91 (m, 3H). HRMS (ESI): *m/z* = 323.1399 $[M+H]^+$.




(a) HATU, $Et_3N$, DCM, rt, 18 h. (b) 1,1,1,3,3,3-hexafluoropropan-2-ol, 100 °C, MW, 10 min. c) Pd/C, $H_2$ (1 atm), MeOH, rt, overnight. (d) $Et_3N$, MeOH, 120 °C, MW, 40 min, 3%.

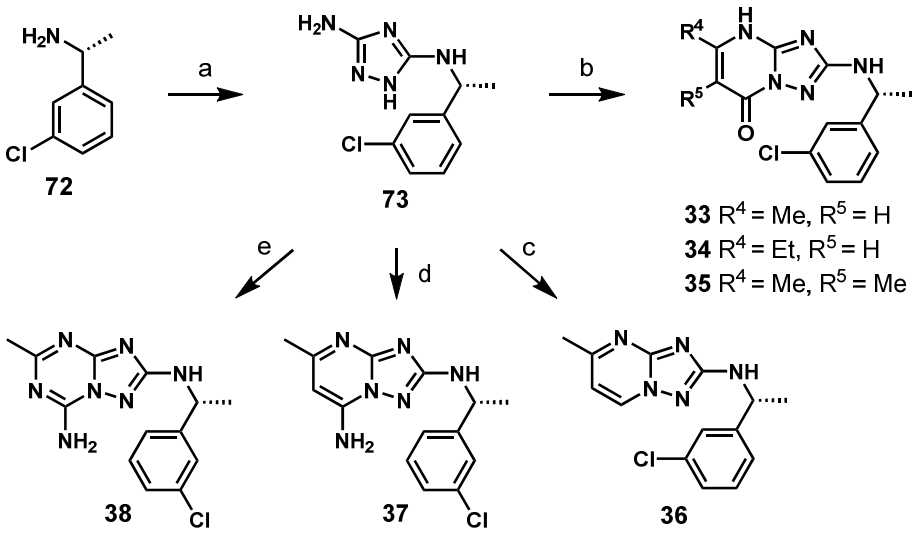
**3-(Benzyloxy)benzene-1,2-diamine** (**68**). Step 1: A solution of 2-amino-3-nitrophenol (2.00 g, 12.98 mmol, 1.0 equiv.) in 70 mL DMF was cooled to 0°C before potassium carbonate (2.69 g, 19.46 mmol, 1.5 equiv.) was added in one portion, followed by benzyl bromide (1.698 ml, 14.27 mmol, 1.1 equiv.) dropwise over 3 min. The reaction mixture was allowed to warm slowly to rt

as the cooling bath expired, then the mixture was stirred at rt overnight. The reaction mixture was quenched with 400 mL saturated aqueous $NaHCO_3$, then extracted 3 x 200 mL $CH_2Cl_2$ and 1 x 100 mL EtOAc. The combined organic extracts were washed sequentially with 100 mL each water and brine, then dried with $MgSO_4$, filtered, and concentrated to a dark red oil. The residue was purified by silica gel flash column chromatography (0→5% MeOH/$CH_2Cl_2$) to give 3.4 g of 2-(benzyloxy)-6-nitroaniline as a red oil. MS (ESI): *m/z* = 245.1 $[M+H]^+$. Step 2: A 20 mL microwave vial was charged with 2-(benzyloxy)-6-nitroaniline (733 mg, 3.00 mmol, 1.0 equiv.), tin(II) chloride (2.84 g, 15.00 mmol, 5.0 equiv.), and 15 mL EtOH. The suspension was vigorously stirred, then heated at 140 °C for 10 min in the microwave. The reaction mixture was cooled to rt, poured into 30 mL aqueous saturated $NaHCO_3$ and extracted 2 x 100 mL EtOAc. The combined organic layers were washed with 50 mL saturated $NaHCO_3$, 30 mL water, and 30 mL brine, then dried with $MgSO_4$, filtered, and concentrated to give 430 mg (67%) of 3-(benzyloxy)benzene-1,2-diamine as a yellow oil. MS (ESI): *m/z* = 425.2 $[M+H]^+$.

**2-(2-Aminoethyl)-1*H*-benzo[*d*]imidazol-4-ol** (**70**). Step 1: A mixture of **69** (229 mg, 1.027 mmol, 1.0 equiv.), HATU (390 mg, 1.027 mmol, 1.0 equiv.) and **68** (220 mg, 1.027 mmol, 1.0 equiv.) in 5.1 mL DCM was treated with $Et_3N$ (143 µl, 1.027 mmol, 1.0 equiv.), and the resulting mixture was allowed to stir at rt overnight. The reaction was quenched with 10 mL water, then extracted with 20 mL $CH_2Cl_2$ and 2 x 20 mL EtOAc. The combined organic extracts were dried with $Na_2SO_4$, filtered, and concentrated to give 680 mg of a white solid. The crude material containing benzyl (3-((2-amino-6-(benzyloxy)phenyl)amino)-3-oxopropyl)carbamate was taken on without further purification. MS (ESI): *m/z* = 420.6 $[M+H]^+$. Step 2: A 2 mL microwave vial containing a solution of crude benzyl (3-((2-amino-6-(benzyloxy)phenyl)amino)-3-

oxopropyl)carbamate (210 mg, 0.5 mmol, 1.0 equiv.) in 2 mL of 1,1,1,3,3,3-hexafluoropropan-2-ol was allowed to stir at rt for 5 min, then the mixture was heated at 100 °C for 10 min in the microwave. The reaction mixture was concentrated on a rotary evaporator to give a dark red oil, which was purified by silica gel flash column chromatography (0→100% EtOAc/heptane) to provide 110 mg (54.8%) of the desired cyclization product, benzyl (2-(4-(benzyloxy)-1*H*-benzo[*d*]imidazol-2-yl)ethyl)carbamate. MS (ESI): *m/z* = 402.6 $[M+H]^+$. Step 3: A solution of benzyl (2-(4-(benzyloxy)-1*H*-benzo[*d*]imidazol-2-yl)ethyl)carbamate in 6.2 mL MeOH was treated with 10% Pd/C (50 wt% water, 133 mg, 0.0625 mmol, 0.05 equiv.). The reaction vessel was purged with nitrogen/hydrogen three times, then stirred rapidly under a hydrogen balloon at rt overnight. The mixture was filtered through Celite® and concentrated to give 350 mg of crude **70**, which was used without further purification. MS (ESI): *m/z* = 178.1 $[M+H]^+$.

**(*R*)-2,4-Dihydroxy-*N*-(2-(4-hydroxy-1*H*-benzo[*d*]imidazol-2-yl)ethyl)-3,3-dimethylbutanamide** (**29**). Triethylamine (0.15 mL, 1.076 mmol, 1.91 equiv.) and **71** (88 mg, 0.677 mmol, 1.2 equiv.) were added to a microwave vial charged with a solution of **70** (100 mg, 0.564 mmol, 1.0 equiv.) in 2.82 mL MeOH. The vial was sealed, then heated in the microwave at 100 °C for 15 min, then at 120 °C for 40 min. The reaction mixture was allowed to cool to rt, concentrated to an oil, then dissolved in DMSO, filtered and purified by RP-HPLC to give 7 mg (3%) of **29** as a white powder. $^1$H NMR (400 MHz, Methanol-$d_4$) δ 7.34 (t, *J* = 8.2 Hz, 1H), 7.14 (d, *J* = 8.2 Hz, 1H), 6.89 (d, *J* = 8.0 Hz, 1H), 3.89–3.77 (m, 2H), 3.72-3.59 (m, 1H), 3.38 (s, 2H), 3.36–3.32 (m, 2H), 0.87 (s, 3H), 0.81 (s, 3H). HRMS (ESI): *m/z* = 308.1609 $[M+H]^+$.




(a) i) Diphenyl *N*-cyanocarbonimidate, 2-propanol, 60 °C, 2 h; ii) $N_2H_4 \cdot H_2O$, rt, 2 days. (b) $R^4COCH(R^5)CO_2Et$, AcOH, 165 °C, MW, 10–30 min; **33**: 15%, **34**: 6%, **35**: 16%. (c) 4,4-dimethoxybutan-2-one, NaOEt, EtOH, rt, 24 h, 32%. (d) 3-oxobutanenitrile, *p*-$TsOH \cdot H_2O$, mesitylene, 200 °C, MW, 30 min, 16%. (e) ethyl *N*-cyanoacetimidate, DME-NMP (4:1), 200 °C, MW, 30 min, 10%.

**(*R*)-*N*3-(1-(3-Chlorophenyl)ethyl)-4*H*-1,2,4-triazole-3,5-diamine** (**73**). Step 1: A suspension of diphenyl *N*-cyanocarbonimidate (9.94 g, 40.5 mmol, 1.5 equiv.) in 85 mL 2-propanol was treated with **72** (4.2 g, 27.0 mmol, 1.0 equiv.). The resulting mixture was allowed to stir at 60 °C for 45 min, then the solution was allowed to stir at rt overnight, during which time a white solid crashed out. The solid was collected by filtration and washed with 2-propanol and heptane, then dried under vacuum at 40 ºC to provide 5.56 g (69%) of pure phenyl (*R*)-*N*-(1-(3-chlorophenyl)ethyl)-*N′*-cyanocarbamimidate, which was used without further purification. MS (ESI): *m/z* = 300.1 $[M+H]^+$. Step 2: A suspension of phenyl (*R*)-*N*-(1-(3-chlorophenyl)ethyl)-*N′*-cyanocarbamimidate (5.56 g, 18.55 mmol, 1.0 equiv.) in 50 mL MeOH was treated with hydrazine hydrate (2.276 mL, 46.4 mmol, 2.5 equiv.) and the resulting mixture was allowed to stir at 50 °C for 4 h. The mixture was concentrated to provide 7.898 g of crude **73** as a sticky oil. The crude product was used directly in next step. MS (ESI): *m/z* = 238.1 $[M+H]^+$.

General procedure for the synthesis of triazolopyrimidinones.

A 0.5 M solution of **73** (1.0 equiv.) in AcOH was treated with β-keto ester (1.2–1.5 equiv.) and the mixture was heated in a microwave reactor at 165 °C for 10–30 minutes. Water was added and a white solid crashed out. The solid was collected by filtration and washed with water, then dissolved in DMSO and purified by RP-HPLC. The desired fractions were combined and neutralized with Agilent StratoSpheres™ PL-$HCO_3$ MP SPE resin to remove TFA.

**(*S*)-5-methyl-2-((1-phenylethyl)amino)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (***S*-Me-31**). Ethyl 3-oxobutanoate (293 mg, 2.25 mmol, 1.2 equiv.) was used to provide 456 mg (87%) of ***S*-Me-31** after trituration with MeOH-water. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.36 (d, $J$ = 7.09 Hz, 2H), 7.30–7.24 (m, 2H), 7.19–7.09 (m, 2H), 5.59 (s, 1 H), 4.80–4.71 (m, 1H), 2.18 (s, 3H), 1.40 (d, $J$ = 6.99 Hz, 3H). $[\alpha]_D^{20}$ – 132.6 (*c* 0.1, MeOH). HRMS (ESI): *m/z* = 270.1357 $[M+H]^+$.

**(*R*)-5-methyl-2-((1-phenylethyl)amino)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (***R*-Me-31**). Ethyl 3-oxobutanoate (342 mg, 2.62 mmol, 1.4 equiv.) was used to provide 344 mg (58%) of ***R*-Me-31** after trituration with MeOH-water. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.38–7.33 (m, 2H), 7.27 (t, $J$ = 7.58 Hz, 2H), 7.19–7.14 (m, 1H), 7.11 (d, $J$ = 8.61 Hz, 1H), 5.59 (s, 1H), 4.80–4.71 (m, 1H), 2.18 (s, 3H), 1.40 (d, $J$ = 6.99 Hz, 3H). $[\alpha]_D^{20}$ + 135.2 (*c* 0.1, MeOH). HRMS (ESI): *m/z* = 270.1357 $[M+H]^+$.

**(*R*)-2-((1-(3-Chlorophenyl)ethyl)amino)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (**33**). Ethyl 3-oxobutanoate (92 mg, 0.704 mmol, 1.2 equiv.) was used to provide 27 mg (15%) of **33**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.37 (s, 1H), 7.30–7.21 (m, 2H), 7.17 (dt, $J$ = 6.70,

2.18 Hz, 1H), 7.08 (d, *J* = 8.51 Hz, 1H), 5.52 (s, 1H), 4.65–4.76 (m, 1H), 2.12 (s, 3H), 1.34 (d, *J* = 6.94 Hz, 3H). HRMS (ESI): *m/z* = 304.0967 $[M+H]^+$.

**(*R*)-2-((1-(3-Chlorophenyl)ethyl)amino)-5-ethyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (**34**). Ethyl 3-oxopentanoate (0.106 mL, 0.704 mmol, 1.2 equiv.) was used to provide 11 mg (6%) of **34**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.43 (s, 1H), 7.28–7.35 (m, 2H), 7.26–7.21 (m, 1H), 7.15 (d, *J* = 8.51 Hz, 1H), 5.59 (s, 1H), 4.84–4.68 (m, 1H), 2.48–2.43 (m, 2H), 1.40 (d, *J* = 6.94 Hz, 3H), 1.15 (t, *J* = 7.56 Hz, 3H). HRMS (ESI): *m/z* = 318.1118 $[M+H]^+$.

**(*R*)-2-((1-(3-Chlorophenyl)ethyl)amino)-5,6-dimethyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (**35**). Ethyl 2-methyl-3-oxobutanoate (110 mg, 0.742 mmol, 1.5 equiv.) was used to provide 26 mg (16%) of **35**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.37 (s, 1H), 7.30–7.22 (m, 2H), 7.19–7.15 (m, 1H), 7.08 (d, *J* = 8.56 Hz, 1H), 4.77–4.67 (m, 1H), 2.14 (s, 3H), 1.80 (s, 3H), 1.34 (d, *J* = 6.94 Hz, 3H). $[\alpha]_D^{20}$ + 139.8 (*c* 0.5, MeOH). HRMS (ESI): *m/z* = 318.1119 $[M+H]^+$.

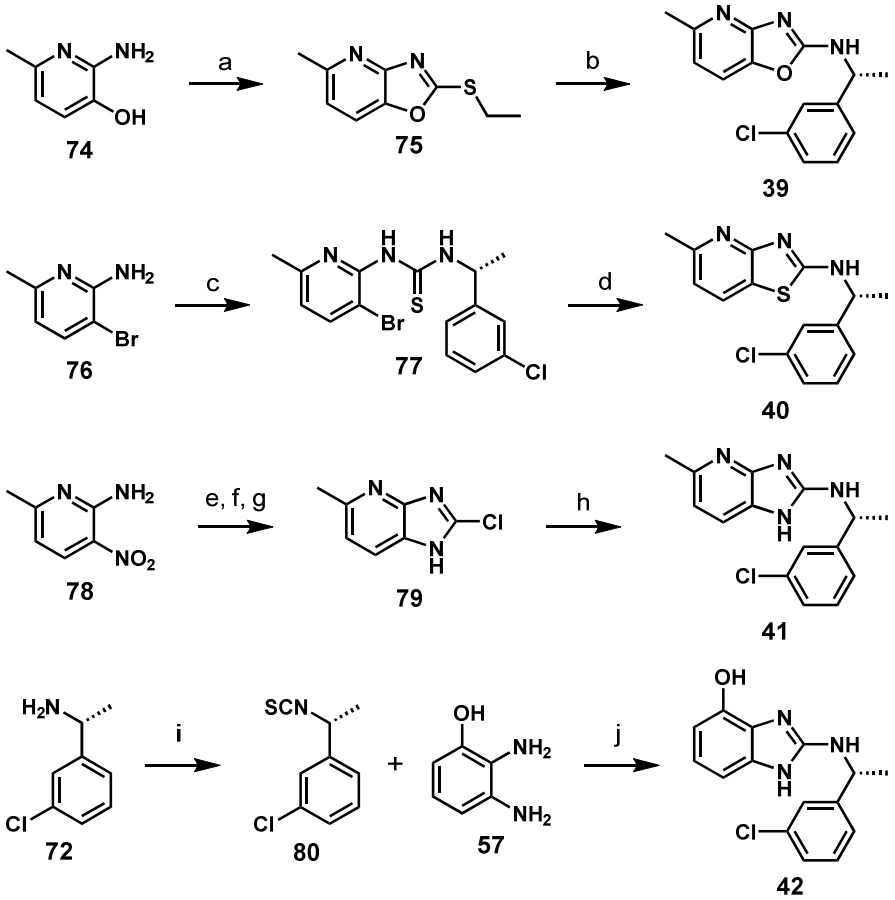
**(*R*)-*N*-(1-(3-Chlorophenyl)ethyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine** (**36**). A solution of NaOEt in EtOH was prepared by dissolving sodium metal (7.35 mg, 0.320 mmol, 0.5 equiv.) in 1.5 mL of absolute EtOH, then **73** (217 mg, 0.639 mmol, 70% purity, 1.0 equiv.) was added in one portion. After stirring at rt for 10 min, a solution of 4,4-dimethoxybutan-2-one (0.094 mL, 0.703 mmol, 1.1 equiv.) in 1 mL of absolute EtOH was added dropwise. The solution took on a light pink color and was allowed to stir at rt overnight. The reaction was quenched with saturated aqueous $NH_4Cl$ until pH ≈ 8, then it was extracted with EtOAc. The combined organic extracts were washed with water and brine, dried with $Na_2SO_4$, filtered and concentrated. The crude material was purified by RP-HPLC to provide 60 mg (33%) of **36**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.78 (d, *J* = 6.75 Hz, 1H), 7.49 (d, *J* = 8.51 Hz, 1H), 7.44 (t, *J* =

1.83 Hz, 1H), 7.36–7.32 (m, 1H), 7.32–7.27 (m, 1H), 7.24–7.20 (m, 1H), 6.85 (d, $J$ = 6.75 Hz, 1H), 4.90–4.75 (m, 1H), 2.45 (s, 3H), 1.42 (d, $J$ = 6.99 Hz, 3H). $[\alpha]_D^{20}$ + 126.2 (*c* 1.0, MeOH). HRMS (ESI): *m/z* = 288.1013 $[M+H]^+$.

**(*R*)-*N*2-(1-(3-Chlorophenyl)ethyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2,7-diamine (37)**. A mixture of **73** (200 mg, 0.589 mmol, 70% purity, 1.0 equiv.) and 3-oxobutanenitrile (48.9 mg, 0.589 mmol, 1.0 equiv.) in 2 mL mesitylene was heated in a microwave reactor at 200 °C for 30 min. LCMS analysis showed only ~50% conversion of the starting material. NMP (0.5 mL) was added to improve the solubility of the starting material, then another 80 μL of 3-oxobutanenitrile and a bit of *p*-TsOH were added. The mixture was heated at 210 °C in a microwave reactor for 30 min to bring the reaction to completion. The mixture was diluted with water, then saturated aqueous $Na_2CO_3$ was added until pH ≈ 8. Extracted with EtOAc, washed the combined EtOAc extracts with water, brine, dried with $Na_2SO_4$, filtered and concentrated. The residue was dissolved in DMSO, filtered and purified by RP-HPLC to provide 31 mg (16%) of **37**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.46 (t, $J$ = 1.79 Hz, 1H), 7.38–7.34 (m, 1H), 7.30 (t, $J$ = 7.73 Hz, 2H), 7.24–7.20 (m, 1H), 7.09 (d, $J$ = 8.95 Hz, 1H), 5.90 (s, 1H), 4.87 (dd, $J$ = 8.75, 7.14 Hz, 1H), 2.23 (s, 3H), 1.42 (d, $J$ = 6.99 Hz, 3H). HRMS (ESI): *m/z* = 303.1128 $[M+H]^+$.

**(*R*)-*N*2-(1-(3-Chlorophenyl)ethyl)-5-methyl-[1,2,4]triazolo[1,5-*a*][1,3,5]triazine-2,7-diamine (38)**. A mixture of **73** (200 mg, 0.589 mmol, 70% of purity, 1.0 equiv.) and ethyl *N*-cyanoacetimidate (0.143 mL, 1.178 mmol, 2.0 equiv.) in 2.5 mL 4:1 DME-NMP was heated in a microwave reactor at 200 °C for 30 min. The mixture was diluted with water and extracted with EtOAc. The combined EtOAc extracts were washed with water and brine, dried with $Na_2SO_4$ and concentrated. The residue was dissolved in DMSO, filtered and purified by RP-HPLC to afford 19 mg (10%) of **38** as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.45 (s, 1H), 7.42–

7.27 (m, 4H), 7.25–7.20 (m, 1H), 4.92–4.81 (m, 1H), 2.29–2.26 (m, 3H), 1.42 (d, $J$ = 6.99 Hz, 3H). HRMS (ESI): $m/z$ = 304.1078 $[M+H]^+$.




(a) i) $EtOCS_2K$, EtOH, 90 °C, 3 h; ii) EtI, $K_2CO_3$, DMF, rt, 5 min, 84%. (b) **72**, MW, 200 °C, 30 min, 62%. (c) i) 1,1'-thiocarbonyldiimidazole, THF, rt, 3 h; ii) **72**, overnight, rt. (d) $Pd_2dba_3$, DPPF, $Cs_2CO_3$, 1,4-dioxane, 85 °C, overnight, 18%. (e) $H_2$ (1 atm), Pd/C, MeOH, 4 h, 100%; (f) CDI, THF, rt, overnight, 92%. (g) $POCl_3$, 95 °C, overnight. (h) **72**, EtOH, 150 °C, 20 min. (i) *i*-$Pr_2NEt$, $SCCl_2$, $CH_2Cl_2$, 0 °C → rt, 20 h, 80%. (j) i) 130 °C, MW, 10 min; ii) EDC, 100 °C, MW, 10 min, 5%.

**2-(Ethylthio)-5-methyloxazolo[4,5-*b*]pyridine** (**75**). A yellow mixture of **74** (248 mg, 1.998 mmol, 1.0 equiv.) and potassium ethyl xanthogenate (640 mg, 4.00 mmol, 2.0 equiv.) in 6 mL EtOH was allowed to heat at 90 °C in a capped vial for 3 h. In process LC-MS showed complete conversion to the desired thiol ($m/z$ 167), so allowed the reaction mixture to cool to rt, then quenched with 2 mL 10% aqueous citric acid, diluted with water and filtered to give 5-

methyloxazolo[4,5-*b*]pyridine-2-thiol as a tan solid, which was dried by pulling air through the filter for 1 h, then it was set directly into the alkylation. The tan solid was suspended in 20 mL DMF, then $K_2CO_3$ (1.38 g, 9.99 mmol, 5.0 equiv.) and ethyl iodide (0.323 mL, 4.00 mmol, 2.0 equiv.) were added sequentially at rt. The reaction mixture was sonicated to break up and distribute some clumps of the thiol in the reaction mixture. In process LC-MS after 5 min showed complete conversion to the desired thioether (*m/z* 195). Diluted the reaction mixture with 80 mL EtOAc, washed three times with water, once with brine, dried with $MgSO_4$, filtered and evaporated to give 325 mg (84%) of crude **75** as a brown solid. MS (ESI): *m/z* = 195.1 $[M+H]^+$.

**(*R*)-*N*-(1-(3-Chlorophenyl)ethyl)-5-methyloxazolo[4,5-*b*]pyridin-2-amine** (**39**). A microwave vial was charged with a neat mixture of **75** (97 mg, 0.499 mmol, 1.0 equiv.) and **72** (155 mg, 0.999 mmol, 2.0 equiv.), then sealed and subjected to microwave irradiation at 200 °C for 30 min. Diluted with $CH_2Cl_2$ and dry loaded on $SiO_2$ before purification by silica gel flash column chromatography (0→10% MeOH/$CH_2Cl_2$). The starting material eluted near the middle of the gradient, followed closely by the desired product that co-eluted with a product whose mass (*m/z* 443) was consistent with the guanidine derived from amine over-addition. Collected all product-containing fractions, dry loaded onto $SiO_2$ and repurified by silica gel flash column chromatography (0→100% EtOAc/heptane) to provide 94 mg (62%) of **39** as a colorless foam. $^1$H NMR (500 MHz, $CDCl_3$) δ 7.40 (t, *J* = 1.8 Hz, 1H), 7.34–7.26 (m, 4H), 6.78 (d, *J* = 8.0 Hz, 1H), 5.49 (d, *J* = 6.2 Hz, 1H), 5.11 (p, *J* = 6.9 Hz, 1H), 2.54 (s, 3H), 1.65 (d, *J* = 6.9 Hz, 3H). HRMS (ESI): *m/z* = 288.0907 $[M+H]^+$.

**(*R*)-1-(3-Bromo-6-methylpyridin-2-yl)-3-(1-(3-chlorophenyl)ethyl)thiourea** (**77**). A brown solution of 1,1'-thiocarbonyldiimidazole (214 mg, 1.200 mmol, 1.2 equiv.) in 3 mL $CH_2Cl_2$ was

allowed to cool to 0 °C, then it was treated with **76** (187 mg, 1.000 mmol, 1.0 equiv.) in one portion. The brown reaction mixture was allowed to allowed to slowly warm to rt and stir at that temperature overnight. **72** (156 mg, 1.000 mmol, 1.0 equiv.) was then added and the resulting brown solution was allowed to stir at rt for 1 h. In process LC-MS showed essentially complete conversion to the desired thiourea (*m/z* 384/6) along with some undesired symmetrical thioureas from each amine component. Diluted with $CH_2Cl_2$, dry loaded onto $SiO_2$ and purified by silica gel flash column chromatography (0→25% EtOAc/heptane). The product eluted near the middle of the gradient. Obtained 315 mg (78%) of **77** as a colorless, feathery solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 7.99 (s, 1H), 7.72 (d, *J* = 8.6 Hz, 1H), 7.39 (s, 1H), 7.33–7.27 (m, 4H), 6.40 (d, *J* = 8.6 Hz, 1H), 5.57 (p, *J* = 6.9 Hz, 1H), 2.55 (s, 3H), 1.66 (d, *J* = 6.9 Hz, 3H). MS (ESI): *m/z* = 384.1 $[M+H]^+$.

**(*R*)-*N*-(1-(3-Chlorophenyl)ethyl)-5-methylthiazolo[4,5-*b*]pyridin-2-amine** (**40**). A mixture of $Pd_2dba_3$ (83 mg, 0.091 mmol, 0.5 equiv.), DPPF (111 mg, 0.200 mmol, 1.1 equiv.) and $Cs_2CO_3$ (119 mg, 0.364 mmol, 2.0 equiv.) under nitrogen was diluted with 1 mL dioxane and allowed to stir at rt for 5 min before **77** (70 mg, 0.182 mmol, 1.0 equiv.) was added. The vial was placed under a nitrogen atmosphere, then capped and the dark red mixture was allowed to stir well at 85 °C overnight. In process LC-MS showed conversion to the desired mass (*m/z* 304). Diluted with $CH_2Cl_2$, dry loaded onto $SiO_2$ and purified by silica gel flash column chromatography (0→100% EtOAc/heptane). The desired product eluted near the middle of the gradient and was obtained as an orange oil that contained some impurities. Diluted with $CH_2Cl_2$, dry loaded onto $SiO_2$ and repurified by silica gel flash column chromatography (0→75% EtOAc/heptane). Obtained 10 mg (18%) of **40** as a yellow solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 7.70 (d, *J* = 7.9 Hz, 1H), 7.40

(s, 1H), 7.34–7.27 (m, 3H), 6.86 (d, $J$ = 7.9 Hz, 1H), 5.69 (d, $J$ = 4.8 Hz, 1H), 5.10 (p, $J$ = 6.7 Hz, 1H), 2.57 (s, 3H), 1.65 (d, $J$ = 6.8 Hz, 3H). HRMS (ESI): $m/z$ = 304.0677 $[M+H]^+$.

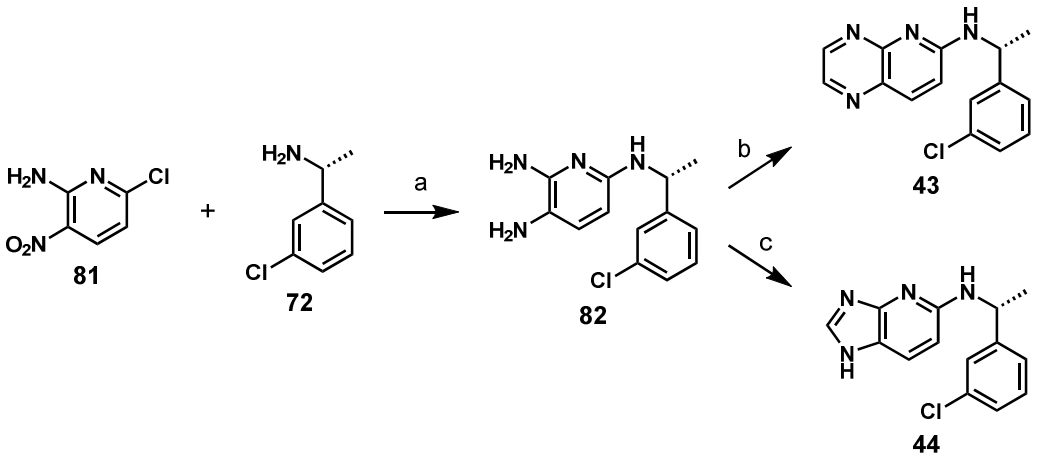
**2-Chloro-5-methyl-1*H*-imidazo[4,5-*b*]pyridine** (**79**). Step 1: A solution of **78** (15 g, 98 mmol, 1.0 equiv.) in 90 mL MeOH was flushed with nitrogen, then 10% Pd/C (0.75 g, 7.05 mmol, 0.07 equiv.) was added. The black reaction mixture was flushed with hydrogen, then the mixture was stirred at rt overnight under a balloon of hydrogen. LCMS analysis only showed starting material, so the mixture was flushed with nitrogen and more 10% Pd/C (0.75 g, 7.05 mmol, 0.07 equiv.) was added. The mixture was flushed with hydrogen again and then stirred at rt under a hydrogen atmosphere for 4 h. LCMS indicated the reaction was not complete, so it was left to stir overnight with the reaction flask wrapped with aluminum foil. LCMS analysis revealed that the reaction was complete and clean. The reaction was filtered through Celite® and the filtrate was evaporated under reduced pressure to provide 12.7 g (quant.) of 6-methylpyridine-2,3-diamine as a brown, sticky oil. The material got darker with time. Step 2: A mixture of 6-methylpyridine-2,3-diamine (12.1 g, 98 mmol, 1.0 equiv.) and carbonyldiimidazole (18.48 g, 114 mmol, 1.16 equiv.) in 50 mL THF was stirred at rt overnight. The solid that precipitated was isolated by filtration, washed with THF, and dried under vacuum at 50 °C. Obtained 16.6272 g (92%) of 5-methyl-1*H*-imidazo[4,5-*b*]pyridin-2(3*H*)-one that was 81% pure (contaminated with imidazole). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 11.11 (br s, 1 H), 10.63 (s, 1 H), 7.08 (d, $J$ = 7.78 Hz, 1H), 6.76 (d, $J$ = 7.78 Hz, 1H), 2.34 (s, 3H). MS (ESI): $m/z$ = 150.0 $[M+H]^+$. Step 3: A mixture of 5-methyl-1*H*-imidazo[4,5-*b*]pyridin-2(3*H*)-one (2.13 g, 11.57 mmol, 81% purity, 1.0 equiv.) in $POCl_3$ (10.78 mL, 116 mmol, 10.0 equiv.) was stirred at 95 °C overnight. The reaction solution was chilled in an ice bath, then diluted with ice water and neutralized with 6 N NaOH until pH ≈ 7. A sticky yellow oil precipitated from the black aqueous solution. Extracted

with EtOAc, then washed the combined EtOAc extracts with water and brine, dried over $Na_2SO_4$, filtered, and concentrated to afford 376 mg (19%) of **79**. The balance of the desired product remained in the aqueous layer. MS (ESI): *m/z* = 168.0 $[M+H]^+$.

**(*R*)-*N*-(1-(3-Chlorophenyl)ethyl)-5-methyl-1*H*-imidazo[4,5-*b*]pyridin-2-amine** (**41**). A mixture of **79** (100 mg, 0.597 mmol, 1.0 equiv.) in 1 mL EtOH was treated with **72** (186 mg, 1.193 mmol, 2.0 equiv.), and the resulting mixture was heated in a microwave reactor at 150 °C for 20 min. The mixture was concentrated and the residue was purified by RP-HPLC. The product-containing fractions were passed through Agilent StratoSpheres™ PL-$HCO_3$ MP SPE resin to remove TFA. Obtained 2 mg (1%) of **41**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.41 (br s, 1H), 7.34–7.25 (m, 2H), 7.20 (d, *J* = 7.14 Hz, 2H), 4.89 (t, *J* = 7.51 Hz, 1H), 2.30 (d, *J* = 10.03 Hz, 4H), 1.40 (br s, 3H). MS (ESI): *m/z* = 287.1 $[M+H]^+$.

**(*R*)-1-Chloro-3-(1-isothiocyanatoethyl)benzene** (**80**). DIPEA (5.39 mL, 30.8 mmol, 2.4 equiv.) was added to a stirring mixture of **72** (2 g, 12.85 mmol, 1.0 equiv.) in 30 mL $CH_2Cl_2$ under nitrogen. After 15 min of stirring, the reaction mixture was cooled to 0 °C in an ice bath and a solution of thiophosgene (1.625 g, 14.14 mmol, 1.1 equiv.) in 5 mL $CH_2Cl_2$ was added dropwise. The mixture was stirred at 0°C for 30 min and at rt overnight. More DIPEA (3 mL) was added and the resulting mixture was stirred for a further 2 h. The mixture was washed twice with water, once with 1 N HCl, and again with water. The organic layer was dried with $Na_2SO_4$, filtered and the solvent was evaporated. The residue was then co-evaporated with toluene and purified by silica gel flash column chromatography (15% $CH_2Cl_2$/heptane) to give 2.024 g (80%) of **80** as a yellow oil.

**(*R*)-2-((1-(3-Chlorophenyl)ethyl)amino)-1*H*-benzo[*d*]imidazol-4-ol** (**42**). To a microwave vial that contained a solution of **80** (63.7 mg, 0.322 mmol, 1.0 equiv.) in 1.3 mL DMF was added **57** (40 mg, 0.322 mmol, 1.0 equiv.). The mixture was stirred at 130 °C for 10 min in the microwave, then kept at rt overnight. EDC·HCl (100 mg, 0.522 mmol, 1.62 equiv.) was added, the vial was resealed and heated at 100 °C in the microwave for 10 min. The reaction mixture was cooled to rt, diluted with 20 mL water, and extracted 3 x 40 mL EtOAc. The combined organic extracts were concentrated, dissolved in DMSO, filtered and purified by RP-HPLC to provide 5 mg (5%) of **42**. $^1$H NMR (400 MHz, Methanol-$d_4$) δ 7.47–7.43 (m, 1H), 7.38–7.29 (m, 2H), 7.27–7.22 (m, 1H), 6.84 (t, $J$ = 8.0 Hz, 1H), 6.76–6.72 (m, 1H), 6.49 (dd, $J$ = 8.0, 1.1 Hz, 1H), 4.95 (q, $J$ = 6.9 Hz, 1H), 1.58 (d, $J$ = 6.9 Hz, 3H). MS (ESI): $m/z$ = 288.1 $[M+H]^+$.




(a) i) EtOH, 100 °C, 3 h; ii) Zn, aq. $NH_4Cl$, THF, rt, 20 min. (b) glyoxal, THF, rt, 1 h, 48%. (c) $HC(OCH_3)_3$, 100 °C, 18 h, 18%.

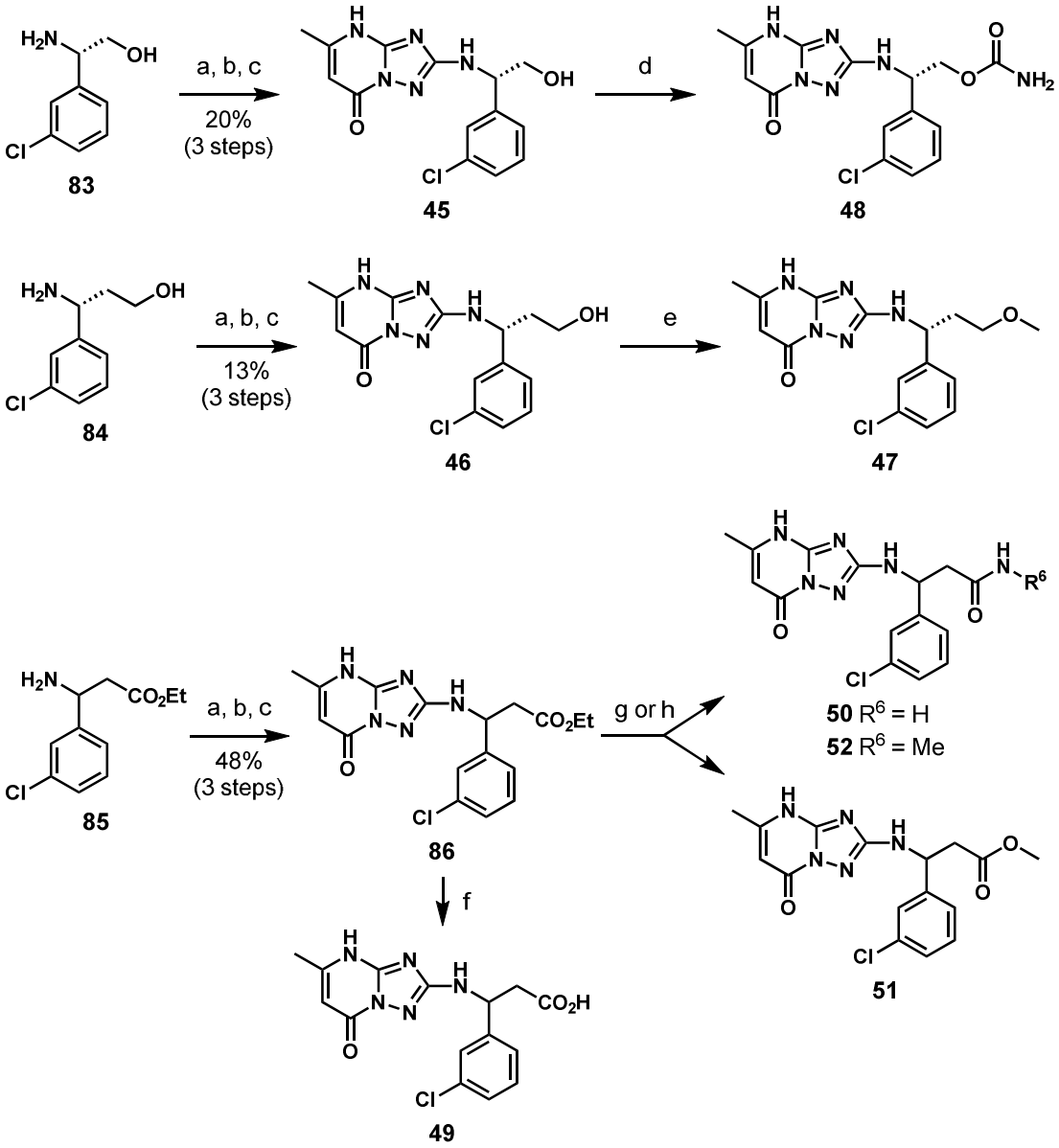
**(*R*)-*N*6-(1-(3-Chlorophenyl)ethyl)pyridine-2,3,6-triamine** (**82**). A yellow suspension of **81** (174 mg, 1.003 mmol, 1.0 equiv.) and **72** (311 mg, 2.006 mmol, 2.0 equiv.) in 1 mL EtOH was allowed to stir in a capped vial at 100 °C for 3 h, during which time dissolution occurred. In process LC-MS showed near complete conversion to the desired $S_NAr$ product ($m/z$ 293).

Allowed the reaction mixture to cool to rt, then diluted with 3 mL saturated $NH_4Cl$ and 2 mL THF before zinc powder (328 mg, 5.01 mmol, 5.0 equiv.) was added portionwise with efficient stirring (heat generation and gas evolution). In process LC-MS of the resulting green, biphasic mixture after 5 min at rt showed near complete reduction to the diamine (*m/z* 263). Allowed the biphasic mixture to stir at rt 15 min more, during which time it took on an indigo color. Filtered the reaction mixture through Celite®, and rinsed the filter cake well with EtOAc. Removed the aqueous layer, then washed the organic layer once each with water and brine. Dried with $MgSO_4$, filtered and evaporated to give 300 mg (115%) of crude **82** as an indigo foam. Took on without further purification.

**(*R*)-*N*-(1-(3-Chlorophenyl)ethyl)pyrido[2,3-*b*]pyrazin-6-amine** (**43**). An indigo solution of **82** (75 mg, 0.285 mmol, 1.0 equiv.) in 1 mL THF was treated with 40% glyoxal in water (104 mg, 0.714 mmol, 2.5 equiv.) and allowed to stir in a capped vial at rt for 1 h. In process LC-MS showed near complete conversion to the desired pyridopyrazine (*m/z* 285). Diluted with $CH_2Cl_2$, dry loaded onto $SiO_2$ and purified by silica gel flash column chromatography (0→100% EtOAc/heptane). The product eluted near the end of the gradient. Obtained 41 mg (48%) of **43** as a tan solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.75 (d, *J* = 2.0 Hz, 1H), 8.53 (d, *J* = 2.0 Hz, 1H), 8.02 (d, *J* = 9.0 Hz, 1H), 7.42 (s, 1H), 7.34 (d, *J* = 7.5 Hz, 1H), 7.31–7.21 (m, 2H), 6.85 (d, *J* = 9.1 Hz, 1H), 5.49 (s, 1H), 5.37 (s, 1H), 1.64 (d, *J* = 6.7 Hz, 3H). HRMS (ESI): *m/z* = 285.0910 $[M+H]^+$.

**(*R*)-*N*-(1-(3-Chlorophenyl)ethyl)-1*H*-imidazo[4,5-*b*]pyridin-5-amine** (**44**). An indigo solution of **82** (75 mg, 0.285 mmol, 1.0 equiv.) in 1 mL trimethylorthoformate was allowed to stir in a capped vial at 100 °C for 1 h. In process LC-MS showed near complete conversion to the desired benzimidazole (*m/z* 273). Diluted with $CH_2Cl_2$, dry loaded onto $SiO_2$ and purified by

silica gel flash column chromatography (0→100% EtOAc/heptane). The product eluted with 100% EtOAc. Obtained 15 mg (18%) of **44** as a tan solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.79–7.68 (m, 1H), 7.38 (s, 1H), 7.25–7.18 (m, 4H), 6.28 (d, *J* = 8.7 Hz, 1H), 4.92 (p, *J* = 6.7 Hz, 1H), 4.81 (s, 1H), 1.57 (s, 3H). HRMS (ESI): *m/z* = 273.0909 $[M+H]^+$.




(a) Diphenyl *N*-cyanocarbonimidate, 2-propanol, 60 °C, 0.75–2.5 h.; b) $N_2H_4 \cdot H_2O$, 50 °C, 1–4 h. (c) ethyl acetoacetate, AcOH, reflux or 165 °C, MW, 10–20 min. (d) KOCN, TFA, $CH_2Cl_2$, rt, 19%. (e) i) MsCl, $Et_3N$, THF, –40 °C → rt, then rt, 3 h; ii) NaOMe, DMSO-MeOH (10:1), rt, 2

h, 13%. (f) LiOH, THF-EtOH-$H_2O$ (3:1:1), rt, overnight, 97%. (g) $NH_3$, MeOH, rt → 50 °C; **50**: 19%, **51**: 23%. (h) $MeNH_2$, MeOH, 50 °C; **52**: 10%, **51**: 20%.

**(*S*)-2-((1-(3-Chlorophenyl)-2-hydroxyethyl)amino)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (**45**). Diphenyl *N*-cyanocarbonimidate (0.336 g, 1.412 mmol, 1.3 equiv.) was suspended in 10 mL 2-propanol and treated with **83**·HCl (0.226 g, 1.086 mmol, 1.0 equiv.), then $Et_3N$ (0.151 mL, 1.086 mmol, 1.0 equiv.) was added and the mixture was heated at 60 °C for 2.5 h. After cooling to rt, hydrazine hydrate (0.169 mL, 5.43 mmol, 5.0 equiv.) was added and the mixture was heated at 50 °C for 1 h, then stirred at rt overnight. The mixture was concentrated to an oil, which was dissolved in 2.4 mL AcOH and treated with ethyl acetoacetate (0.161 mL, 1.272 mmol, 1.2 equiv.) prior to heating in the microwave for 10 minutes at 165 °C. The mixture was concentrated and the residue was diluted with DMSO, filtered and purified by RP-HPLC to give 68 mg (20%) of **45**, as well as the corresponding acetate ester (44 mg, 11%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.67 (br s, 1H), 7.42 (s, 1H), 7.34–7.30 (m, 2H), 7.27–7.23 (m, 1H), 7.01 (d, $J$ = 8.51 Hz, 1H), 5.60 (s, 1H), 4.67 (q, $J$ = 6.88 Hz, 1H), 3.59 (qd, $J$ = 10.97, 6.41 Hz, 2H), 2.19 (s, 3H). HRMS (ESI): $m/z$ = 320.0913 $[M+H]^+$.

**(*S*)-2-(3-Chlorophenyl)-2-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)ethyl carbamate** (**48**). A suspension of **45** (0.0323 g, 0.101 mmol, 1.0 equiv.) in 15 mL $CH_2Cl_2$ was treated with potassium cyanate (0.016 g, 0.202 mmol, 2.0 equiv.), then a solution of TFA (0.016 mL, 0.212 mmol, 2.1 equiv.) in 5 mL $CH_2Cl_2$ was added to the reaction mixture dropwise via pipette over 8 min. The resulting solution was stirred at rt overnight, then more potassium cyanate (0.050 g, 0.616 mmol, 6.1 equiv.) was added. The mixture was concentrated to give a solid that was dissolved in DMSO, filtered and purified by RP-HPLC to provide 7 mg (19%) of **48**. HRMS (ESI): $m/z$ = 363.0970 $[M+H]^+$.

**(*R*)-2-((1-(3-Chlorophenyl)-3-hydroxypropyl)amino)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (**46**). Diphenyl *N*-cyanocarbonimidate (0.30 g, 1.259 mmol, 1.2 equiv.) was suspended in 7 mL 2-propanol and treated with **84** (0.195 g, 1.049 mmol, 1.0 equiv.), then heated at 60 °C for 1.75 h and stirred at rt overnight. The mixture was stripped to an oil and dissolved in 5 mL MeOH, treated with hydrazine hydrate (0.065 mL, 2.098 mmol, 2.0 equiv.), then heated at 50 °C for 2.25 h and stirred over the weekend at rt. The mixture was concentrated to an oil that solidified upon standing at rt. A portion of the crude triazole (0.148 g, 0.553 mmol, 1.0 equiv.) was dissolved in 1 mL AcOH and treated with ethyl acetoacetate (0.085 mL, 0.663 mmol, 1.2 equiv.), then heated in the microwave at 165 °C for 10 min to give a mixture of the desired product along with the corresponding acetate ester. The mixture was concentrated to give an oil that was dissolved in DMSO, filtered and purified by RP-HPLC to provide 24 mg (13%) of **46**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.65 (s, 1H), 7.43–7.40 (m, 1H), 7.34–7.30 (m, 2H), 7.28–7.22 (m, 1H), 7.22–7.17 (m, 1H), 5.61 (s, 1H), 4.86–4.72 (m, 1H), 2.02–1.87 (m, 1H), 1.84–1.71 (m, 1H). HRMS (ESI): *m/z* = 334.1068 $[M+H]^+$.

**(*R*)-2-((1-(3-Chlorophenyl)-3-methoxypropyl)amino)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(4*H*)-one** (**47**). Step 1: A solution of **46** (0.0572 g, 0.171 mmol, 1.0 equiv.) in 5 mL THF was cooled to -40 °C before methanesulfonyl chloride (0.016 mL, 0.206 mmol, 1.2 equiv.) was added dropwise. Triethylamine (0.053 mL, 0.377 mmol, 2.2 equiv.) was added and the reaction was allowed to warm to rt and stir at that temperature for 3 h. The mixture was diluted with 100 mL EtOAc and washed 2 x 40 mL water. The EtOAc layer was dried over $MgSO_4$, filtered and concentrated to give a yellow oil that was stored in the freezer. LCMS showed the desired mesylate [(*R*)-3-(3-chlorophenyl)-3-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)propyl methanesulfonate], as well as the presence of

an equimolar amount of the terminal olefin product resulting from mesylate elimination. MS (ESI): *m/z* = 412.1 $[M+H]^+$. Step 2: A solution of the mesylate from above (0.037 g, 0.090 mmol, 1.0 equiv.) in 1 mL DMSO was treated with a freshly prepared solution of 25% NaOMe in MeOH (0.1 ml, 0.416 mmol, 4.6 equiv.) and stirred at rt for 2 h. The solution was filtered and purified by RP-HPLC to give 4 mg (13%) of **47**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.66 (br s, 1H), 7.46–7.38 (m, 1H), 7.36–7.28 (m, 2H), 7.28–7.19 (m, 2H), 5.61 (s, 1H), 4.79–4.70 (m, 1H), 3.21 (s, 3H), 2.06–1.94 (m, 1H), 1.92–1.80 (m, 1H). MS (ESI): *m/z* = 348.1 $[M+H]^+$.

**Ethyl 3-(3-chlorophenyl)-3-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)propanoate** (**86**). A suspension of diphenyl *N*-cyanocarbonimidate (0.67 g, 2.81 mmol, 1.4 equiv.) in 10 mL EtOH was treated with **85**·HCl (0.531 g, 2.01 mmol, 1.0 equiv.) and $Et_3N$ (0.280 mL, 2.01 mmol, 1.0 equiv.), then heated at 60 °C for 2 h and stirred at rt overnight. The mixture was concentrated to an oil that was suspended in 5 mL EtOH, treated with hydrazine hydrate (0.069 mL, 2.219 mmol, 1.1 equiv.) and heated at 50 °C for 1.25 h, then stirred at rt overnight. The entire mixture was filtered sequentially through four 2 g $SiO_2$ plugs, washing with 10% EtOH/$CH_2Cl_2$. The 4 plugs were then washed with 50 mL 1:1:3 (2 M $NH_3$ in EtOH)-EtOH-$CH_2Cl_2$ and then with 2 M $NH_3$ in EtOH. The combined filtrates were concentrated to an oil (0.43 g, 1.388 mmol, 1.0 equiv.) that was dissolved in 4 mL AcOH, treated with ethyl acetoacetate (0.213 mL, 1.666 mmol, 1.2 equiv.) and heated to reflux. The mixture was concentrated and the crude residue was dissolved in DMSO, filtered and purified by RP-HPLC to give 100 mg (19%) of **86**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.71 (br s, 1H), 7.49–7.45 (m, 1H), 7.39–7.33 (m, 2H), 7.32–7.26 (m, 2H), 5.63 (s, 1H), 5.12–5.04 (m, 1H), 4.02 (q, *J* = 7.1 Hz, 2H), 2.88 (dd, *J* = 15.4, 8.6 Hz, 1H), 2.76 (dd, *J* = 15.4, 6.5 Hz, 1H), 2.20 (d, *J* = 0.9

Hz, 3H), 1.10 (t, $J$ = 7.1 Hz, 3H). MS (ESI): $m/z$ = 376.0 $[M+H]^+$. The corresponding carboxylic acid (**49**) (75 mg, 15%) was also isolated.

**3-(3-Chlorophenyl)-3-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)propanoic acid** (**49**). A solution of **86** (0.05 g, 0.133 mmol, 1.0 equiv.) in 5 mL 3:1:1 THF-EtOH-water was treated with $LiOH \cdot H_2O$ (0.033 g, 0.798 mmol, 6.0 equiv.) and the mixture was stirred at rt overnight. The mixture was concentrated, then 2 mL water was added, followed by 6 M HCl to produce a well-defined white solid. The solid was collected by filtration, washed with water and air-dried on the funnel for two days to provide 45 mg (97%) of **49**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.70 (s, 1H), 7.47–7.44 (m, 1H), 7.39–7.31 (m, 2H), 7.30–7.25 (m, 2H), 5.63 (s, 1H), 5.10–5.01 (m, 1H), 2.79 (dd, $J$ = 15.7, 8.6 Hz, 1H), 2.71–2.64 (m, 1H), 2.20 (s, 3H). HRMS (ESI): $m/z$ = 348.0865 $[M+H]^+$.

**3-(3-Chlorophenyl)-3-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)propanamide** (**50**) and **methyl 3-(3-chlorophenyl)-3-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)propanoate** (**51**). A solution of **86** (0.110 g, 0.293 mmol, 1.0 equiv.) in 2.0 M $NH_3$ in EtOH (3.0 mL, 6.00 mmol, 20.5 equiv.) was stirred overnight at rt, then heated at 50 °C for 24 h. More 2.0 M $NH_3$ in EtOH (1.0 mL, 2.00 mmol, 6.83 equiv.) was added and stirring was continued for 3 days. 7 N $NH_3$ in MeOH (1.2 mL, 8.4 mmol, 28.67 equiv.) was added and the mixture was stirred overnight then concentrated. The residue was dissolved in 7 N $NH_3$ in MeOH (3 mL, 21 mmol, 71.67 equiv.) to give a pink solution that was stirred at rt. Concentrated, dissolved the residue in DMSO, filtered and purified by RP-HPLC to give 19 mg (19%) of **50**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.69 (br s, 1H), 7.43 (s, 1H), 7.36–7.29 (m, 3H), 7.27–7.22 (m, 2H), 6.82 (br s, 1H), 5.62 (s, 1H), 5.13–5.02 (m, 1H), 2.60 (dd, $J$ = 14.4, 7.6 Hz, 1H), 2.20 (s, 3H). HRMS (ESI): $m/z$ = 347.1022 $[M+H]^+$. Methyl ester **51** (25 mg,

23%) was also obtained. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.69 (s, 1H), 7.45 (s, 1H), 7.36–7.31 (m, 2H), 7.31–7.24 (m, 2H), 5.61 (s, 1H), 5.08 (td, $J$ = 8.91, 6.48 Hz, 1H), 3.55 (s, 3H), 2.91–2.73 (m, 2H), 2.19 (s, 3H). HRMS (ESI): $m/z$ = 362.1021 $[M+H]^+$.

**3-(3-Chlorophenyl)-*N*-methyl-3-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)propanamide** (**52**) and **methyl 3-(3-chlorophenyl)-3-((5-methyl-7-oxo-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)amino)propanoate** (**51**). A solution of **86** (0.051 g, 0.136 mmol, 1.0 equiv.) in 2.5 mL MeOH was treated with 2.0 M $MeNH_2$ in MeOH (0.5 mL, 1.000 mmol, 7.4 equiv.) and stirred at 50 °C for 41 h, then more 2.0 M $MeNH_2$ in MeOH (0.5 mL, 1.000 mmol, 7.4 equiv.) was added and heating was resumed. The reaction mixture was concentrated to a dark yellow oil that was dissolved in DMSO, filtered and purified by RP-HPLC to give 5 mg (10%) of **52**. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.69 (s, 1H), 7.80–7.74 (m, 1H), 7.45–7.40 (m, 1H), 7.31 (d, $J$ = 5.0 Hz, 2H), 7.27–7.21 (m, 2H), 5.62 (s, 1H), 5.14–5.04 (m, 1H), 2.60 (dd, $J$ = 14.5, 7.5 Hz, 1H), 2.20 (s, 3H). HRMS (ESI): $m/z$ = 361.1179 $[M+H]^+$. Methyl ester **51** (10 mg, 20%) was also obtained.

**Crystallographic methods**

Crystallization of PPAT

The full length *E. coli* phosphopantetheine adenylyltransferase (PPAT, CoaD) was overexpressed in *E.coli* and purified as described.[1] The apo crystals were grown by the vapor diffusion method at 291K in hanging drops by incubating equal volumes of protein with a well solution of 1.3 M ammonium sulfate, 0.2 M potassium thiocyanate, and 0.2 M potassium bromide. The crystals were soaked overnight in cryosolution of 20 mM Tris pH 7.5, 40% PEG400, and 5 mM compound.

Data processing and refinement

Data were collected at beam-lines 5.0.1 and 5.0.2 of the Advanced Light Source (Berkeley, CA) and processed with autoPROC.[2] Structures were solved by molecular replacement using PHASER[3] and refined with BUSTER.[4] All models were built with COOT.[5]

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