## **Electronic Supplementary Information for**

# PMP-diketopiperazine adducts form at the active site of a PLP dependent enzyme involved in formycin biosynthesis.

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# Supplementary figures



Figure S1 SEC-MALS chromatograph showing elution of the protein from gel filtration column. Changes in the refractive index (blue) show protein-mediated scattering of light. The root-mean-square radius (orange) allows estimation of the molecular size, Table S1.

3K28	MRKFDKSIAAFEEAQDLMPGGVNSPVRAFKSVGMNPLFMERGKGSKVYDIDGNEYI	56
ForI	MITEKSAALYARAVEVMPGGNSRTAVYSSPYPVYVRSGSGARVTDVDGVERI	52
GSAAT	VTSSPFKTIKSDEIFAAAOKLMPGGVSSPVRAFKSVGGOPIVFDRVKDAYAWDVDGNRYI	60
Mt.TlvE	~ ~	0
NeoB	MTKNSSI.I.AFFDTCDBDFKDBDBUFTAASCAWI.TDFSCFBWT	42
NCOD		72
27720		114
3K28	DYVLSWGPLIHGHANDRVVEALKAVAERGTSFGAPTEIENKLAKLVIERVPSIEIVRM	114
ForI	DFLNNSTTLIHGHAHPEMVEAIAAAVGHGTSFGMPTPVEVEYAEALSARNETFEHVRF	110
GSAAT	DYVGTWGPAICGHAHPEVIEALKVAMEKGTSFGAPCALENVLAEMVNDAVPSIEMVRF	118
MtIlvE	MTSGSLQFTVLRA	13
NeoB	DFDNARGSILLGHGDPVVAEAVARAATGADGTATGWSRRVDAVLERLHALCGGEVVGL	100
3K28	VNSGTEATMSALRLARGYTGRNKILKFICCYHGHGDSLLIKAGSGVATLGLPDSPGVPEG	174
ForT	TSSCREAVMMAVOAARAYTERPKTAKTACAYHCAYDAVAVNND-GSGNLTSHAVTGNPEG	169
CSAAT	UNS CHEACMAUL DI MDAVTCDDKT I KEECCYHCHADMEI UKACSCUATI CI DSSDCUDKK	178
MATINE		62
MULIVE		02
Neob	FRSEMAAVRAAVLAVREATGRPLLLSAGYIIGYDPMWYPSEABLE	144
3K28	VAKNTITVAYNDLESVKYAFEQFGDDIACVIVEPVAGNM-GVVPPQPGFLE	224
ForI	VVANTVVIPFNDPEGSLEVLRRHADDLACVLIDPVPWRI-GLLPASKEWLD	219
GSAAT	TTANTLTTPYNDLEAVKALFAENPGEIAGVILEPIVGNS-GFIVPDAGFLE	228
MtIlvE	LDPSAIVLHYAOEVFEGLKA-YRWADGSIVSFRADANAARLRSSARRLAIPELPDAVFIE	121
NeoB	PNADGWVDFFFDLGLLRE-LLRAPERWAAVVWSPDHMHLSPGWYR	188
		200
3828		269
JK20		209
FOIL	AUREFCDASGAVIISDBV-GSYRVGYHGAMQLLG4EADITVMGKV	203
GSAAT	GUREITLEHDALLVFDEVMTGFRIAYGGVQEKFGVTPDLTTLGKI	273
MtIlvE	SURQLIAVDKAWVPGAGGEEALYLRPFIFATDPGLGVRPATQYRYLLIASPAGAY	176
NeoB	EDRRLCSAAGWVIIVADEVKVGLRYAPGLSTA-EIIIAPDVWVVIKG	232
3K28	IGGGL-PVGAYGGKAEIMRQVAPSGPIYQAGTLSGNPLAMAAGYETLVQLT-P	320
ForI	IAACM-PIGAVAGRRRFMSVFDPSKGKPALPHSGSYNANPVSMSSGIASLRLLT-P	317
GSAAT	IGGCL-PVGAYGGKREIMOLVAPAGPMYOAGTLSGNPLAMTAGIKTLELLROP	325
MtIlvE	FKGCIAPVSVW-VSTEYVRAC-P-GGTGAAKFGGNYAASLLAOAEAAENGCDOVVWLD-A	232
NeoB	MANCH-AVSAVCGSRRLLKPLKEVSFT-SFFEPTILAAADAAT ARWATG	279
RCOD		275
3838		360
JAZO		209
FOLL	QAHERIGQLGEQARGSMRTALGEAGLDWEVNGLGSLFRVVANS-APGI	202
GSAAT	GTYEYLDQ1TKRLSDGLLAIAQETGHAACGGQVSGMFGFFFTE-GPVHNY	3/4
MtIlvE	VERRYIEESGGMNIFFVLGSGGSARLVTPELSGSLL	268
NeoB	EPQRAVREAGDRFLRHARKALDDASLPVEIAGDGTFFQFVPAT-EE	330
3K28	DA-AKSSNLQFFAAYYREMVEQGVFLPPSQFEGLFLSTVHSD	410
ForI	DS-AAAAMKALYWKLLENGIHIGDSGLGCISTPMGE	400
GSAAT	ED-AKKSDLOKFSRFHRGMLEOGTYLAPSOFEAGFTSLAHTE	415
M+T1vE	PGTTRDSLLOLATDAGFAVEBREIDTDEWOKKAAAGETTEVFACCTAAVTTPVARVRHGA	328
NooP		250
NEOP	GR-ANAEGLLF IAGDNQGWSAMT DEAVLGE	339
27220		0
3K28	AUTEKA 42	.9
ForI	EEIAEYAVAFAKSLGQV-LAEGRA 42	23
GSAAT	EDID 43	32
MtIlvE	SEFRIADGQPGEVTMALRDTLTGIQRGTFADTHGWMARLG 36	58
NeoB	A RFARVCERLAPYAGGEPVGDAARYRVAWNVMDGLR-QAPRDREETTGLLARLLDD 41	L6

Figure S2 Sequence based alignment of Forl with 3K28, GSAAT, *Mt*Ilve and NeoB. Forl shows 30 %, 10 % and 24 % sequence identity with GSAAT, NeoB and *Mt*Ilve, respectively. 3K28 shows 50 % sequence similarity with Forl.



Figure S3 Structural analysis of active site residues of Forl (slate) and GSAAT (orange). (a) There is a  $\alpha$ -helix that closes up the active site in GSAAT. While in Forl, the loop flips away opening the active site. PLP in Forl is colored in yellow and PMP in GSAAT colored in orange. (b) Met 248 in active site of GSAAT takes more space than Gly 238 in Forl. Met 248 from GSAAT in orange, Gly 238 in yellow. (c) The loop containing functionally important residue Glu 406 is close to the co-factor in GSAAT but kinks away from the active site in Forl.



Figure S4 UV spectrum of ForI upon incubation with L-Glu. The decrease in intensity at the wavelength of 400 nm corresponding to the consumption of PLP and increase at the wavelength of 330 nm corresponding to the accumulation of PMP.



Figure S5 Active site structural analysis of ForI (a) L-configured diketopiperazine adduct hydrogen bonds with water molecules. (b) When GSAAT (PDB: 3GSB) and ForI are superimposed, Arg 23 of ForI would clash with gabaculing. (c) Ser 163, Asn 217, Glu 406 of GSAAT would block diketopiperazine binding at its active site. Residues from GSAAT are shown in orange and those from ForI are shown in yellow, with oxygen atoms in red and nitrogen atoms in blue.



Figure S6 Structure analysis of NeoB (PDB: 6CBL) (pale orange) compared with ForI-LCS complex (slate). (a) Structure alignment of NeoB and ForI-LCS complex shows a high structural similarity with r.m.s.d. = 1.94 Å over 337 residues. (b) The six-membered carbohydrate ring of neosamine attached to the cofactor, sits in the catalytic pocket of ForI, while the other ring clashes with Asn 152 of ForI. Residues from NeoB are shown in pale orange and those from ForI are shown in yellow, with oxygen atoms in red and nitrogen atoms in blue.



Figure S7 SDS-PAGE analysis of ForI. Coomassie blue stained SDS-PAGE of fractions from gel filtration. Lane 1-8 are elution fractions.



Figure S8 Mass spectra showing the presence of an ion (m/z = 431) corresponding to the anion of the diketopiperazine observed by X-ray crystallography. (a) and (b) shows the reaction of ForI (with 50  $\mu$ M external PLP added) incubated with LCS and DCS, respectively. The details of the reaction are in materials. (c) In a solution of L-cycloserine (102 Da), no peak corresponding to diketopiperazine (204 Da) was observed.



Figure S9 In a repeat of the experiment in Figure S8, the reaction was carried out in  $D_2O$ . The resulting mass spectrum shows the presence of an ion (m/z = 431) corresponding to the anion of the diketopiperazine but no incorporation of deuterium was observed, although the experiment was set up in  $D_2O$ .

## Supplementary tables

Table S1 Experimentally derived mass for Forl in each SEC-MALS fraction. A Forl monomer has calculated molecular mass of 44kDa.

Fraction	Elution volume (ml)	Mw (kDa)	Uncertainty
1	70-71	353	± 5 %
2	71-72	113	± 17 %
3	72-73	87	±7%
4	73-74	83	± 3 %
5	74-75	81	± 3 %
6	75-76	80	±4%
7	76-77	90	± 13 %
8	77-78	200	± 60 %

Table S2 Data collection and refinement statistics.

	ForI-PLP	Forl-PMP	Forl-LCS	Forl-DCS
Beamline	i24 (DLS)	i04 (DLS)	i24 (DLS)	i24 (DLS)
Wavelength (Å)	0.9686	0.9795	0.9686	0.9686
Space group	P4 <sub>3</sub> 2 <sub>1</sub> 2	P212121	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
Cell dimensions				
a, b, c (Å)	92.3 92.3 118.8	58.3 125.8	57.9 126.8	58.1 127.1
		142.8	141.2	141.2
α, β, γ (°)	90 90 90	90 90 90	90 90 90	90 90 90
Resolution range	43.02-1.18	94.39 - 1.56	61.67 - 1.48	61.73 - 1.47
	(1.21 - 1.18)	(1.60 - 1.56)	(1.52 - 1.48)	(1.51 - 1.47)
<b>R</b> <sub>meas</sub>	0.070 (1.89)	0.182 (2.702)	0.160 (2.709)	0.173 (2.453)
CC <sub>1/2</sub>	1.0 (0.5)	1.0 (0.5)	1.0 (0.7)	1.0 (0.7)
I/ σ (I)	22.3 (1.5)	8.0 (1.1)	16.8 (1.3)	13.9 (1.2)
Completeness (%)	93.1 (61.7)	98.8 (93.3)	98.0 (96.3)	97.9 (96.1)
Multiplicity	23.6 (16.5)	9.7 (9.7)	33.1 (29.6)	33.1 (28.7)
<u>Refinement</u>				
No. reflections	148310 (4974)	140719 (6922)	161265 (8227)	165228(8486)
R <sub>work</sub>	0.112 (0.256)	0.167 (0.286)	0.142 (0.318)	0.145 (0.306)
R <sub>free</sub>	0.129 (0.275)	0.192 (0.297)	0.173 (0.334)	0.169 (0.312)
<u>Number of atoms</u>				
Total	4051	7448	7473	7428
Protein	3385	6500	6502	6537
Ligands / ions	5	41	59	59
waters	661	907	912	832
<u>B-factor</u>				
Average	19.28	21.95	23.38	23.82
Protein only	15.1	18.4	20.2	20.6
Ligands/Ion/water	22.4	25.1	26.2	26.7
R.m.s deviations				
Bond lengths (Å)	0.012	0.013	0.005	0.005
Bond angles (°)	1.59	1.76	1.34	1.32

Values in parentheses are for the highest-resolution shell. Structure and data have been deposited with RCSB.

### Method and materials

#### **General methods**

Unless specified, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Oligonucleotides were purchased from ThermoFisher Scientific. Restriction enzymes were purchased from New England Biolab. dNTP and DNA polymerase were purchased as part of the EMD Millipore Novagen KOD Hot Start DNA Polymerase kit. DNA sequencing was performed by GATC.

#### **Cloning and expression**

Streptomyces kaniharaensis Shomura and Niida (ATCC<sup>®</sup> 21070<sup>™</sup>) was purchased from ATCC (Middlesex, UK). The freeze-dried cell sample was directly dissolved in autoclaved Tryptic Soy Broth media containing 25% glycerol in biological safety cabinet. 10 µl of the cell culture was plated on the TSB agar and incubate at 26 °C for 15 days. Four 0.5-inch diameter mycelia appeared on the plate, and one of the mycelia was picked for inoculating 50 mL TSB liquid media. The cell culture was incubated at 26 °C for 5 days before harvesting by centrifugation at 3500 rpm at 4 °C. The cell pellets were washed by deionized water twice before store in -80 °C overnight. The genomic DNA extraction was performed using PureLink™ Genomic DNA Mini Kit (ThermoFisher, UK) and the manufacture protocol for Gram-positive bacteria. To increase the purity, the yield genomic DNA was purified again using the same procedure. This yielded 17  $\mu$ g genomic DNA (260nm/280nm = 1.9, 260nm/230nm = 2.0) for sequencing. Preparation of PacBio DNA library from the genomic DNA sample, data generation on RSII SMRT cell, and genome assembly using HGAP were performed at University of Liverpool Centre for Genomic Research. The final assembly of the genome of S. kaniharaensis contained 19 contigs. The genome was annotated through the PATRIC software pipeline (https://www.patricbrc.org/).<sup>1</sup> The final assembly of the genome of S. kaniharaensis contained 19 contigs. The genome sequence has been deposited with accession number is: SAMN12859417. We tentatively identified the gene cluster encoding by searching for the homologs of the known formycin synthesis protein ForH (PDB id: 6NKO) using the internal PATRIC BlastP tool and then analyzing the surrounding CDSs for homologs of previously identified forA-X genes.<sup>2</sup> The Forl protein was predicted based on the membership to the PLP-dependent aminotransferase family (fig|212423.3.peg.657). The corresponding codon optimised synthetic gene Forl was purchased from Integrated DNA Technologies, Europe and then cloned into a pEHISTEV vector<sup>3</sup> resulting in a plasmid with the coding DNA sequence below.

The codon optimised synthetic gene Forl<sup>2</sup> was purchased from Integrated DNA Technologies, Europe and then cloned into a pEHISTEV vector<sup>3</sup> resulting in a plasmid with the coding DNA sequence below.

1	ATGTCGTACT	ACCATCACCA	TCACCATCAC	GATTACGACA	TCCCAACGAC	CGAAAACCTG
61	TATTTTCAGG	<b>GCGCC</b> ATGAT	AACAGAGAAG	TCTGCCGCCT	TATACGCCCG	GGCCGTAGAG
121	GTCATGCCTG	GCGGTAATTC	GAGAACTGCC	GTATATTCTT	CTCCATATCC	GGTATATGTA
181	CGTAGCGGCT	CTGGAGCGAG	AGTTACGGAC	GTTGACGGAG	TTGAAAGAAT	CGACTTTTTG
241	AACAATTCGA	CCACTTTGAT	TCACGGGCAC	GCCCACCCGG	AAATGGTCGA	GGCAATCGCG
301	GCCGCTGTTG	GACATGGCAC	GTCTTTTGGA	ATGCCTACAC	CGGTTGAAGT	TGAATACGCT
361	GAGGCTTTAA	GCGCGCGCAA	CGAAACATTC	GAACATGTGA	GATTTACGTC	GAGCGGAACA
421	GAAGCTGTAA	TGATGGCTGT	GCAAGCTGCG	CGGGCATATA	CGGAGCGGCC	AAAAATTGCT
481	AAGATTGCTG	GCGCCTATCA	TGGCGCGTAT	GATGCTGTCG	CCGTCAACAA	CGATGGTTCC
541	GGGAACCTTA	TCAGCCATGC	AGTGACCGGG	AATCCCGAAG	GGGTCGTAGC	TAACACTGTC
601	GTAATTCCGT	TTAACGACCC	AGAAGGTAGC	TTGGAAGTAT	TACGTCGTCA	CGCCGATGAT
661	CTGGCGTGTG	TATTAATTGA	CCCCGTACCA	TGGCGCATCG	GCCTTTTGCC	TGCATCGAAG
721	GAGTGGTTAG	ACGCTCTTCG	TGAGTTCTGT	GATGCCTCTG	GAGCCGTGTT	GATTAGCGAT
781	GAGGTAGGTT	CATATCGGGT	AGGCTACCAT	GGAGCCATGC	AACTTCTGGG	CGCCGAGGCG
841	GATATTACCG	TGATGGGAAA	AGTAATAGCC	GCAGGTATGC	CTATAGGAGC	TGTAGCTGGC
901	AGACGGCGTT	TCATGTCCGT	ATTCGATCCG	AGCAAAGGCA	AGCCTGCCTT	GCCACACAGT
961	GGATCGTACA	ATGCAAATCC	TGTTTCCATG	TCTTCAGGAA	TCGCTAGTCT	GCGGCTTTTG
1021	ACTCCTCAAG	CGCATGAGAG	AATCGGTCAA	TTGGGTGAGC	AGGCTAGAGG	TAGTATGCGC
1081	ACAGCTTTGG	GTGAGGCGGG	ATTGGACTGG	GAAGTGAATG	GTTTGGGTAG	CTTGTTTCGC
1141	GTCGTAGCTA	ATTCGGCACC	AGCCGGATAC	GACAGTGCAG	CAGCCGCTAT	GAAGGCTCTT
1201	TATTGGAAGT	TACTGGAGAA	CGGAATCCAC	ATAGGTGATT	CGGGGGCTTGG	GTGCATTTCC
1261	ACGCCGATGG	GAGAAGAGGA	AATAGCGGAG	TACGCCGTAG	CGTTTGCGAA	GAGTTTGGGC
1321	CAGGTATTGG	CGGAAGGCCG	TGCCTAG			

(highlighted in cyan are the His tag and TEV cleavage site).

The plasmid has been deposited with ADDGENE. This construct has an N terminal  $His_6$  tag followed by a tobacco etch virus cleavage site before the start of the protein. The resulting protein was expressed in *Escherichia coli* BL21(DE3) cells grown in the autoinduction media described by Studier<sup>4</sup> for 48 h at 20 °C.

#### **Protein purification**

*E. coli* cells overexpressing ForI were resuspended in lysis buffer [500 mM NaCl, 20 mM Tris-HCl (pH 8.0), 20 mM imidazole (pH 8.0), and 3 mM 2-mercaptoethanol] and EDTA-free protease inhibitor tablets (Roche) and DNase at 0.4 mg/g of wet cell pellet. The resuspension was lysed by being passed through a cell disruptor at 30K psi (Constant Systems). The lysate was cleared by centrifugation (17,000 rpm, 4 °C, 20 min) and then loaded onto a Ni Sepharose 6 Fast Flow column (GE Healthcare) equilibrated with lysis buffer. The protein was eluted with elution buffer [500 mM NaCl, 20 mM Tris-HCl (pH 8.0), 250 mM imidazole (pH 8.0), and 3 mM 2-mercaptoethanol] and passed over a desalting column (16/10 Desalting, GE Healthcare) exchanging into desalting buffer [100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 3 mM 2-mercaptoethanol]. TEV protease was added at a mass ratio of 1:10, and

the protein was digested for 3 h at 20 °C before being loaded onto a second nickel column preequilibrated with desalting buffer. The eluted protein was applied directly to an anion-exchange column (HisTrap Q Sepharose FF, GF Healthcare) where it was eluted with a 0.1 to 1 M NaCl gradient. The peak fraction was then concentrated to 7.5 mL (Vivaspin concentrators, 30 kDa molecular weight cutoff) and applied to a Superdex 200 gel filtration column (GE Healthcare) equilibrated with gel filtration buffer [150 mM NaCl, 20 mM HEPES (pH 7.4), and 1 mM TCEP]. The integrity, identity and purity of Forl was confirmed by SDS gel electrophoresis (Figure S7) and MS. 50 μM external PLP is added in the protein before it was fast frozen by liquid nitrogen.

#### UV – vis absorbance spectroscopy of Forl.

All UV absorbance spectra were recorded on a SpectraMax 2e microplate reader (Molecular Devices) and analysed using Graphpad Prism 6. An additional 1  $\mu$ M PLP was added to enzyme to aid protein stability. For UV - vis absorbance assays, the concentration of recombinant protein was 50  $\mu$ M. The plate reader was blanked with 100 mM HEPES (pH 7.5) and spectra were collected from 300 nm to 500 nm. The screening experiments used a total sample volume of 100  $\mu$ l. The instrument was set to record spectra at a wavelength step of 2 nm. Changes in the spectrum were monitored after addition of 0.5 mM L-glutamate or 5 mM cycloserine (D/L).

#### **Oligomeric status of Forl determination**

ForI was analysed by size-exclusion chromatography multi-angle light scattering (SEC-MALS)<sup>5</sup> for determination of molecular mass. Purified protein was loaded onto a GE Health Superdex 200 column, equilibrated in Gel filtration buffer (150 mM NaCl, 10 mM HEPES, 1 mM TCEP), attached to the Wyatt Dawn Heleos II Multi-Angle Light Scattering detector and Wyatt Optilab T-rex Refractive Index detector. The protein elution peak was characterised by the differential refractive index (dRI).

#### Crystallography

The enzyme was screened for suitable crystallisation conditions. 1 mM PLP was added into protein to ensure a complete loading prior to crystallisation. Crystals were obtained in 200 mM  $Li_2SO_4$ , 100 mM Tris-HCl pH 8.0 and 30 % (w/v) PEG 4000 with 35 mg/ml protein at 4 °C. The original hit was optimised in hanging drop plates (EasyXtal 15-well DG-Tool X-Seal) using 1 µl of the protein solution (35 mg/ml) and 1-2 µl of well solution in the hanging drop. The PMP form of ForI was produced by soaking the crystals in the mother liquor plus 10 mM L-glutamic acid for 5 min at 4 °C or incubating 10 mM L-glutamic acid with enzyme for 16 hours at 4 °C prior to crystallisation. For the preparation of L- and D-cycloserine crystals, 10 mM of the corresponding cycloserine was incubated with the protein at 4 °C for 16 hours before setting up crystallisation plates. Crystals were harvested at 3 days later. The crystallisation conditions were identical to those optimised for the ForI-PLP but the space group was different.

The crystals were mounted in a cryo-loop (Molecular Dimensions) and cryo-protected in solutions containing mother liquor brought to 20 % (v/v) glycerol. The crystals were then frozen by plunging them into liquid nitrogen and sent in a cryogenic Dewar to Diamond Light Source for data collection. The data sets were collected at two different beam lines (I04 and I24) and all data processing used the Diamond online automated software XIA2<sup>6</sup> DIALS<sup>7</sup>. The resolution limit of data is determined where  $CC_{1/2} \ge 0.5^{8}$  and intensity fall off. Structures were determined using molecular replacement in PHASER<sup>9</sup> (the native ForI used the GSAAT as search model, subsequent and refined in REFMAC5<sup>10</sup> with anisotropic B-factors as implemented in CCP4.<sup>11</sup> Ligands were introduced into density during refinement when the Fo-Fc map was judged unambiguous in COOT.<sup>12</sup> The maps that we introduced the ligands into are the same omit maps that we showed in the paper. Thus the phases for these Fo-Fc maps were derived from models that never had the ligand present. The final refined coordinates of the ligand were then shown in this Fo-Fc map. This method of calculating the omit maps is unbiased. The geometry of the diketopiperazine was restrained guided by parameters from the PRODRG server.<sup>13</sup> Data and structures have been deposited with the RCSB.

#### Mass spectrometric detection of diketopiperazines

Forl, dissolved in 20 mM HEPES buffer, pH 7.4, containing 150 mM NaCl was passed through a desalting column (GE Healthcare) to exchange the protein into 100 mM NH<sub>4</sub>OAc, pH 8.0. 2 mM of either L- or D-cycloserine and 100  $\mu$ M Forl dissolved in 100 mM NH<sub>4</sub>OAc, pH 8.0, (50  $\mu$ L total volume) were then incubated overnight at 4 °C before the addition of MeOH (50  $\mu$ L) to precipitate the enzyme. Centrifugation then gave a supernatant that was analysed by mass spectrometry. Control samples lacking either the enzyme or cycloserine were prepared in a similar manner. Liquid chromatography mass spectrometry (LC-MS) was performed on a Waters Acquity UPLC coupled to a Waters Synapt G2-Si QTOF mass spectrometer. The column used was a Waters Acquity CSH C18, 130 Å, 1.7  $\mu$ m (2.1 mm x 30 mm) and held at 40 °C throughout the run. Mobile phase A was H<sub>2</sub>O (0.1% CHOOH) and B was ACN (0.1% CHOOH). The flow rate was 0.3 mL/min and the gradient employed began at 98% A with a final of composition of 2% A over 10 minutes. The mass spectrometer was operated in electrospray ionisation positive or negative mode. Negative ion mass spectrometric analysis of both Forl/cycloserine reaction mixtures clearly showed ions with m/z =

431 corresponding to the anion of the corresponding diketopiperazine distereoisomer (Figure S8). This peak was absent in the corresponding mass spectra of the control samples of protein nor was any peak corresponding to diketopiperazine observed.

For deuterium experiment, Forl was exchanged into 100 mM NH<sub>4</sub>OAc, pH 8.0 in D<sub>2</sub>O. 2 mM Lcycloserine (in D<sub>2</sub>O) and 100  $\mu$ M Forl dissolved in 100 mM NH<sub>4</sub>OAc, pH 8.0, (50  $\mu$ L total volume) were then incubated overnight at 4 °C before the addition of MeOH (50  $\mu$ L) to precipitate the enzyme. Centrifugation then gave a supernatant that was analysed by mass spectrometry.

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