Supporting Information Appendix

Revisiting the Biosynthesis of Dehydrophos Reveals a tRNA-dependent Pathway

Despina J. Bougioukou, Subha Mukherjee, and Wilfred A. van der Donk*

Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206 West Gregory Drive, Urbana, IL 61801 Phone, 217 244 5360 Fax, 217 244 8533

E-mail: vddonk@illinois.edu

*Author to whom correspondence should be addressed

Table of Contents

Molecular Biology **4**

Reagents *4*

Cloning procedures *4*

Enzymology **4**

Reagents *4*

Analytical Methods *5*

Expression and Purification of His_6-DhpH, His_6-DhpH-N, and His_6-DhpD *5*

Expression and Purification of His_6-DhpH-C *5*

Expression and Purification of His_6-LeuRS and His_6-GlyRS *5*

Expression and Purification of MBP-DhpJ and MBP-DhpK *5*

Typical Reconstitution of PLP-dependent DhpH and DhpD Activity *6*

Typical Reconstitution of DhpJ Activity *6*

Typical Reconstitution of tRNA-dependent Activity of DhpH *6*

Enzymatic preparation of L-Leu-L-Ala(P) on mg-scale using DhpH-C *6*

Extraction of nucleic acids from DhpH for labeling with ^{14}C-amino acids *7*

Aminoacylation assay of nucleic acids extracted from DhpH *7*

Enzymatic preparation of Gly-L-Leu-L-Ala(P) by DhpK *7*

Organic Chemistry **8**

General remarks *8*

Synthesis of benzyl (2-((tert-butyldimethylsilyl)oxy)-1-(dimethoxyphosphoryl)ethyl)carbamate *8*

Synthesis of rac-Ser(P) *8*

Synthesis of rac-pSer(P) *9*

Synthesis of protected L-Leu-Ser(P) *9*

Synthesis of protected L-Leu-Ser(P) with free alcohol moiety *9*

Synthesis of L-Leu-Ser(P) *9*

Synthesis of protected precursor of L-Leu-ΔAla(P) *10*

Synthesis of L-Leu-ΔAla(P) *10*

Synthesis of protected L-Leu-Ala(P) *11*

Synthesis of L-Leu-Ala(P) *11*

Synthesis of Gly-L-Leu-Ala(P) *11*

Synthesis of protected Gly-L-Leu-Ser(P) *11*

Synthesis of Gly-L-Leu-Ser(P) *12*

Supplementary Figures **12**

Fig. S1. *Representative phosphonopeptides.* *12*

Fig. S2. *Proposed biosynthesis of ΔAla(P) starting from DH-EP based on single gene deletion experiments.* *13*

Fig. S3. *Organization of the two domains in DhpH.* *13*

Fig. S4. *SDS-PAGE analysis of His_6-DhpH after IMAC purification.* *14*

Fig. S5. *Synthetic scheme towards rac-pSer(P).* *14*

Fig. S6. *^{31}P NMR analysis of DhpD activity with rac-Ser(P).* 15

Fig. S7. *Michaelis-Menten plot showing the dependence of the rates of DhpD-catalyzed formation of Ala(P) on the concentrations of AP and MAP.* 15

Fig. S8. *^{31}P NMR analysis of DhpH and DhpH-N activity with rac-Ser(P).* 16

Fig. S9. *Michaelis-Menten plot for conversion of L-pSer to pyruvate by DhpH and DhpH-N.* 16

Fig. S10. *^{31}P NMR spectra of DhpH-N activity with L-Ala(P) in the presence of different α-ketoacids.* 17

Fig. S11. *UV-Vis Analysis of DhpH and 14C labeling of extracted nucleic acids.* 18

Fig. S12. *Schemes for the syntheses of peptides.* 19

Fig. S13. *HPLC traces and NMR analysis of synthetic L-Leu-ΔAla(P).* 20

Fig. S14. *HPLC traces and ^{1}H NMR of synthetically and enzymatically prepared L-Leu-L-Ala(P).* 21

Fig. S15. *Radioactive TLC analysis of rac-pSer(P) conversion to L-[^{14}C(U)]-Leu-Ala(P) by the activity of DhpH and DhpD in a one-pot reaction.* 21

Fig. S16. *^{31}P NMR analysis of enzymatic formation of L-Leu-L-Ala(P) by DhpH.* 22

Fig. S17. *^{31}P NMR analysis of control experiments of enzymatic formation of L-Leu-L-Ala(P) by DhpH-C in the absence of tRNA.* 23

Fig. S18. *^{31}P NMR analysis of DhpH activity after RNase treatment.* 24

Fig. S19. *UV-Vis spectrophotometric assay for calculating kinetic parameters for DhpH and DhpH-C.* 25

Fig. S20. *List of oligopeptides screened as putative substrates of MBP-DhpJ.* 26

Fig. S21. *^{1}H NMR analysis of the DhpJ activity in the presence of L-Leu-L-Ala(P).* 27

Fig. S22. *^{31}P NMR analysis of the DhpJ activity in the presence of L-Leu-L-Ala(P).* 28

Fig. S23. *NMR analysis of HPLC-purified major product of DhpJ reaction with L-Leu-L-Ala(P).* 29

Fig. S24. *^{1}H NMR spectrum of HPLC-purified side-product of DhpJ reaction with L-Leu-L-Ala(P).* 30

Fig. S25. *^{1}H NMR spectra of control experiments in which Fe(II)-reconstituted DhpJ was incubated with α-KG/O_2 and L-ascorbate in the absence of substrate.* 31

Fig. S26. *^{1}H NMR analysis of DhpJ incubation with the hydroxylated side-product.* 32

Fig. S27. *^{31}P NMR analysis of DhpJ incubation with the hydroxylated side-product.* 33

Fig. S28. *Enzymatic preparation of L-Leu-L-Ala(POMe). A. Reaction scheme.* 34

Fig. S29. *^{1}H NMR analysis of the DhpJ activity in the presence of L-Leu-L-Ala(POMe).* 35

Fig. S30. *Expanded region of the ^{1}H NMR spectrum of the DhpJ activity with L-Leu-L-Ala(POMe).* 36

Fig. S31. *^{31}P NMR analysis of the DhpJ activity with L-Leu-L-Ala(POMe).* 36

Fig. S32. *^{1}H NMR analysis of the HPLC purified product L-Leu-ΔAla(POMe) after incubation of DhpJ with L-Leu-L-Ala(OMe).* 37

Fig. S33. *HPLC traces of Gly-L-Leu-L-Ala(P).* 37

Fig. S34. *^{1}H NMR spectra of enzymatically (DhpK) and chemically prepared Gly-Leu-Ala(P) tripeptides.* 38

Fig. S35. *Enzymatic formation of DHP from L-Leu-ΔAla(P) in one-pot cascade reaction with DhpK and DhpI.* 39

Fig. S36. *Proposed catalytic cycle for DhpH-N.* 40

Fig. S37. *^{31}P NMR analysis of the DhpJ activity on L-Leu-L-Ala(P) in the presence or absence of L-ascorbic acid.* 41

Supplementary Tables **41**

Table S1. *List of strains and plasmids used in this study.* 41

Table S2. *List of oligonucleotides used in this study.* 42

References **43**

Molecular Biology

Reagents

E. coli strains and plasmids used in this study are listed in Table S1. Restriction enzymes (*Nde*I, *Hind*III, *Dpn*I), Phusion polymerase, Taq DNA ligase and 100 mM stock solutions of dATP, dTTP, dGTP and dCTP were purchased from New England Biolabs. Fail Safe PCR system and T5 exonuclease were provided by Epicenter. DTT was provided by Promega. KOD hot start DNA polymerase, dNTPs and $MgSO_4$ were obtained from EMB Millipore. Oligonucleotides were purchased from IDT in standard, desalted form and used without further purification (Table S2). All other reagents were purchased from Sigma-Aldrich unless otherwise specified. LB medium contained yeast extract (5 g/L), tryptone (10 g/L), NaCl (10 g/L). Antibiotics were used at the following concentration: ampicillin (AMP): 100 µg/mL, chloramphenicol: (CAM) 12 µg/mL, apramycin: (APR) 34 µg/ mL.

Cloning procedures

Plasmids were purified with Qiagen or EconoSpin columns. PCR products were purified with Promega Wizard® PCR Clean-Up System. Electro-competent cells were prepared in-house according to standard protocols (1). Dehydrophos biosynthetic genes (Table S1) were PCR amplified from fosmid 17E11-4 (2) using the Fail Safe polymerase in buffer G. All cloning manipulations were based on the *in vitro* ligation isothermal assembly protocol described by Gibson et al. (3) Briefly, after the vector was linearized with the appropriate restriction enzyme, the vector was PCR amplified using primers listed in Table S2, re-digested with *Dpn*I and purified, before it was mixed (ca. 100 ng) with the appropriate insert (ca. 100 ng), in a final volume of 5 µL of distilled water. The solution of vector and insert was mixed with 15 µL of assembly master mixture and incubated at 50°C, for 1 h in a PCR thermocycler. An aliquot (1-3 µL) from the ligation reaction mixture was used to transform electro-competent cells. The assembly master mixture was prepared by adding into 218 µL of distilled water the following reagents: 98.2 µL isothermal reaction buffer (5 ×), 6.8 µL Phusion Polymerase, 2 µL T5 exonuclease (10 × diluted in the T5 exonuclease buffer) and 50 µL Taq DNA ligase. Isothermal reaction buffer (6 mL final volume; 5×) was prepared by mixing the following reagents (final concentrations are indicated in parenthesis): 3 mL of 1 M Tris-HCl (500 mM; pH 7.5), 1.5 g PEG-4000 (25% (w/v)), 300 µL of 1 M DTT (50 mM), 300 µL of 1 M $MgCl_2$ (50 mM), 300 µL of 100 mM NAD^+ (5 mM), and 60 µL of 100 mM stock solution of each of the four dNTPs (800 µM) in the appropriate amount of distilled water.
E. coli glycyl-tRNA-synthetase gene, *glyS*, was amplified by colony-PCR using DH5α strain as a template and primers listed in Table S2 and cloned into a pET-15b vector as described above.

Enzymology

Reagents

Nickel(2^+)-nitrilotriacetic acid (Ni-NTA) agarose was purchased from Qiagen. Amylose resin was obtained from New England Biolabs. Immobilized metal affinity chromatography (IMAC) resin (bead size 80-100 µm) was purchased from PALL Life Science. InVision™ His-tag In-gel Stain was supplied by Invitrogen. Amicon ultracentrifugal filters were purchased from EMB Millipore. Media components and salts were purchased from Fisher Scientific. Isopropylthio-β-D-galactoside (IPTG) was obtained from IBI Scientific. O-Phospho-D-serine was obtained from MB Biochemicals. RNase free water, RNase cocktail enzyme mix and RNase-free DNase were purchased from Ambion. Thermostable inorganic pyrophosphatase (TIPP) was supplied by NEB. Total tRNA from *E. coli* MRE 600 was obtained from Roche Applied Science. L-[^{14}C(U)]-leucine, [^{14}C(U)]-glycine and Ultima Gold cocktail were purchased from Perkin-Elmer. A mixture of pyruvate kinase/lactic acid dehydrogenase from rabbit muscle and myokinase from chicken muscle as well as all other chemicals and reagents were purchased from Sigma-Aldrich. Lysis buffer consisted of 50 mM NaPi, 300 mM NaCl, 10 mM imidazole, 20% glycerol, pH 8.0. Wash buffer consisted of 50 mM NaPi, 300 mM NaCl, 20 mM imidazole, 20% glycerol, pH 8.0. Elution buffer consisted of 50 mM NaPi, 300 mM NaCl, 250 mM imidazole, 20% glycerol, pH 8.0. Storage buffer consisted of 50 mM KPi, 10 mM NaCl, 20 mM $MgCl_2$, 20% glycerol, pH 7.5. Alternatively, the phosphate buffers were replaced by 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (Na-HEPES), pH 8.0.

Analytical Methods

NMR experiments were carried out using one of the following instruments: Agilent 600 MHz equipped with OneNMR probe, Varian Unity 400 MHz equipped with 5 mm Nalorac QUAD probe or Varian Unity 500 MHz with a 5 mm Nalorac QUAD probe. Proton-decoupled ^{31}P NMR spectra were recorded at room temperature in 20% - 90% D_2O in H_2O using 85% phosphoric acid (referenced to 0.0 ppm) as external standard. Water suppression was applied when necessary. UV-Vis measurements were recorded on a Cary 4000 spectrophotometer. HPLC analysis was carried out using an Agilent HPLC 1200 series equipped with a UV-Vis detector and a Synergi 4 µ Fusion-RP 80A semi-preparative column (250 x 10 mm, 4 µm, Phenomenex Torrance, CA). Radioactive TLC sheets were exposed to phosphor imaging plates (IP) (Fujifilm, BAS-MS2040) for 36 to 48 h and radioactivity was detected by scanning the IP on a Molecular Dynamics STORM™ 860 phosphorimager. Radioactivity of liquid samples was measured by a Tri-Carb 2910TR Liquid Scintillation Analyzer. DNA sequencing reactions were carried out at the W.M. Keck Center for Biotechnology at the University of Illinois at Urbana-Champaign. The purity of the isolated proteins was assessed by SDS-PAGE analysis. Coomassie (Bradford) protein assay (Thermo Scientific) was used for calculating protein concentrations. Sequencing data were analyzed by Vector NTI Advance 11.0 (Invitrogen Co.). NMR data were analyzed by MestReNova 8.0 (MestreLab Research). Kinetic data analyses were performed using GraphPad Prism 6 software. Phosphorimager radioactivity data were analyzed by ImageQuant software.

Expression and Purification of His_6-DhpH, His_6-DhpH-N, and His_6-DhpD

An overnight culture of *E. coli* Rosetta 2(DE3) pLysS freshly transformed with the appropriate plasmid and grown in $LB^{AMP, CAM}$ medium was diluted 1 : 100 into 1 L of the same medium in a 4 L flask. The culture was shaken at 37 °C until the optical density at 600 nm reached 0.6-0.8, then the flask was placed in an ice/water bath for ca. 30 min before the addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 200 µM. The culture was shaken for an additional 10-12 h at 18 ºC. All the following purification steps were carried out at 4 °C. The cells (5-7 g wet mass from 2 L culture) were collected by centrifugation, washed once with phosphate-buffered saline solution (PBS), pH 7.4 and then resuspended in 30 mL of lysis buffer supplemented with 100 µM pyridoxal-5'-phosphate (PLP) and 1000 U DNase. The cells were lysed by passage twice through a French pressure cell and debris was removed by centrifugation at 37,000 x g for 45 min at 4 °C. The supernatant was loaded onto a column containing 5-7 mL of Ni-NTA resin previously equilibrated with lysis buffer. After equilibration of the resin with the lysate in a rocking platform for ca. 30 min, the flow-through was discarded and the resin was washed with 2 × 40 mL of wash buffer supplemented with 100 µM PLP. Resin-bound protein was eluted with elution buffer supplemented with 100 µM PLP. Fractions of 2 mL were collected and the absorbance at 280 nm was measured by a NanoDrop spectrophotometer. Fractions with strong absorbance at 280 nm were pooled and concentrated in an Amicon Ultra centrifugal filter unit with 30 kDa molecular weight cut off (MWCO) to a final volume of 2.5 mL. Imidazole and excess salt was removed by passing the protein solution through a PD10 desalting column previously equilibrated with storage buffer. Protein was eluted with 3.5 mL of storage buffer and stored in aliquots at -80 ºC. Typical yields: i) His_6-DhpH, 30 mg/L, ii) His_6-DhpH-N, 30 mg/L, iii) His_6-DhpD, 25 mg/L.

Expression and Purification of His_6-DhpH-C

The expression and purification of His-tagged DhpH-C was carried out as described above for DhpH. PLP was omitted from all buffers. Typical yield: 25 mg/L.

Expression and Purification of His_6-LeuRS and His_6-GlyRS

The expression and purification of His-tagged LeuRS and GlyRS was carried out as described above for DhpH except that PLP was omitted from all buffers. Storage buffer consisted of 10 mM Tris-HCl, 100 mM NaCl, 5 mM NaH_2PO_4, 50% glycerol, pH 7.5. The plasmid for expression of LeuRS was obtained from the laboratory of Dr. Susan Martinis (UIUC); the plasmid for GlyRS was generated in this work (see Tables S1 and S2).

Expression and Purification of MBP-DhpJ and MBP-DhpK

The expression of MBP-DhpJ and MBP-DhpK was carried out as described above for DhpH with the following modification: 2% glucose was added to the LB solution prior to inoculation. All the purification

steps were carried out at 4 °C. The cells (5-7 g wet mass from 2 L of culture) were collected by centrifugation, washed once with phosphate-buffered saline solution (PBS), pH 7.4 and then resuspended in 30 mL amylose resin buffer (50 mM KPi, 200 mM NaCl, 10 % glycerol, pH 7.5) supplemented with 1000 U DNase. The cells were lysed by passage twice through a French pressure cell and debris was removed by centrifugation at 37,000 x g for 45 min at 4 °C. The supernatant was diluted twice in the same buffer and loaded onto a column containing 10-15 mL of amylose resin previously equilibrated with the amylose resin buffer. After equilibration of the resin with the lysate in a rocking platform for ca. 1 h, the flow-through was discarded and the resin was washed with 2 × 50 mL of amylose resin buffer. Resin-bound protein was eluted with the amylose resin buffer supplemented with 10 mM maltose. Fractions of 2 mL were collected and the absorbance at 280 nm was measured by a NanoDrop spectrophotometer. Fractions with strong absorbance at 280 nm were pooled and concentrated in an Amicon Ultra centrifugal filter unit (30 kDa MWCO) to a final volume of 2.5 mL. Maltose and excess salt was removed by passing the protein solution through a PD10 desalting column previously equilibrated with storage buffer. Protein was eluted with 3.5 mL of storage buffer and stored in aliquots at -80 ºC. Typical yields: i) MBP-DhpJ, 25 mg/L, ii) MBP-DhpK, 15 mg/L.

Typical Reconstitution of PLP-dependent DhpH and DhpD Activity.

The reaction mixture (500 μL) contained 5 mM of the appropriate aminophosphonate, 10 mM of an amino-acceptor (pyruvate, α-ketoglutarate, or oxaloacetate), 100 μM PLP, and ca. 50 μM of DhpH, DhpH-N or DhpD in 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (Na-HEPES), pH 8.0. When DhpH was incubated with rac-pSer(P), the presence of an α-ketoacid was not necessary. After 6-8 h incubation at ambient temperature the protein was removed by passing the reaction mixture through an Amicon spin column (30 kDa MWCO) and 150 μL D_2O was added to the sample prior to NMR analysis.

Typical Reconstitution of DhpJ Activity

The reaction mixture (500 μL) contained 5 mM of the appropriate phosphonic acid, 10 mM α-ketoglutarate, 1 mM L-ascorbic acid, 0.2 mM $(NH_4)_2Fe(SO_4)_2$ and 50-100 μM DhpA in 50 mM Na-HEPES, pH 8.0. Typically the enzyme had been reconstituted in an anaerobic glove box (Coy, Grass Lake, MI) with 1.2 equivalent of Fe(II) for ca. 10 min on ice prior to its use. After 5-6 h incubation at ambient temperature ca. 100 μL of Chelex resin was added to the reaction tube. The resin was left to chelate the excess of iron species by mixing the solution at room temperature for ca. 20 min. The protein was removed by passing the reaction mixture through an Amicon spin column (30 kD MWCO) and 150 μL D_2O was added to the flow-through before NMR analysis. We found that the addition of L-ascorbic acid (1-3 mM) enhanced the conversion of the reaction (Fig. S35). In enzymatic assays during which the progress of the reaction was followed by ^1H NMR spectroscopy, the buffer in which the protein was stored, was first exchanged with 50 mM KPi and/or the glycerol was omitted altogether from the protein storage buffer.

Typical Reconstitution of tRNA-dependent Activity of DhpH

The assay mixture (500 μL) contained 5 mM of the appropriate aminophosphonic acid, 2 mg of total tRNA from *E. coli* MRE 600, 2-6 mM L-Leu, 4-6 mM ATP, 5 μM LeuRS, 20 Units of thermostable inorganic pyrophosphatase (TIPP), 30-50 μM DhpH or DhpH-C in 100 mM HEPES, 10 mM KCl, 20 mM $MgCl_2$, pH 7.5. After 5-6 h incubation at ambient temperature ca. 100 μL of Chelex resin was added to the reaction tube. The resin was allowed to chelate any divalent cations to minimize line broadening of ^{31}P NMR signals of nucleoside species. Prior to NMR analysis, the protein and the nucleic acids were removed by passing the reaction mixture through an Amicon spin column (10 kDa MWCO) and 150 μL D_2O was added to the flow through.

Enzymatic preparation of L-Leu-L-Ala(P) on mg-scale using DhpH-C

Bulk tRNA from *E. coli* MRE 600 (10 mg) was dissolved in 3.3 mL of 100 mM ammonium bicarbonate solution containing 20 mM $MgCl_2$ and 20 mM KCl. To this solution was added: 1 mL PEP (50 mM stock, 10 mM final), 50 μL ATP (100 mM stock, 1 mM final), 275 μL L-Leu (100 mM stock, 5.5 mM final) and 250 μL L-Ala(P) (100 mM stock, 5 mM final). The reaction was initiated by the addition of ca. 100 μL of enzyme mixture that had been concentrated through a spin Amicon filter (10 kDa MWCO)

and contained DhpH-C (30 μM final), EcLeuRS (5 μM final), pyruvate kinase (PK, 150 Units), adenylate kinase (ADK, 200 Units) and 100 Units TIPP. The total volume of the reaction was ca. 5 mL. The reaction was monitored by ^{31}P NMR spectroscopy. After completion of the reaction, proteins and nucleic acids were removed via an Amicon device. After lyophilization, the solid was resuspended in 0.1% acetate to a final volume of 1.5 mL, the pH was adjusted to ~ 5 with acetic acid, and the solution was loaded on 5 mL of Fe-IMAC resin equilibrated with 0.1% AcOH. After gentle mixing, the unbound compounds were eluted and the resin was washed with 15 mL of 0.1% AcOH. Compounds bound to the resin were eluted with 0.1 M NH_4HCO_3 (15 mL; fraction I) and 0.5 M NH_4HCO_3 (15 mL, fraction II). Fraction II contained the desired dipeptide which was further purified by HPLC to afford 3.2 mg of white solid.
HPLC Conditions: Column: Synergi 4 μ Fusion-RP 80A column (250 x 10 mm, 4 μm, Phenomenex) Elution Gradient: 0-15 min 100 % Solvent A (0.1 % formic acid (FA) in water), 15-30 min 25 % A, 75 % solvent B (0.1 % FA in methanol), 30 to 33 min 75 % Solvent B, 38 to 43 min 100 % Solvent A. Flow: 4mL / min. Detection: @ 210 nm, 4 mL per fraction.

Extraction of nucleic acids from DhpH for labeling with ^{14}C-amino acids
An aliquot of DhpH preparation (ca. 1.5 mL; 13 mg/mL) was concentrated through a spin Amicon filter (10 kDa MWCO) to 500 μL and extracted twice (2 × 500 μL) with acidic phenol and once with chloroform/isoamyl alcohol (24:1). The aqueous layer was supplemented with sodium acetate to a final concentration of 0.3 M and nucleic acids were precipitated with 2 volumes of abs. ethanol overnight at –20 ºC. The pellet was washed with 80% ethanol and resuspended in 50 μL deionized H_2O.

Aminoacylation assay of nucleic acids extracted from DhpH
Nucleic acids extracted from DhpH (375 μg) were dissolved in 30.6 μL of His-tagged LeuRS (3x) charging buffer (180 mM Tris, 30mM KCl, 30 mM $MgCl_2$, 3 mM DTT pH 7.5). To this solution, an aliquot of 16.4 μL of [^{14}C(U)]-L-Leu (0.1 μCi/μL, 328 mCi/mmol, 0.1 mM final conc.) was added. LeuRS was diluted in LeuRS charging buffer containing 5 μg BSA and 1 μL (~1 μM final conc.) was added to the reaction mixture. An aliquot of 10 μL was removed (t=0 min) and spotted on a dry 1×1 cm 3MM CHR blotting paper (Whatman; pre-soaked in 5% trichloroacetic acid). The reaction was started with 2 μL ATP (4 mM final concentration) and incubated at room temperature. Aliquots (10 μL) were removed and spotted on paper pads (Whatman™ chromatography, grade 3MM CHR) paper at different time points. When His_6-GlyRS was used for charging, LeuRS was replaced by 2 μM GlyRS and leucine was replaced by [^{14}C(U)]-Gly (0.1 μCi/μL, 88 mCi/mmol, final conc. 0.1 mM).
As control experiment, total tRNA from *E. coli* MRE 600 was labeled either with ^{14}C-Leu/LeuRS or with ^{14}C-Gly/GlyRS. In all cases, after the completion of the reaction, the pads were washed three times with 5% cold TCA (5 mL) for 10-15 min, once with 80% cold EtOH, for 15 min and once with ether before they were dried in the hood for 15 min. Each pad was placed in a liquid scintillation (LSC) vial containing 6 mL LSC cocktail and radioactivity was measured for 1 min.

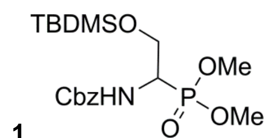
Enzymatic preparation of Gly-L-Leu-L-Ala(P) by DhpK
The reaction mixture (1 mL) contained: 5 mM L-Leu-Ala(P), 2 mg of total tRNA from *E. coli* MRE 600, 6 mM glycine, 6 mM ATP, 10 μM GlyRS, 10 Units of TIPP, and 50 μM MBP-DhpK in 50 mM HEPES, 10 mM KCl, 20 mM $MgCl_2$, pH 7.5. After 6 h incubation at ambient temperature ~ 200 μL of Chelex resin was added to the reaction tube. The resin was allowed to chelate any divalent cations by mixing the solution at room temperature. The protein and the nucleic acids were removed by passing the reaction mixture through an Amicon spin column (10 kDa MWCO) and the tripeptide was purified by HPLC.

Organic Chemistry

General remarks

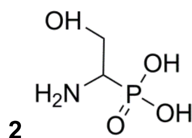
Commercially available reagents were used without further purification. All reactions were performed under nitrogen atmosphere unless otherwise noted. Reaction progress and fractions from flash-chromatography purifications were monitored by thin layer chromatography (TLC) on silica-gel-coated glass plates with a F254 fluorescent indicator. Visualization was achieved by fluorescence quenching during UV irradiation, ninhydrin stain (0.5% ninhydrin in ethanol), or permanganate stain (1.5 g $KMnO_4$, 10 g K_2CO_3, 1.25 mL 10% NaOH in 200 mL of H_2O). Flash chromatography was performed using Silicycle SiliaFlash P60, 230-400 mesh silica gel. Cation exchange resin AG 50W-X8, hydrogen form (100-200 mesh) was purchased from Bio-Rad and used for compound purification when noted. Reversed-phase high performance liquid chromatography (RP-HPLC) was performed using an Agilent 1200 series quad pump system equipped with a diode array detector and a G1956B mass spectrometer with a multimode-electrospray/atmospheric pressure chemical ionization (MM-ES+APCI) source. For analytical scale HPLC, a Synergi 4 μ Fusion-RP 80A column (150 x 4.6 mm, 4 μm, Phenomenex Torrance, CA) was used with a flow rate of 0.5 mL/min (column A). For preparative HPLC, a Synergi 4 μ Fusion-RP 80A semi-preparative column (200 x 10 mm, 4 μm, Phenomenex Torrance, CA) was used with a flow rate of 4 mL/min, (column B). Elution Gradient: 0-15 min 100 % Solvent A (0.1 % formic acid (FA) in water), 15-30 min 25 % A, 75 % solvent B (0.1 % FA in methanol), 30 to 33 min 75 % Solvent B, 38 to 43 min 100 % Solvent A. Flow: 4mL / min. Detection: @ 210 nm, 4 mL per fraction. NMR data are represented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and/or multiple resonance), coupling constant (if determined), integration, assignment. Mass spectrometry (except LC-MS) was performed at the University of Illinois Mass Spectrometry Center.

Synthesis of benzyl (2-((tert-butyldimethylsilyl)oxy)-1-(dimethoxyphosphoryl)ethyl)carbamate



Racemic protected Ser(P) **1** was synthesized in three steps from N-Cbz-Ser-OH following a literature procedure (4).
^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 7.36-7.32 (m, 5H; arom), 5.27-5.25 (d, J = 10 Hz, 1H; NH), 5.13 (s, 2H; CH_2-Bn), 4.25-4.18 (m, 1H; Hα), 3.92-3.78 (m, 2H; Hβ), 3.76 (d, J = 12.5 Hz, 3H; OCH_3), 3.74 (d, J= 12.5 Hz, 3H; OCH_3), 0.88 (s, 9H, $SiC(CH_3)_3$), 0.05 (s, 6H, $Si(CH_3)_2$). ^{31}P NMR (202 MHz, $CDCl_3$) δ/ppm = 26.7. MS (ESI): m/z = 418.2 ($M+H^+$).

Synthesis of *rac*-Ser(P)

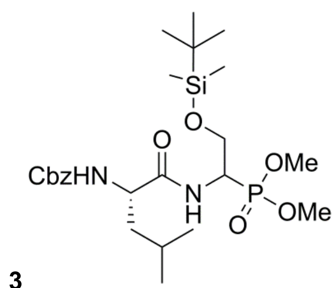


The protected precursor **1** (0.25 g, 0.6 mmol) was placed in a round bottom flask and stirred in 9 mL of toluene under N_2 atmosphere and cooled on an ice-bath. To the reaction mixture, a 1 M solution of boron tribromide in hexanes (2.4 mL, 2.4 mmol) was added dropwise and stirred on an ice bath for 10 min and a white suspension formed. The reaction flask was subsequently transferred to an oil bath preheated to 70 ˚C and stirred for 5 h. The brown reaction mixture was quenched by adding 3.0 mL of dry MeOH (5) and the reaction mixture turned nearly colorless. The reaction mixture was diluted by adding EtOAc (15 mL) and extracted twice with 10 mL of H_2O. The aqueous layers were back extracted with 10 mL of EtOAc and the aqueous fraction was lyophilized to generate a yellow powder. The crude mass was passed through a column loaded with cation exchange resin and eluted with water to obtain a final yield of 95 mg (71.5%) of bromide salt of the product as white solid.
^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 3.89-3.85 (ddd, J = 5 Hz, 1H; CH-β), 3.63-3.58 (ddd, J = 5 Hz, 1H; CH-β), 3.27-3.21 (ddd, J = 5 Hz, 1H; CH-α). ^{31}P NMR (202 MHz, $CDCl_3$) δ 10.98

Synthesis of *rac*-pSer(P)

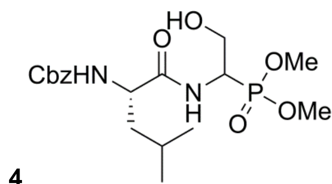
Phosphorus oxychloride (1 mL) was added dropwise to H_2O (0.36 mL) and the mixture was stirred until the evolution of HCl ceased. Then, 110 mg of *rac*-Ser(P) was added and the mixture was heated at 60 ºC with stirring. The reaction was followed by ^{31}P NMR spectroscopy and after 3 h a second portion of $PO(OH)_2Cl$ was added and allowed to react with the remaining starting material for 2 h at 60 ºC with stirring. The reaction was quenched with addition of water at 25 ˚C. The pH was adjusted to 4.5 and the crude mixture was loaded on 20 mL of Fe-IMAC resin equilibrated with 0.1% AcOH. The column was washed with 0.1 % AcOH and the bound compounds were eluted with 0.1 M NH_4HCO_3. Fractions containing the desired product were lyophilized and passed through a 10 mL cation exchange column. The desired product was found in the flow through and the purest fractions based on ^{31}P NMR analyses were combined and lyophilized to afford 50 mg of yellow powder out of which 3.5 mg was *rac*-pSer(P). The content of *rac*-Ser(P) was calculated by ^{31}P NMR spectroscopy by comparing the peak integrations of a D_2O solution containing a measured amount of the material containing *rac*-Ser(P) and a known concentration of L-Ala(P). ^1H NMR (600 MHz, D_2O) δ/ppm = 4.13 (m, 1H; CH-β), 3.91 (m, 1H; CH-β), 3.36 (m, 1H; CH-α). ^{31}P NMR (242 MHz, D_2O-HEPES buffer pH 7.5) δ/ppm 9.1 (**P-**C), 4.6 (**P-**O).

Synthesis of protected L-Leu-Ser(P)



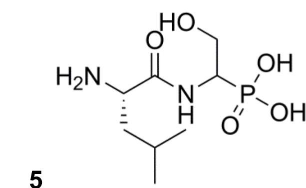
The protected Leu-Ser(P) molecule was synthesized as previously reported (4, 6).
^1H NMR (500 MHz, $CDCl_3$) δ/ppm = 7.38-7.30 (m, 5H; arom), 6.48 (dd, *J* = 10 Hz, 1H; NH), 5.2 (d, *J* = 10 Hz, 1H; NH), 5.11 (d, *J* = 12.5 Hz, 1H; CH_2Bn), 5.09 (d, *J* = 12.5 Hz, 1H; CH_2Bn), 4.55-4.46 (m, 1H), 4.28-4.18 (m, 1H), 4.02-3.96 (m, 1H), 3.73 (m, 6H; OCH_3), 1.68 (m, 4H), 0.95-0.93 (m, 6H; Hδ Leu), 0.89 (s, 9H; $SiC(CH_3)_3$), 0.07 (s, 6H; $Si(CH_3)_2$). ^{31}P NMR (202 MHz, $CDCl_3$) δ 26.4, 26.29 (pair of diastereomers).

Synthesis of protected L-Leu-Ser(P) with free alcohol moiety



Compound **3** (1.0 g, 1.88 mmol) was treated with 4 mL of 1 M TBAF solution in THF (4 mmol) with vigorous stirring under nitrogen for 45 min. The reaction mixture was diluted with 100 mL of dichloromethane (DCM) and washed with 0.1 M aqueous HCl (2×25 mL). The acidic layer was back extracted with 20 mL of DCM. The organic layers were collected, dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash chromatography eluting with 3% MeOH in DCM to 4% MeOH in DCM to yield the desired product (0.69 g, 1.66 mmol, 88%). ^1H NMR (500 MHz, $CDCl_3$) δ/ppm = 7.36-7.26 (m, 5H; arom), 5.51-5.44 (m, 1H), 5.11-5.09 (m, 2H, CH_2-Bn), 4.56-4.48 (m, 1H), 4.34-4.24 (m, 1H), 3.98-3.92 (m, 1H), 3.80-3.68 (m, 6H; OCH_3), 1.71-1.62 (m, 2H), 1.58-1.51 (m, 1H), 0.99-0.89 (m, 6H; Hδ Leu). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm = 173, 156, 136, 128.6, 128.3, 67, 61.7, 61.4, 54, 53, 52, 42, 25, 24. ^{31}P NMR (202 MHz, $CDCl_3$) δ/ppm = 26.2, 25.9 (pair of diastereomers). HRMS (ESI): m/z calc. for $C_{18}H_{29}N_2O_7P$ 417.1792, found 417.1791. TLC: R_f = 0.15 (3% MeOH in DCM).

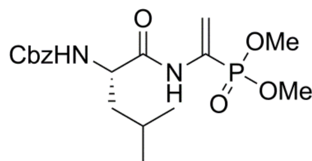
Synthesis of L-Leu-Ser(P)



Compound **4** (52.7 mg, 0.13 mmol) was dissolved in 1.7 mL of toluene and the flask was immersed in an ice-bath. To this solution, 380 µL of a 1 M solution of boron tribromide in hexanes (0.38 mmol) was added dropwise and the reaction was stirred at 0 ˚C for 10 min when the reaction mixture turned from colorless to white. The flask

was transferred to an oil bath heated to 70 ˚C and the reaction was stirred for an additional 3 h, when the reaction mixture turned brown. The reaction mixture was allowed to cool to room temperature and was quenched with 2 mL of dry MeOH, when a clear brownish-black solution formed. The solution was concentrated on a rotary evaporator. The residue was diluted with 6 mL of water and extracted with 5 mL of ethyl acetate. The organic layer was back-extracted with 5 mL of water. The combined aqueous layers were collected and lyophilized to obtain crude product (60 mg). The crude product was purified by Fe-IMAC as described on page S7 for the purification of Leu-Ala(P) and the purest fraction was lyophilized (ca 1.6 mg). ^{1}H NMR δ/ppm = 4.18-4.06 (m, 1H; Hα Ser), 3.95-3.70 (m, 2H; Hβ Ser), 3.60-3.48 (m, 1H; Hα Leu), 1.65-1.45 (m, 3H; Hβ/γ Leu), 0.85-0.72 (m, 6H; Hδ Leu).^{31}P NMR (202 MHz, D_2O) δ/ppm = 17, 16.7 as diastereomers.

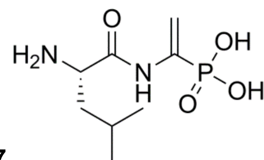
Synthesis of protected precursor of L-Leu-ΔAla(P)



6

Compound **4** (0.15 g, 0.37 mmol) was dissolved in dry DCM (2 mL). The flask was cooled in an ice-bath, and triethylamine (0.1 mL, 0.74 mmol) and methanesulfonyl chloride (0.06 mL, 0.77 mmol) were added. The reaction mixture was allowed to warm to room temperature and stirred for 1 h and 15 min. The solvent was evaporated and ^{31}P NMR spectroscopy indicated complete conversion to the mesylated product. ^{31}P NMR (500 MHz, $CDCl_3$) δ/ppm = 22.76, 22.70 (pair of diastereomers). The mesylated product was dissolved in 5 mL of dry THF, followed by addition of diazabicycloundecene (DBU, 0.14 mL, 0.93 mmol) and the solution was refluxed for 30 min. The reaction mixture was then cooled and the solvent was evaporated. The residue was purified by silica gel flash chromatography with 100 % EtOAc as the eluent to yield the desired product (103 mg, 0.26 mmol, 60 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 7.71 (s, 1H; NH), 7.38-7.32 (m, 5H; arom), 6.73-6.64 (d, J = 42 Hz, 1H; =CH_{trans}), 5.65-5.61 (d, J = 19.5 Hz, 1H; =CH_{cis}), 5.13 (s, 2H; CH_2-Bn), 4.22 (s, 1H; NH), 3.75 (d, J = 7.5 Hz, 3H; OCH_3), 3.73 (d, J = 7.5 Hz, 3H; OCH_3), 1.71-1.66 (m, 2 H; Hβ Leu), 1.54-1.50 (m, 1H; Hγ Leu), 0.96 (s, 6H; Hδ Leu). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm = 172, 156, 136, 129, 128.8, 128.5, 128.2, 116, 67, 54, 53.5, 41, 25, 23, 22.^{31}P NMR (202 MHz, $CDCl_3$). δ/ppm = 15.8. HRMS (ESI) m/z calc. for $C_{18}H_{27}N_2O_6P$ 399.1685, found 399.1686. TLC: R_f = 0.5 (100 % EtOAc).

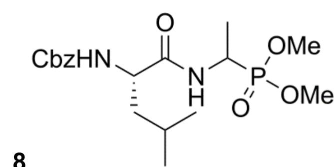
Synthesis of L-Leu-ΔAla(P)



7

Compound **6** (42.3 mg, 0.11 mmol) was dissolved in 1.6 mL of toluene and the flask was immersed in an ice bath. To the solution, a 1 M solution of BBr_3 in hexanes was added (0.32 mL, 0.32 mmol), and the flask was transferred to an oil bath heated to 70 ˚C for 3 h and allowed to cool to 30 ˚C. The reaction mixture was quenched by addition of 2 mL of dry MeOH. The solvent was evaporated and the residue suspended in 4 mL of deionized water after which the mixture was extracted with EtOAc (2×3 mL) and the EtOAc layers were back-extracted with water (3 mL). The combined aqueous layers were lyophilized to generate crude product (~ 31.5 mg). LC-MS purification was performed using analytical HPLC (column A) to optimize the conditions for separation, after which preparative HPLC (column B) was used to purify the compound. Product was collected with R_t=10.5 min, which was checked by MS. The presence of product was confirmed by ^{31}P and ^{1}H NMR spectroscopy.^{1}H NMR (500 MHz, D_2O) δ/ppm = 8.28 (s, 1H; NH), 5.89-5.82 (d, J = 35 Hz, 1H; =CH_{trans}), 5.50-5.47 (d, J = 15.5 Hz, 1H; =CH_{cis}), 3.96-3.93 (t, J = 8 Hz, 1H; Hα Leu), 1.69-1.53 (m, 3H; Hβ/γ Leu), 0.82-0.79 (m, 6H; Hδ Leu). ^{31}P NMR (202 MHz, $CDCl_3$) δ/ppm = 6.44.Yield: 1.2 mg HRMS (ESI) m/z calculated for $C_{18}H_{28}N_2O_6P$ 399.1685, found 399.1686.

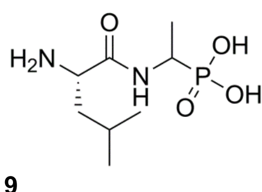
Synthesis of protected L-Leu-Ala(P)



N-Cbz-L-Leu-OH (209 mg, 0.76 mmol) was suspended in 3 mL of dry DCM. To this mixture, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl; 181 mg, 0.94 mmol) was added, followed by addition of dimethyl-1-aminoethyl phosphonate (120 mg, 0.63 mmol) and N-methylmorpholine (0.18 mL, 1.63 mmol); the solution was stirred at room temperature for 18 h. The reaction mixture was diluted with DCM (20 mL) and washed with 10% citric acid (10 mL), followed by sat. aqueous $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried with Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel flash chromatography and the product eluted with 3% EtOAc in hexanes (100.4 mg, 0.25 mmol, 40%).

^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 7.34-7.27 (m, 5H; arom), 5.10-5.05 (m, 2H, CH_2-Bn), 4.55-4.45 (m, 1H), 4.32-4.26 (m, 1H), 3.73-3.65 (m, 6H; OCH_3), 1.7-1.45 (m, 3H; Hβ/γ Leu), 1.35-1.22 (m, 3H; CH_3), 0.94-0.86 (m, 6H; Hδ Leu). ^{31}P NMR (202 MHz, $CDCl_3$) (δ = 28.95, 28.72); product present as two diastereomers. MS (ESI): m/z = 401.4 (M+H$^+$). TLC: R_f = 0.48 (10% MeOH in DCM)

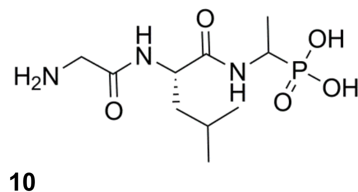
Synthesis of L-Leu-Ala(P)



Compound **8** (283 mg, 0.71 mmol) was dissolved in 9 mL of toluene and the flask was cooled in an ice-bath. To this solution, 2.1 mL of a 1 M solution of BBr_3 in hexanes (2.1 mmol) was added and the reaction was stirred for 10 min at 0 °C resulting in a yellow precipitate. The flask was placed in an oil bath maintained at 70 °C for 4.5 h. The reaction mixture was cooled and 10 mL of dry MeOH was added to quench the reaction. A clear greenish-black solution formed, which was concentrated on a rotary evaporator to remove all solvents, the residue was redissolved in 5 mL of EtOAc and extracted with H_2O (5 mL). The aqueous layer was collected in a vial and lyophilized. Crude yield: 189.6 mg.

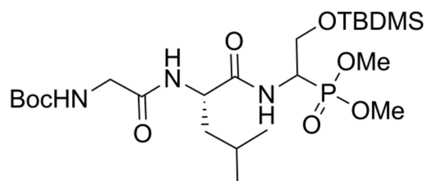
^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 4.10-3.90 (dq, *J* = 10 Hz; Hα Ala^P), 3.85-3.80 (dd, *J* = 10 Hz; Hα Leu), 1.65-1.50 (m, 3H; Hβ/γ), 1.22-1.12 (m, 3H; CH_3), 0.85-0.76 (t, 6H; Hδ Leu). ^{31}P NMR (202 MHz δ = 23.45, 22.81) indicated the presence of the product as two diastereomers. HRMS (ESI) m/z calculated for $C_8H_{19}N_2O_4P$ 239.1161, found 239.1158. The crude product (189.6 mg) was purified by RP-HPLC (column B) to yield 20 mg of each diastereomers.

Synthesis of Gly-L-Leu-Ala(P)



Gly-L-Leu-Ala(P) was synthesized from Boc-Gly-L-Leu-Ala(P) methyl ester using similar chemistry as reported previously (6). ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 4.20-4.17 (t, *J* = 5 Hz, 1H; Hα Leu), 4.08-4.00(m, 1H; Hα Ala^P), 3.70-3.65 (s, 2H; Hα Gly), 1.54-1.37 (m, 3H; Hβ/γ Leu), 1.20-1.12 (m, 3H; Hβ Ala), 0.77-0.67 (dd, *J* = 5 Hz, 6H; Hδ Leu). ^{31}P NMR (202 MHz δ/ppm = 23.45, 22.81) indicated presence of product as two diastereomers. MS (ESI): m/z =237.1 (M−H$^+$). The 33 mg of crude product was purified by RP HPLC (column B) to give 4 mg and 2.4 mg of the two diastereomers.

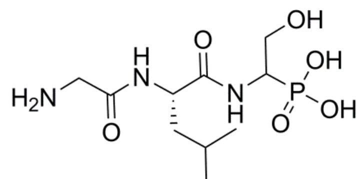
Synthesis of protected Gly-L-Leu-Ser(P)



Compound **11** was synthesized by peptide coupling of Boc-Gly-L-Leu-OH (6) with a Ser(P) analog obtained after deprotection of the N-terminal Cbz group of compound **1**. Boc-Gly-L-Leu-OH (242 mg, 0.84 mmol) was dissolved in 3 mL of dry DCM, to which dimethyl (1-amino-2-((tert-butyldimethylsilyl)oxy)ethyl)phosphonate (198.3 mg, 0.7

mmol) and N-methylmorpholine (0.12 mL,1.09 mmol) were added. To this suspension, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl, 201.2 mg, 1.05 mmol) was added and the reaction was stirred for 8 h 45 min. The reaction mixture was then diluted with DCM and extracted with 10% citric acid (10 mL), sat. aqueous $NaHCO_3$ (20 mL), and brine (20 mL). The organic layer was dried over sodium sulfate, filtered and concentrated. The residue was purified by silica gel flash chromatography eluting with 3% MeOH in DCM. (165 mg, 0.3 mmol, 42%). ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 6.49-6.45 (t, J = 10 Hz, 1H), 4.75-4.45 (m, 1H), 4.03-3.96 (m, 1H), 3.82-3.74 (m, 6H; OCH_3), 1.70-1.52 (m, 4H), 1.45 (s; 9H, $C(CH_3)_3$ Boc), 1.02-0.96 (m, 1H), 0.96-0.91 (t, 6H, Hδ Leu), 0.91-0.89 (d, 9H; $SiC(CH_3)_3$), 0.08 (t, 6H; $Si(CH_3)_3$). ^{31}P NMR (202 MHz, $CDCl_3$) δ/ppm = 26.41, 26.27 indicated product as two diastereomers. MS(ESI) m/z: 554.2 ($M+H^+$). TLC: R_f = 0.3 (10% EtOAc).

Synthesis of Gly-L-Leu-Ser(P)



12

Compound **11** (54.3 mg, 0.098 mmol) was dissolved in 1.5 mL of toluene and the flask was cooled in an ice bath. To this solution, 1 M BBr_3 in hexanes (0.3 mL, 3.06 equiv) was added and the reaction was stirred on an ice bath for 10 min. Then the flask was transferred to an oil bath pre-heated to 70 °C and the reaction was stirred for another 4 h. After cooling the reaction mixture to room temperature, 2 mL of dry MeOH was added to quench the reaction. The resulting clear solution was concentrated on a rotary evaporator after which the residue was taken up in EtOAc (5 mL) and extracted with water (5 mL). The aqueous layer was lyophilized (crude yield: 45.2 mg). The crude material was purified by RP-HPLC (column B) to yield two diastereomers (1.6 mg and 1.5 mg).^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 4.32-4.28 (m, 1H; Hα Leu), 4.10-4.04 (m, 1H; Hα Ser^P), 3.81-3.75 (m, 1H), 3.76-3.72 (d, 2H, Hα Gly), 3.60-3.52 (m, 1H), 1.62-1.46(m, 3H, Hβ/γ Leu), 0.80-0.75 (dd, J = 5 Hz, 6H, Hδ Leu). Both diastereomers had similar ^{1}H NMR pattern. ^{31}P NMR (202 MHz δ = 14.4, 14.7). HRMS (ESI) m/z calculated for $C_{10}H_{22}N_3O_6P$ 312.1324, found 312.1322.

Supplementary Figures

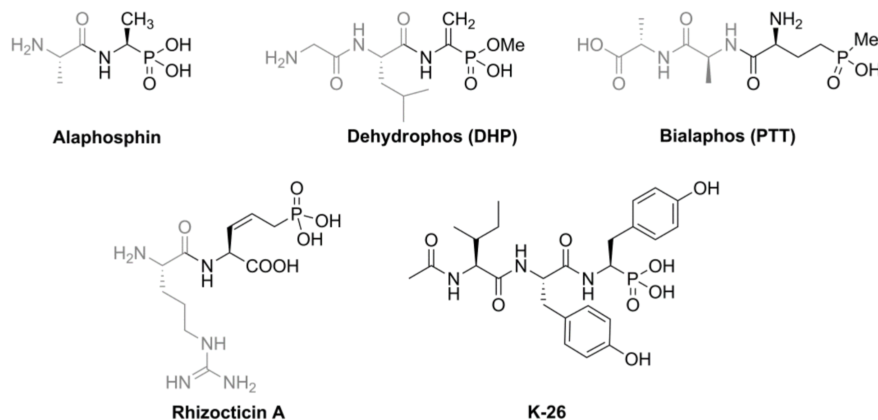


Fig. S1. Representative phosphonopeptides. Man-made and natural phosphonopeptides. For Trojan horse compounds, the active "warhead" of the molecule that is released upon entry into the cell is shown in dark lines.

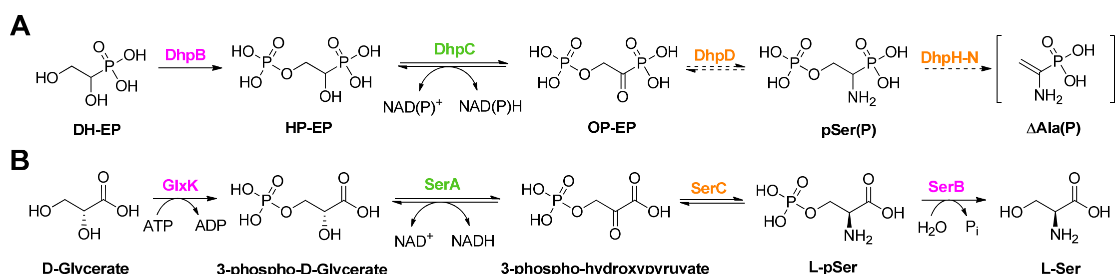


Fig. S2. Originally proposed biosynthetic pathway towards ΔAla(P) starting from DH-EP and based on single gene deletion experiments. A. DhpB, DhpC, DhpD and the N-terminal domain of DhpH were proposed to catalyze the formation of ΔAla(P) using DH-EP as starting material (7). **B.** Biosynthesis of serine in *E. coli*. The three consecutive steps catalyzed by glycerate kinase II (GlxK), 3-D-phosphoglycerate dehydrogenase (SerA) and phosphoserine phosphatase (SerC) are similar to the steps in the proposed biosynthesis of dehydrophos in panel A.

Note: because of the reversibility of the reactions catalyzed by DhpC and DhpD, it is not unexpected that the *dhpC* and *dhpH* mutants accumulate very similar intermediates (i.e. mainly DH-EP and HP-EP). For instance, the reported equilibrium constant for SerA is 7×10^{-11} for the forward oxidation reaction (8). The lack of observation of accumulation of OP-EP in any of the mutants could be because α-oxo phosphonates are not very stable in water and their signals in ^{31}P NMR spectra are expected to be at around zero ppm (9), a region in ^{31}P-NMR usually populated by very large signals of phosphate metabolites. Finally, the observed accumulation of pSer(P) in the *dhpH* mutant (7) was a key reason for the proposed pathway in panel A.

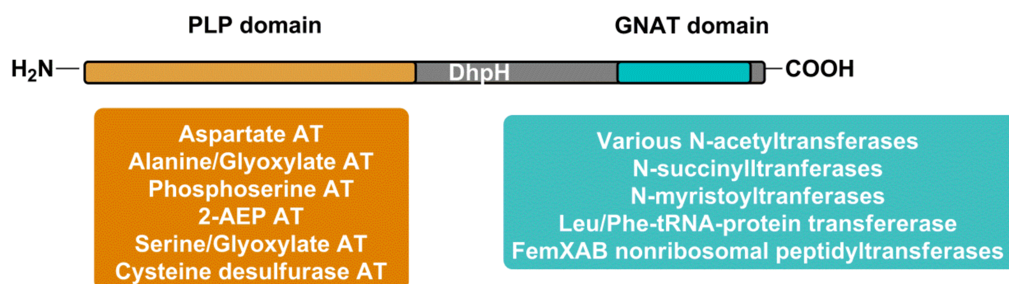


Fig. S3. Organization of the two domains in DhpH. The pyridoxal 5' -phosphate (PLP) domain comprises the N-terminal part of the DhpH sequence and shows similarity with several well-characterized aminotransferases (AT), whereas the C-terminal domain harbors the GCN5-related N-acetyltransferase (GNAT) domain found in numerous acetyl- and peptidyl-transferases.

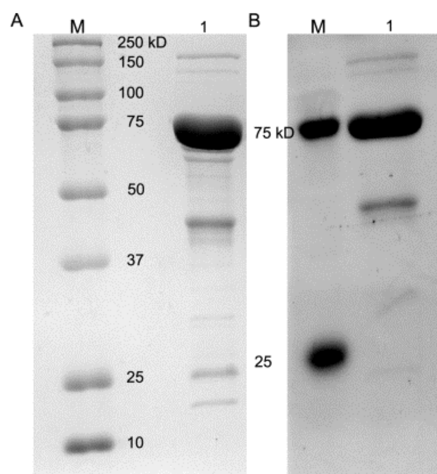


Fig. S4. SDS-PAGE analysis of His_6-DhpH after IMAC purification. A. Coomassie staining**. B**. InVision His-tag in-gel staining. Lane M: Prestained precision Plus Protein Standards (Bio-Rad). Lane 1: His_6-DhpH.

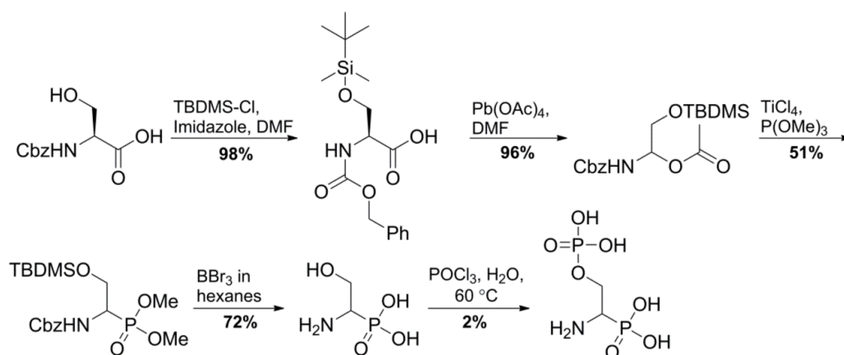


Fig. S5. Synthetic scheme towards *rac*-pSer(P).

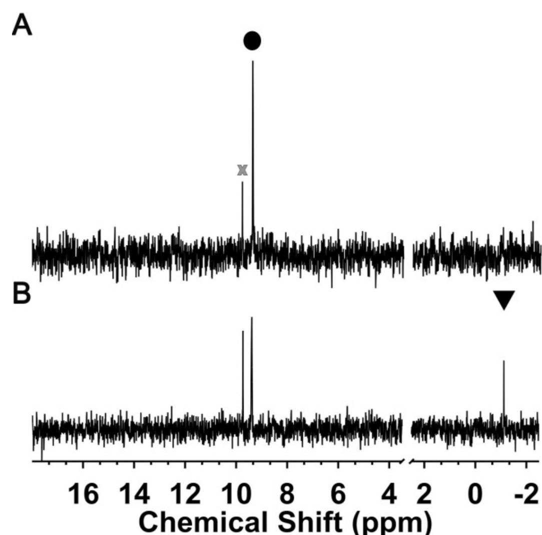


Fig. S6. ^{31}P NMR analysis of DhpD activity with *rac*-Ser(P). A. ^{31}P NMR spectrum after incubation of *rac*-Ser(P) (●) with DhpD in the absence of pyruvate; the peak marked with × corresponds to unknown impurity present in the Ser(P) standard. **B.** ^{31}P NMR spectrum after conversion of *rac*-Ser(P) into 2-hydroxy-1-oxoethylphosphonate (▼) by DhpD in the presence of pyruvate. The phosphate peak (between 2 and 4 ppm) was omitted for clarity.

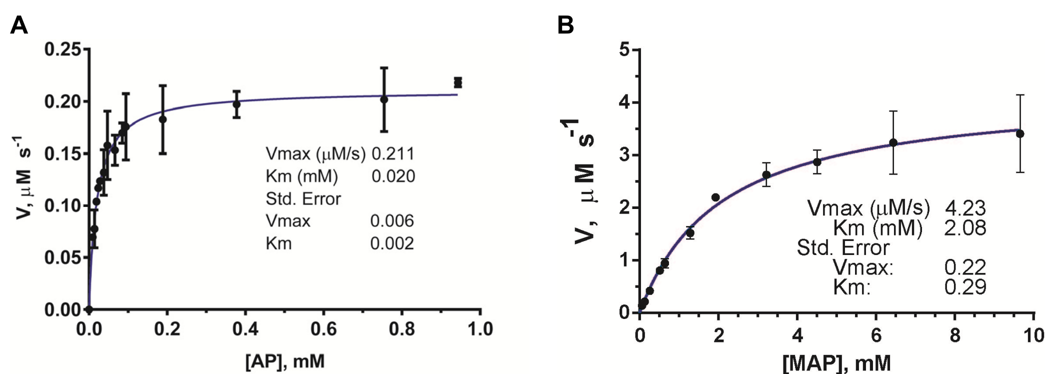


Fig. S7. Michaelis-Menten plot showing the dependence of the rates of DhpD-catalyzed formation of Ala(P) on the concentrations of AP and MAP. A. Reaction assay was carried out at 25 ºC and consisted of: 0.9 M L-Ala, 15 Units of LDH, 0.11 μM DhpD, 300 μM NADH in 200 μL of Na-HEPES, pH 8.0, **B.** Reaction assay was carried out at 25 ºC and consisted of 50 mM HEPES, pH 8.0, 0.9 M L-Ala, 350 μM NADH, 15 Units of LADH, 2 μM DhpD in a final volume of 156 μL. All reactions were started with the addition of DhpD.

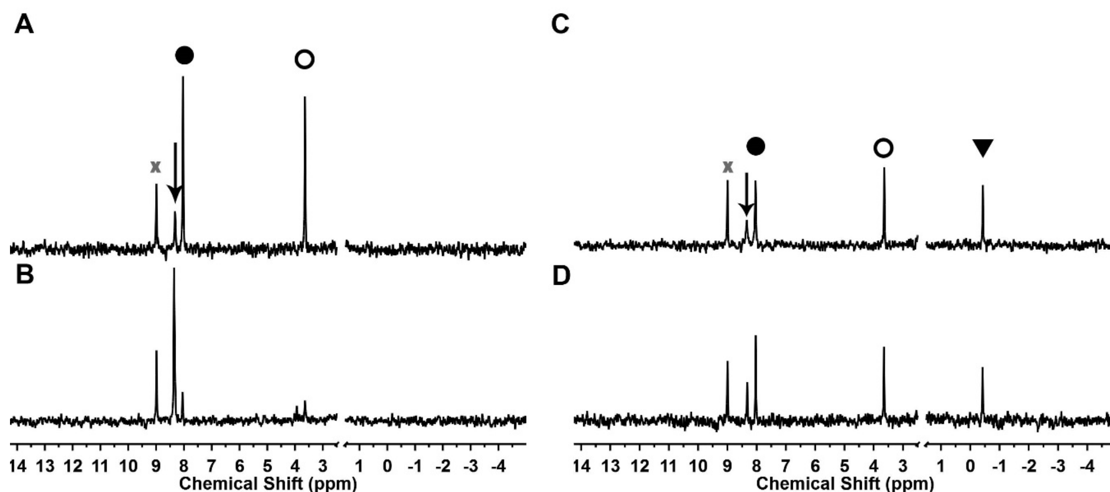


Fig. S8. ^{31}P NMR analysis of DhpH and DhpH-N activity with *rac*-pSer(P). A. ^{31}P NMR spectrum after 12 h incubation of *rac*-Ser(P) (●, signal of P attached to carbon atom, ○, signal of P attached to oxygen atom) in the assay buffer; the peak marked with arrow belongs to SerP (the starting material of pSerP preparation), the peak marked with × corresponds to unknown impurity carried over from the SerP synthesis. **B.** ^{31}P NMR spectrum after treatment of *rac*-Ser(P) with 50 Units CIP. **C** and **D.** ^{31}P NMR spectrum after 12 h incubation of 3 mM *rac*-Ser(P) with DhpH (**C**) or DhpH-N (**D**) (50 μM in 50 mM HEPES, pH 7.5). The signal of the generated product AP is marked with (▼). The phosphate peak (between 2 and 4 ppm) was omitted for clarity.

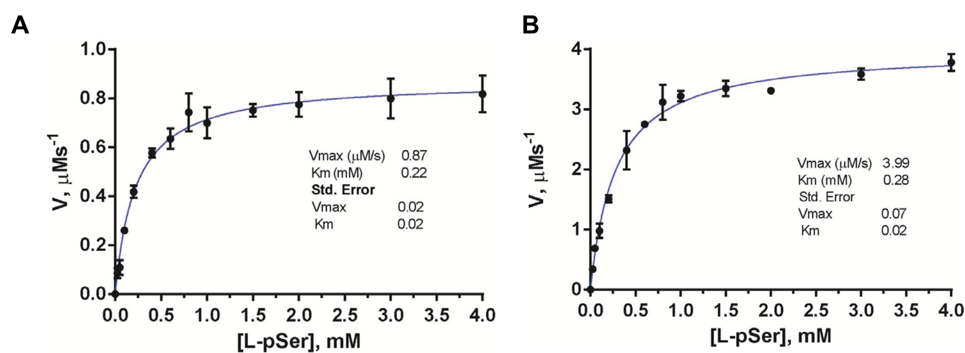


Fig. S9. Michaelis-Menten plot for conversion of L-pSer to pyruvate by DhpH and DhpH-N. A. The assays (157 µL final volume) were carried out at 25 ºC and contained: 0.25 - 4 mM L-pSer, 15 Units of LDH, 1.14 µM DhpH, and 300 µM NADH in 50 mM Na-HEPES, pH 8.0. **B.** The assays (157 µL final volume) were carried out at 25 ºC and contained: 0.025 - 4 mM L-pSer, 15 Units of LDH, 3.6 µM DhpH-N, and 300 µM NADH in 50 mM Na-HEPES, pH 8.0. The reaction assay, conducted in triplicated for each time point, was initiated by the addition of DhpH or DhpH-N to a solution containing all other components.

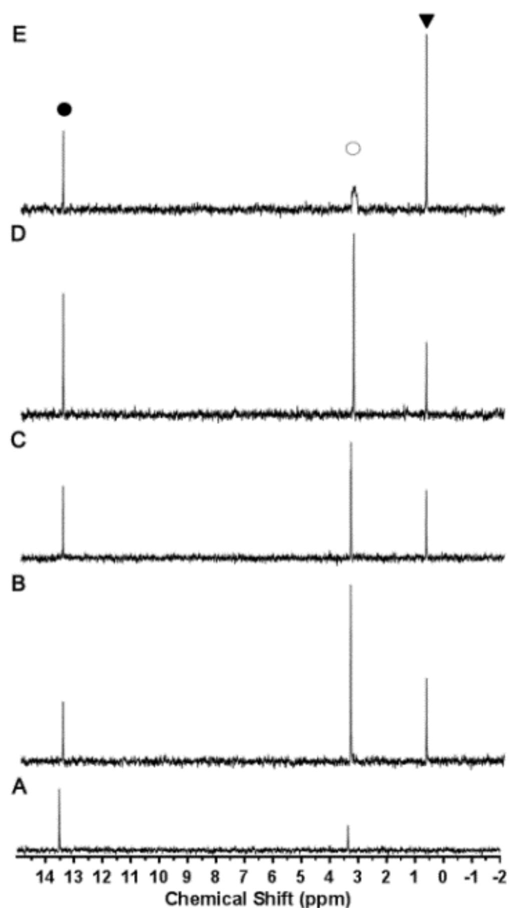
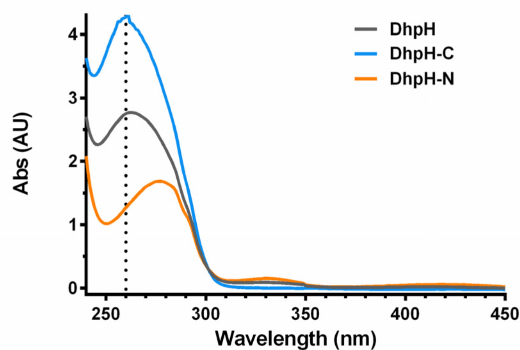


Fig. S10. ^{31}P NMR spectra of DhpH-N activity with L-Ala(P) in the presence of different α-ketoacids. A. Control: L-Ala(P) in the absence of α-ketoacids**. B.** L-Ala(P) in the presence of 10 mM pyruvate (integrated area L-Ala(P)/Pi (1:2.5). **C.** L-Ala(P) in the presence of 10 mM oxaloacetate. **D.** L-Ala(P) in the presence of 10 mM α-ketoglutarate**. E.** Spectrum of reaction B spiked with AP (integrated area L-Ala(P)/Pi (1:3.7). All reactions contained 10 mM L-Ala(P) and 50 μM enzyme (in KPi buffer) in 50 mM Na-HEPES, pH 8.0. Open circle denotes inorganic phosphate (Pi), closed circle denotes L-Ala(P), triangle denotes acetylphosphonate (AP).

A



B

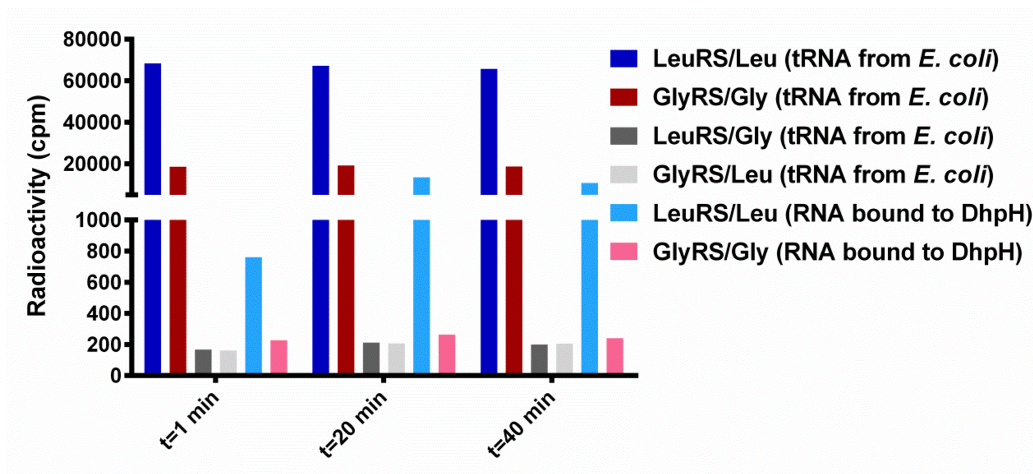


Fig. S11. UV-Vis Analysis of DhpH and 14C labeling of extracted nucleic acids. A. DhpH (full length) and DhpH-C (nucleic acid binding domain) exhibited a significant higher A260/A280 compared to DhpH-N (PLP domain) **B.** Nucleic acids extracted from DhpH were labeled either with ^{14}C-Leu/LeuRS or ^{14}C-Gly/GlyRS as described in the text.

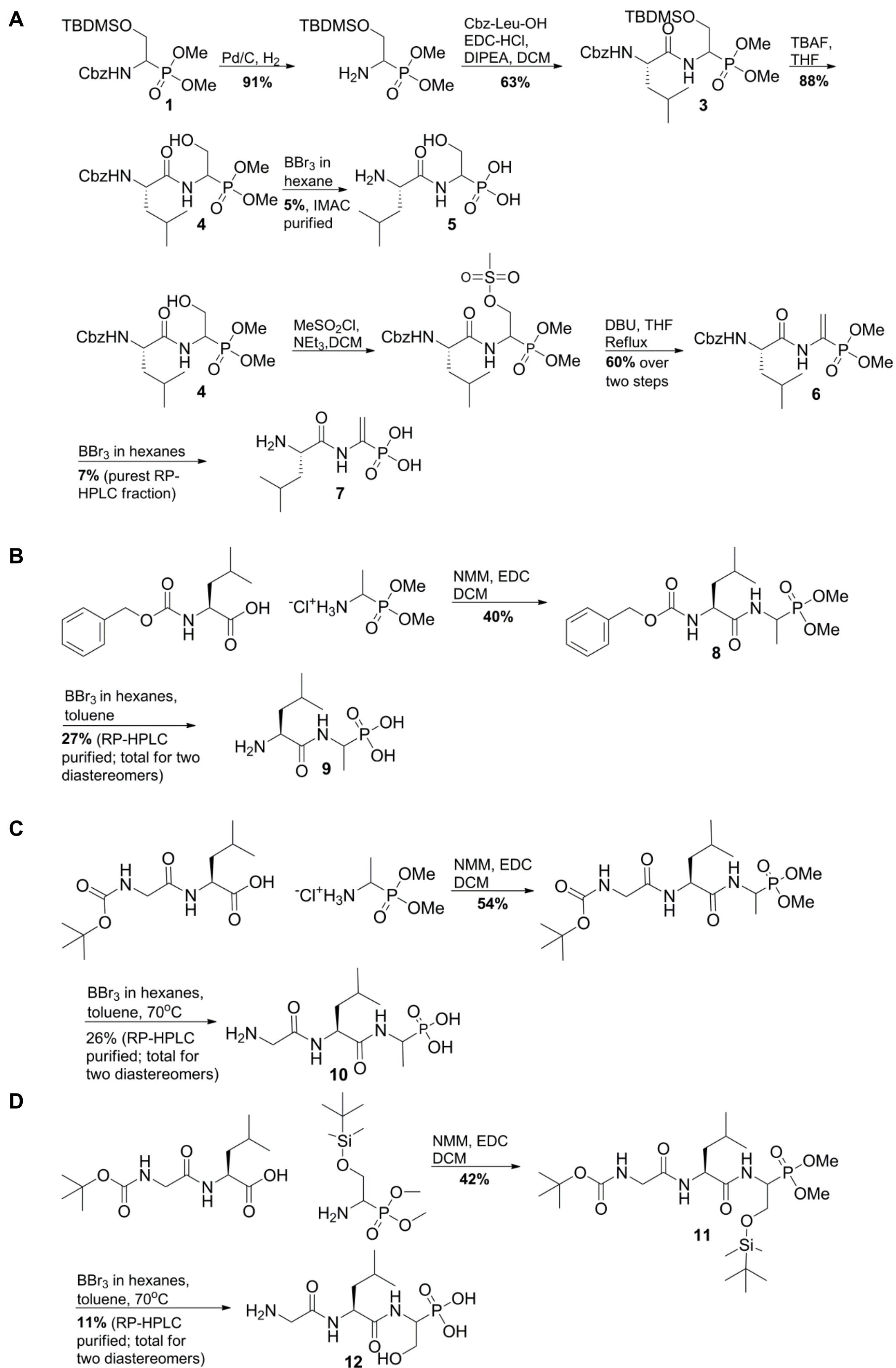


Fig. S12. Schemes for the syntheses of peptides. A. Synthesis of L-Leu-Ser(P) and L-Leu-ΔAla(P) **B.** Synthesis of L-Leu-Ala(P). **C.** Synthesis of Gly-L-Leu-Ala(P). **D.** Synthesis of Gly-L-Leu-Ser(P).

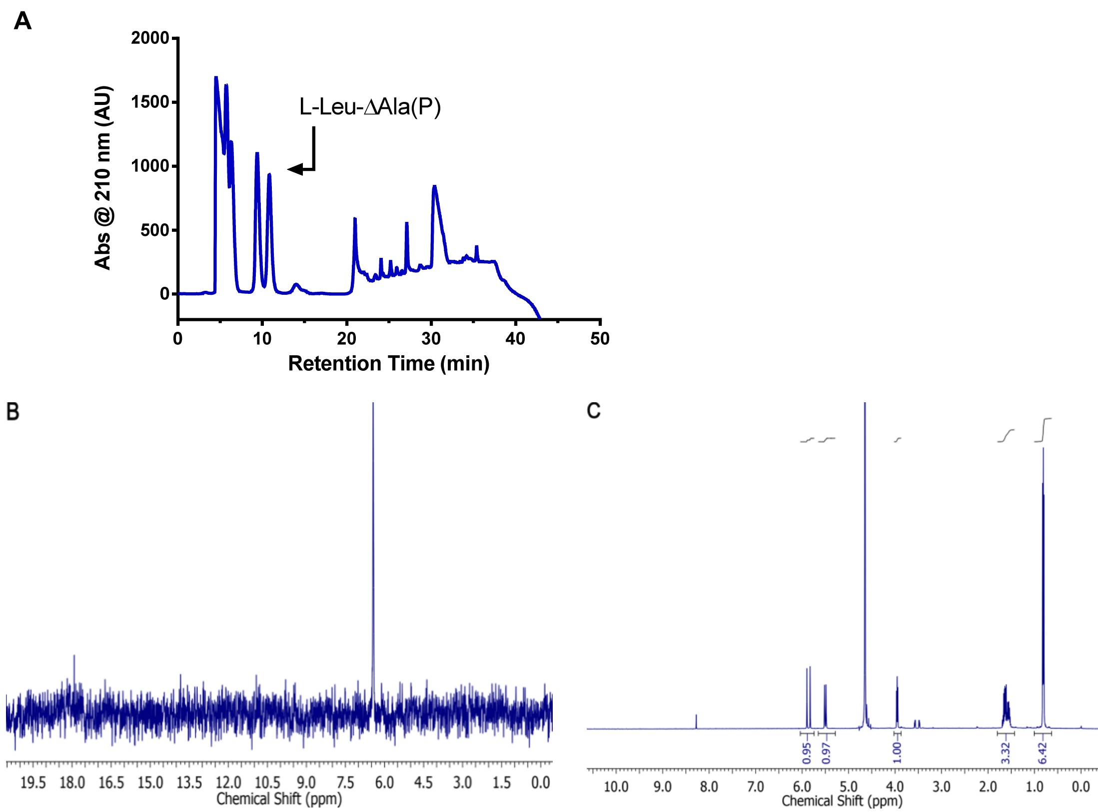


Fig. S13. HPLC traces and NMR analysis of synthetic L-Leu-ΔAla(P). A. Elution from a semi-preparative RP-fusion column. Conditions: 0.1% formic acid in water, isocratic for 20 min, flow 4 mL/min. Peak with retention time of 11 min corresponds to L-Leu-Ala(P). **B.** ^{31}P NMR spectrum of L-Leu-ΔAla(P) in D_2O. **C.** ^{1}H NMR spectrum of L-Leu-ΔAla(P) in D_2O.

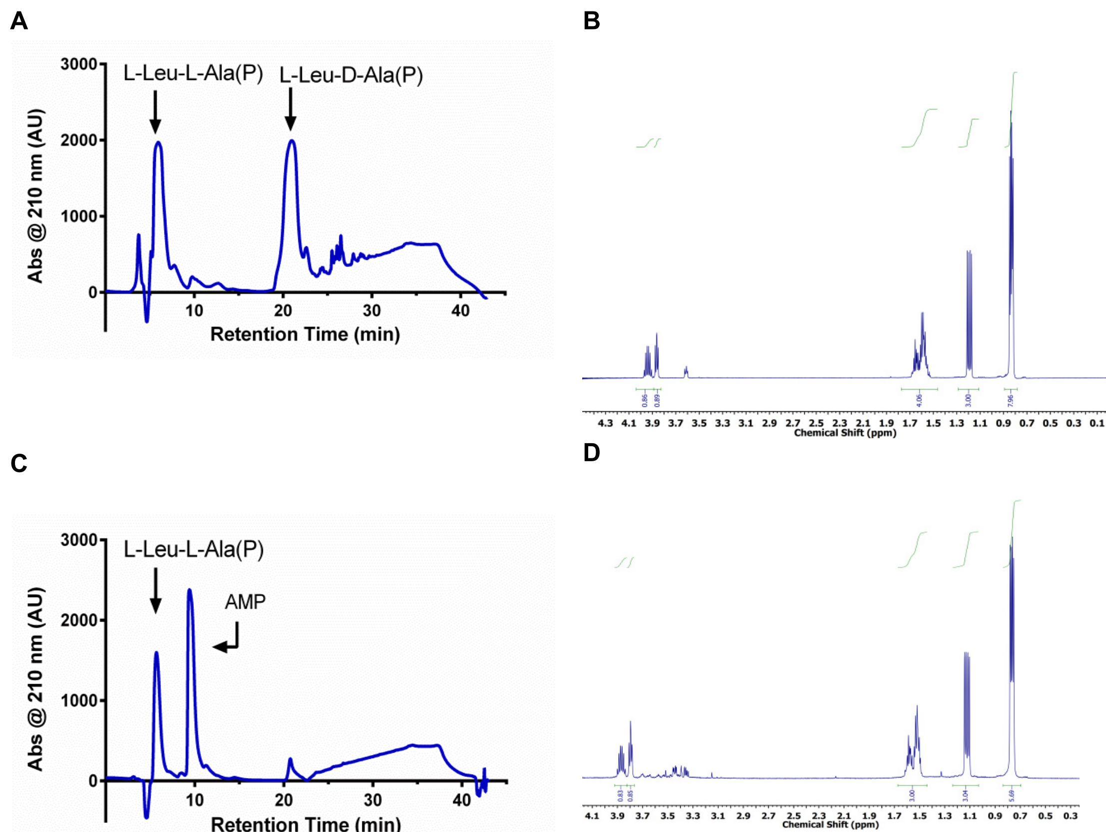


Fig. S14. HPLC traces and ^{1}H NMR of synthetically and enzymatically prepared L-Leu-L-Ala(P). A. HPLC trace of synthetic L-Leu-(L/D)-Ala(P) **B.** ^{1}H NMR spectrum of HPLC fraction containing synthetic L-Leu-L-Ala(P) in D_2O. **C.** HPLC trace of enzymatically prepared L-Leu-L-Ala(P). **D.** ^{1}H NMR spectrum of enzymatically prepared L-Leu-L-Ala(P) in D_2O.

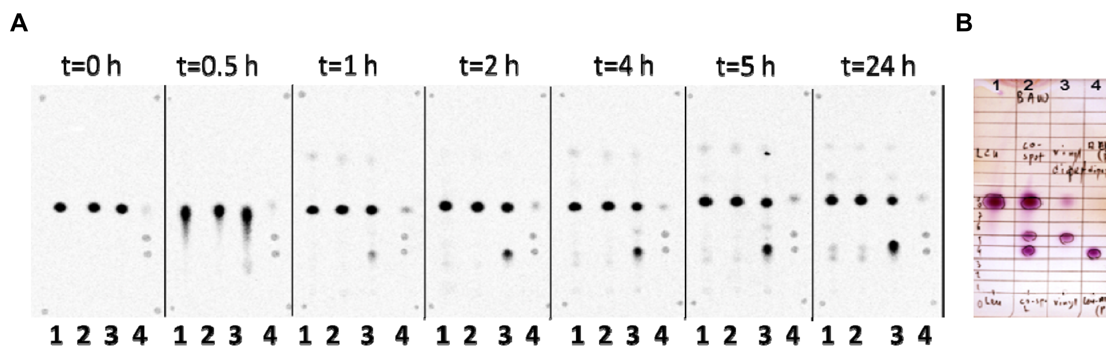


Fig. S15. Radioactive TLC analysis of rac-pSer(P) conversion to L-[^{14}C(U)]-Leu-Ala(P) by the activity of DhpH and DhpD in a one-pot reaction. A. Scanned phosphor imaging plate of a silica TLC sheet spotted with: lane 1, a 0.5 µL aliquot of a reaction containing DhpH, *rac*-pSer(P), L-[^{14}C(U)]-Leu, tRNA and (re)generation components of Leu-$tRNA^{Leu}$; lane 2, a 0.5 µL aliquot of a reaction containing the same components as lane 1 in addition to L-Ala; lane 3, a 0.5 µL aliquot of a reaction containing the same components as lane 1 in addition to L-Ala and DhpD. Lane 4: Spots created after the development of the TLCs corresponding to Rf values of L-Leu-L-Ala(P), L-Leu-ΔAla(P), and leucine. **B.** Ninhydrin-stained TLC of Leu (lane 1), Leu + L-Leu-ΔAla(P) + L-Leu-L-Ala(P) (lane 2), L-Leu-ΔAla(P) (lane 3) and L-Leu-L-Ala(P) (lane 4).

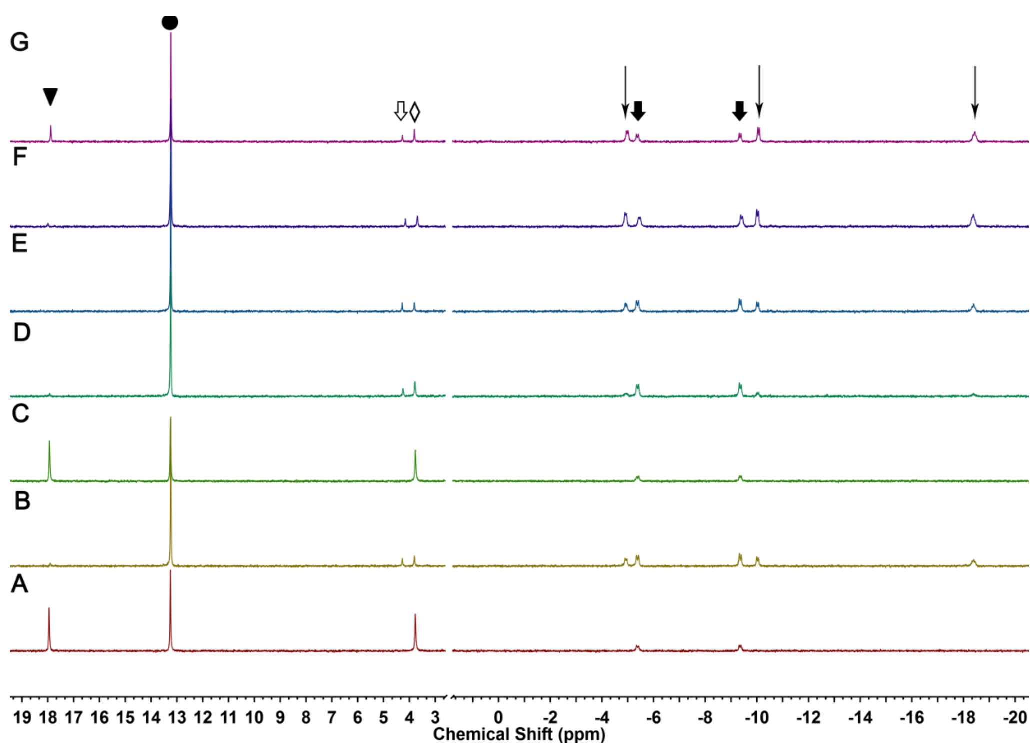


Fig. S16. ^{31}P NMR analysis of enzymatic formation of L-Leu-L-Ala(P) by DhpH. Each reaction contained: 10 mM of the appropriate enantiomer of Ala(P) (circle), 4 mM L-Leu, 4 mM ATP (thin arrows), 2 mg total tRNA (from *E. coli*), 250 µg BSA, 5 µM LeuRS, 20 U of TIPP, and a 50 µL aliquot of the appropriate enzyme in 100 mM Na-HEPES, 10 mM KCl, 20 mM $MgCl_2$, pH 7.5 (500 µL total volume). ADP is denoted by thick arrows AMP is denoted by a diamond. Triangle denotes the product L-Leu-L-Ala(P). Open block arrow denotes an uncharacterized phosphorylated compound related to LeuRS activity by itself in the absence of DhpH. **A.** DhpH incubated with L-Ala(P). **B.** DhpH-N incubated with L-Ala(P) **C.** DhpH-C incubated with L-Ala(P). **D.** DhpH incubated with D-Ala(P). **E.** DhpH-N incubated with D-Ala(P). **F.** DhpH-C incubated with D-Ala(P). **G.** Control experiment in which DhpH-C was incubated with L-Ala(P) in the absence of externally added tRNA. Partial formation of product is due to tRNA that co-purified with DhpH-C. Phosphate peak near 2 ppm is omitted for clarity.

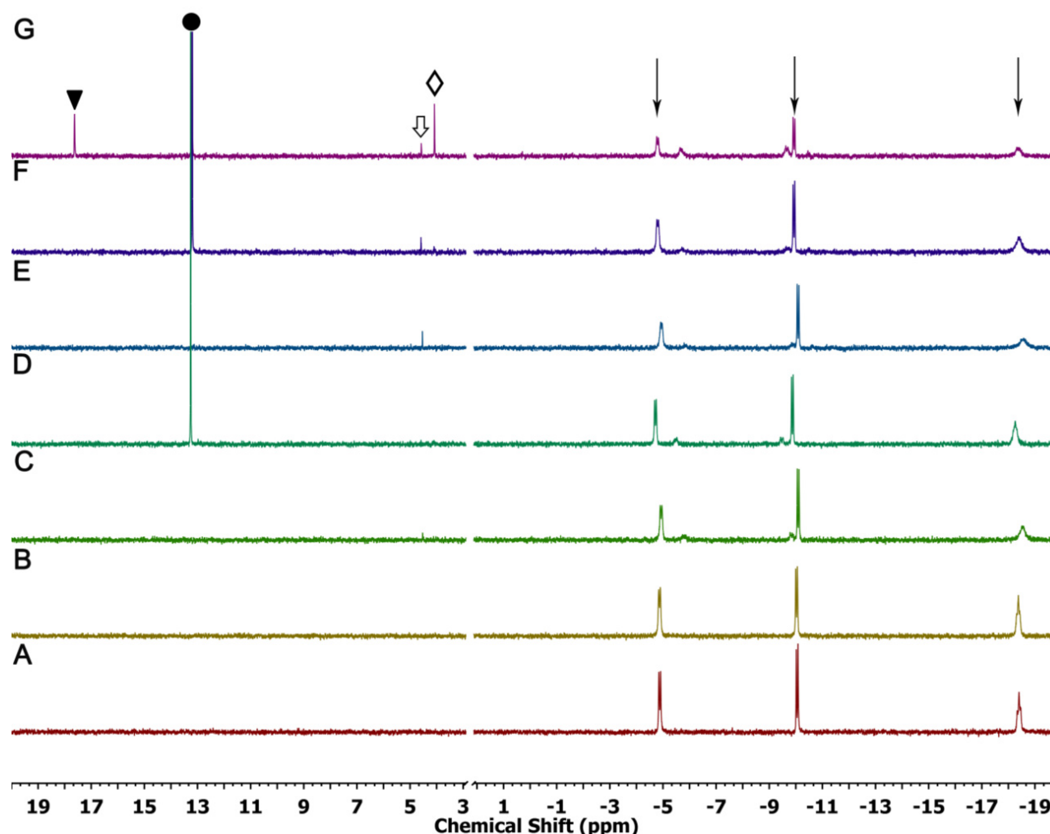


Fig. S17. ^{31}P NMR analysis of control experiments of enzymatic formation of L-Leu-L-Ala(P) by DhpH-C in the absence of tRNA. All reactions were carried out in 100 mM Na-HEPES, 10 mM KCl, 20 mM $MgCl_2$, pH 7.5 (500 µL total volume). L-Leu was added to a final concentration of 4 mM. ATP was added to a final concentration of 4 mM (thin arrows). L-Ala(P) (circle) was added to 10 mM final concentration (when included). DhpH-C was added to a final concentration of 50 µM (when included). LeuRS was added to a final concentration of 5 µM (when included). Triangle denotes the product L-Leu-L-Ala(P). **A.** L-Leu and ATP in reaction buffer. **B.** L-Leu, ATP and TIPP (20 U) in reaction buffer. **C.** L-Leu, ATP, TIPP (20 U) and DhpH-C in reaction buffer. **D.** L-Leu, ATP, TIPP (20 U), L-Ala(P) and DhpH-C in reaction buffer. **E.** L-Leu, ATP, LeuRS in reaction buffer. **F.** L-Leu, ATP, TIPP (20 U), L-Ala(P) and LeuRS in reaction buffer. **G.** L-Leu, ATP, TIPP (20 U), L-Ala(P), LeuRS and DhpH-C in reaction buffer. Phosphate peak between 2 and 3 ppm is omitted for clarity.

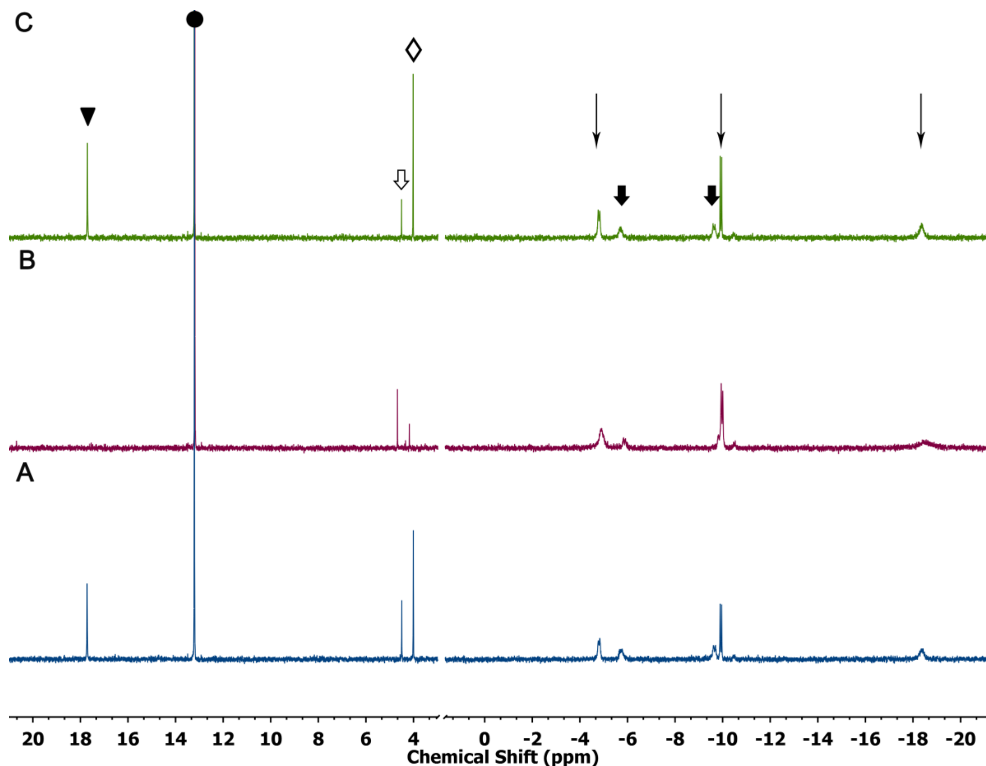


Fig. S18. ^{31}P NMR analysis of DhpH activity after RNase treatment. An aliquot of DhpH-C was treated with either BSA (0.5 mg) or with DNase (20 U) or with RNase cocktail from Ambion (200 U T1, 5 Units A) for 30 min at room temperature. All reactions were carried out in 100 mM Na-HEPES, 10 mM KCl, 20 mM $MgCl_2$, pH 7.5 (500 µL total volume). L-Leu was added to a final concentration of 4 mM. ATP was added to a final concentration of 4 mM (thin arrows). L-Ala(P) (circle) was added to 10 mM final concentration. Pretreated DhpH-C was added to a final concentration of 50 µM. LeuRS was added to a final concentration of 5 µM, 10 U of TIPP was also added to reaction mixture. Triangle denotes L-Leu-L-Ala(P) product. Phosphate peak (break like) is omitted for clarity. **A**. NMR spectrum of the reaction mixture containing BSA-treated DhpH-C. **B.** NMR spectrum of the reaction mixture containing RNase-treated DhpH-C. **C.** NMR spectrum of the reaction mixture containing DNase-treated DhpH-C.

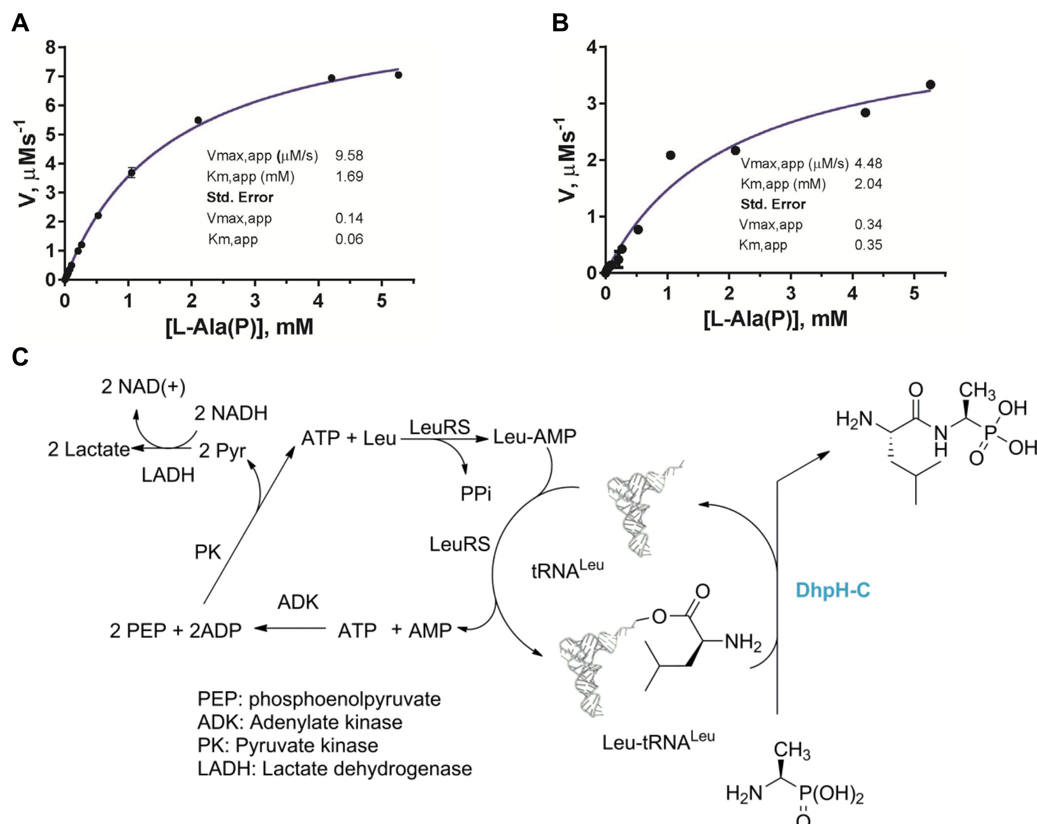


Fig. S19. UV-Vis spectrophotometric assay for calculating kinetic parameters for DhpH and DhpH-C. A. Dependence of the rates of catalysis by DhpH-C on substrate concentration as measured by a coupled assay shown in panel C. The reaction assay were conducted in 50 mM HEPES, 10 mM KCl, 20 mM $MgCl_2$, pH 7.6 containing 2.5 mM ATP, 10 mM L-Leu, 0.25 mM NADH, 1.2 mg total tRNA from *E. coli*, 2.5 mM PEP, 5 Units PK, 5 Units LADH, 15 Units ADK, 5 µM EcLeuRS, and 11 µM DhpH-C, in a final volume of 190 µL at 30 ºC. Rates were corrected for background (in the absence of substrate) uncoupled NADH oxidation and for stoichiometry. **B.** Dependence of the rates of catalysis by DhpH on substrate concentration as measured by a coupled assay shown in panel C. The reaction assays were conducted in 50 mM HEPES, 10 mM KCl, 20 mM $MgCl_2$, pH 7.6 containing 0.25 mM ATP, 10 mM L-Leu, 0.25 mM NADH, 1.2 mg total tRNA from *E. coli*, 2.5 mM, 5 Units PK, 5 Units LADH, 15 Units ADK, 5 µM EcLeuRS, and 6 µM DhpH, in a final volume of 190 µL at 30 ºC. Rates were corrected for background (in the absence of substrate) uncoupled NADH oxidation and for stoichiometry. **C.** General reaction scheme. The spectroscopic assays measured the rates of NADH consumption. Note: we do not know if Vmax,app and Km,app represent the true V_{max} and K_m because we do not know if we achieve saturation in Leu-tRNA^{Leu} under the assay conditions.

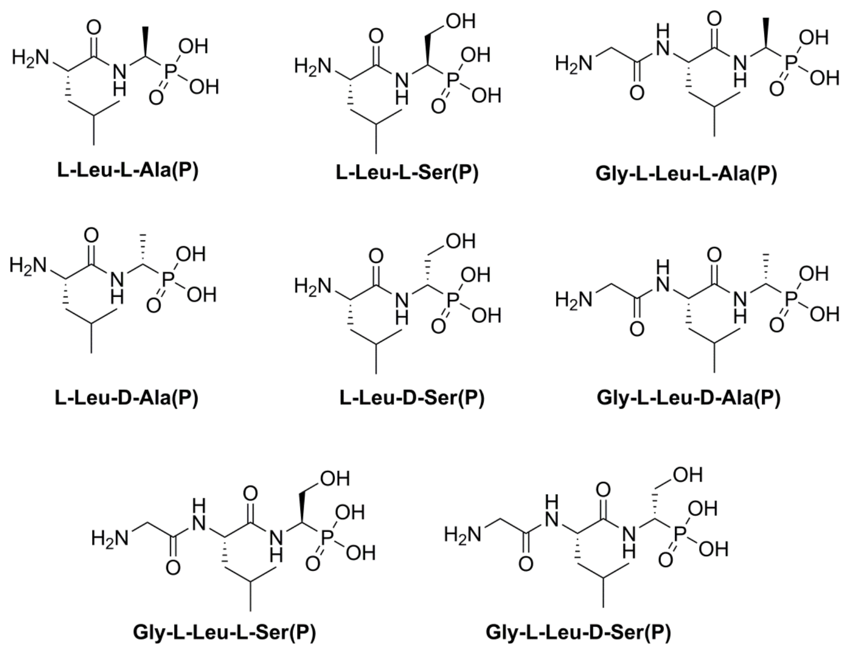


Fig. S20. List of oligopeptides screened as putative substrates of MBP-DhpJ.

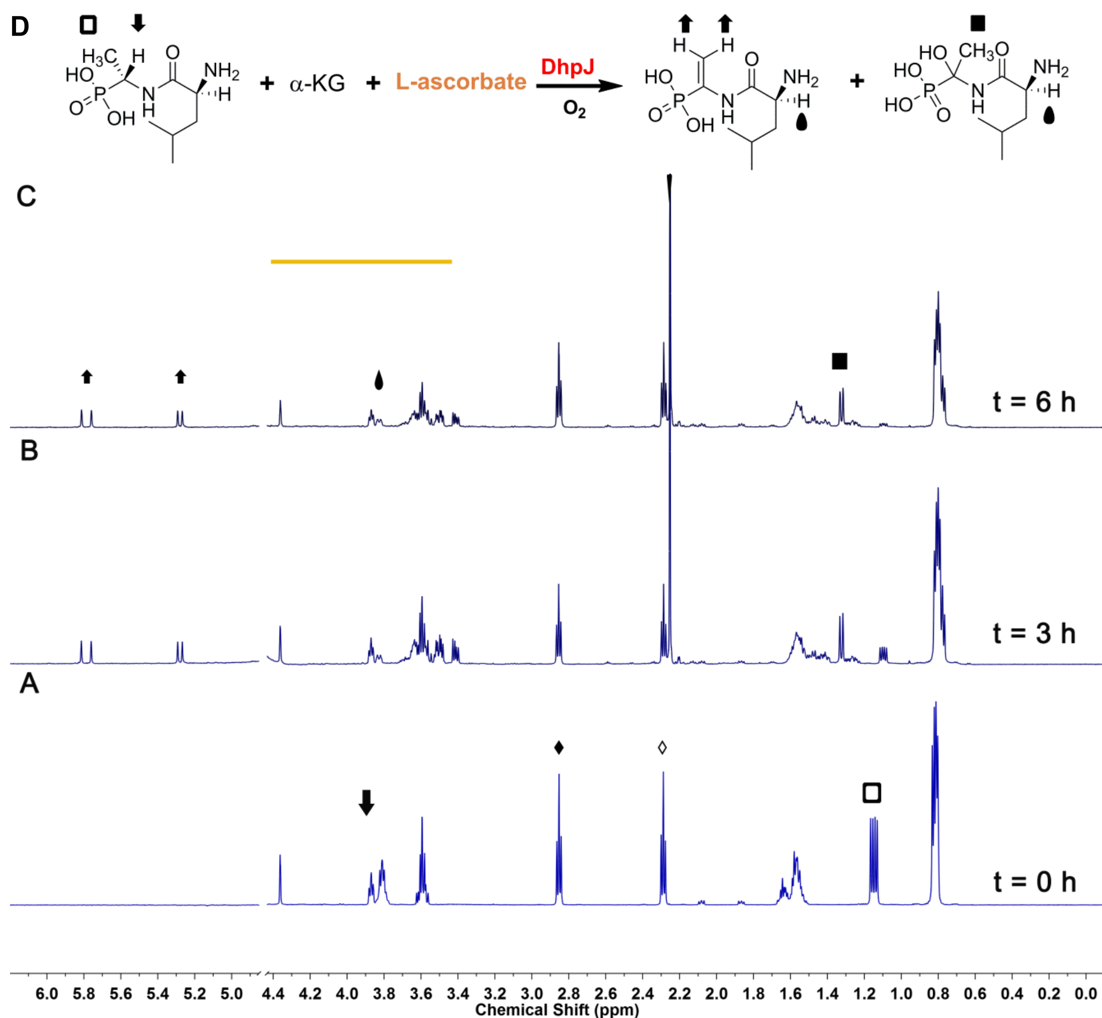


Fig. S21. ^{1}H NMR analysis of the DhpJ activity in the presence of L-Leu-L-Ala(P). The reaction (2 mL final volume) was carried out in 25 mM NaPi, pH 7.5 and contained 2 mM of L-Leu-L-Ala(P), 4 mM α-KG, 2 mM L-ascorbic acid, 0.2 mM $(NH_4)_2Fe(SO_4)_2$, 60 μM DhpJ, previously reconstituted anaerobically with 1.2 equiv. of Fe(II). Additional α-KG (2 mM) was added after 2 and 5 h. Aliquots of 500 μL were removed at different time points, treated with Chelex resin, and passed through an Amicon spin column (30 kDa MWCO) before the addition of D_2O and NMR analysis. Orange bar indicates the region of the spectra in which signals from protons that belong to ascorbate appeared. Diamonds denote signals of α-KG protons. The singlet near 2.3 ppm corresponds to the succinate protons. The water peak is omitted for clarity.

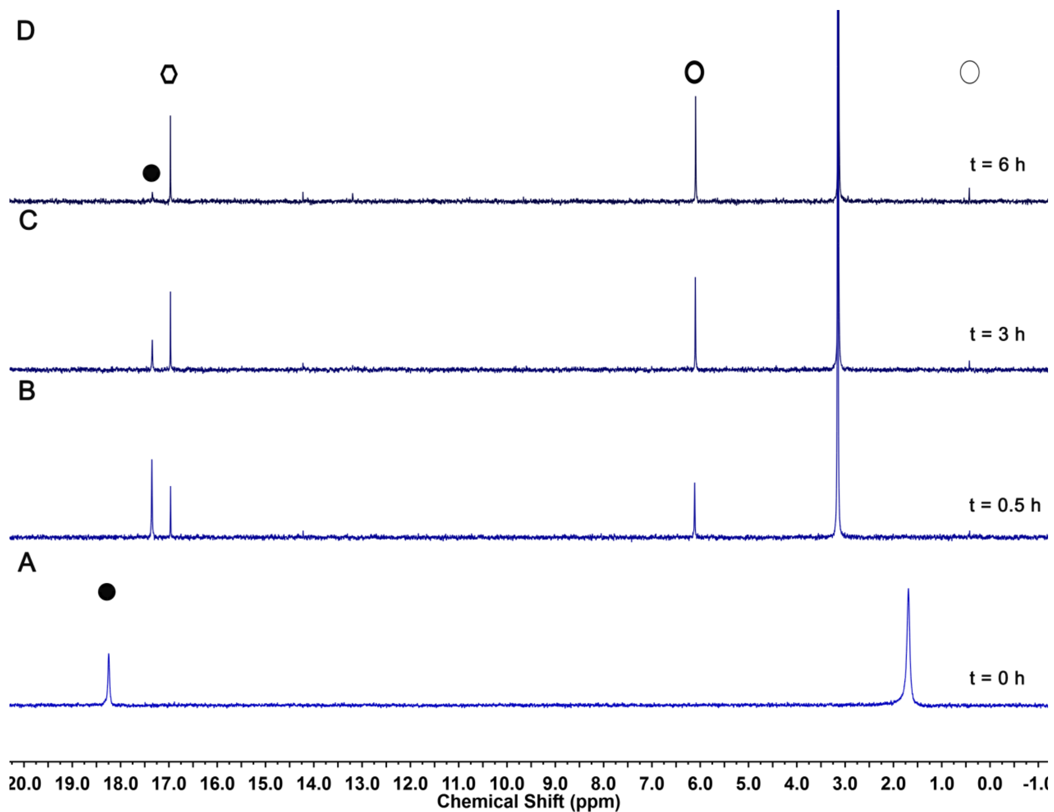


Fig. S22. ^{31}P NMR analysis of the DhpJ activity in the presence of L-Leu-L-Ala(P). See Fig. S21 for experimental details. Closed circles denote L-Leu-L-Ala(P), diamond denotes side-product, open circle (thick line) denotes L-Leu-ΔAla(P), open circle (thin line) denotes AP, presumably due to hydrolysis of L-Leu-ΔAla(P). The unlabeled peak at 1.7 ppm in panel A and at 3.2 ppm in panels B-D belongs to Pi. The differences in chemical shifts in panel A compared to the other panels is the result of small changes in pH that have a relatively large effect on chemical shift of these compounds as they have pK_a values near 7. The identity of all compounds was confirmed by spiking with authentic materials.

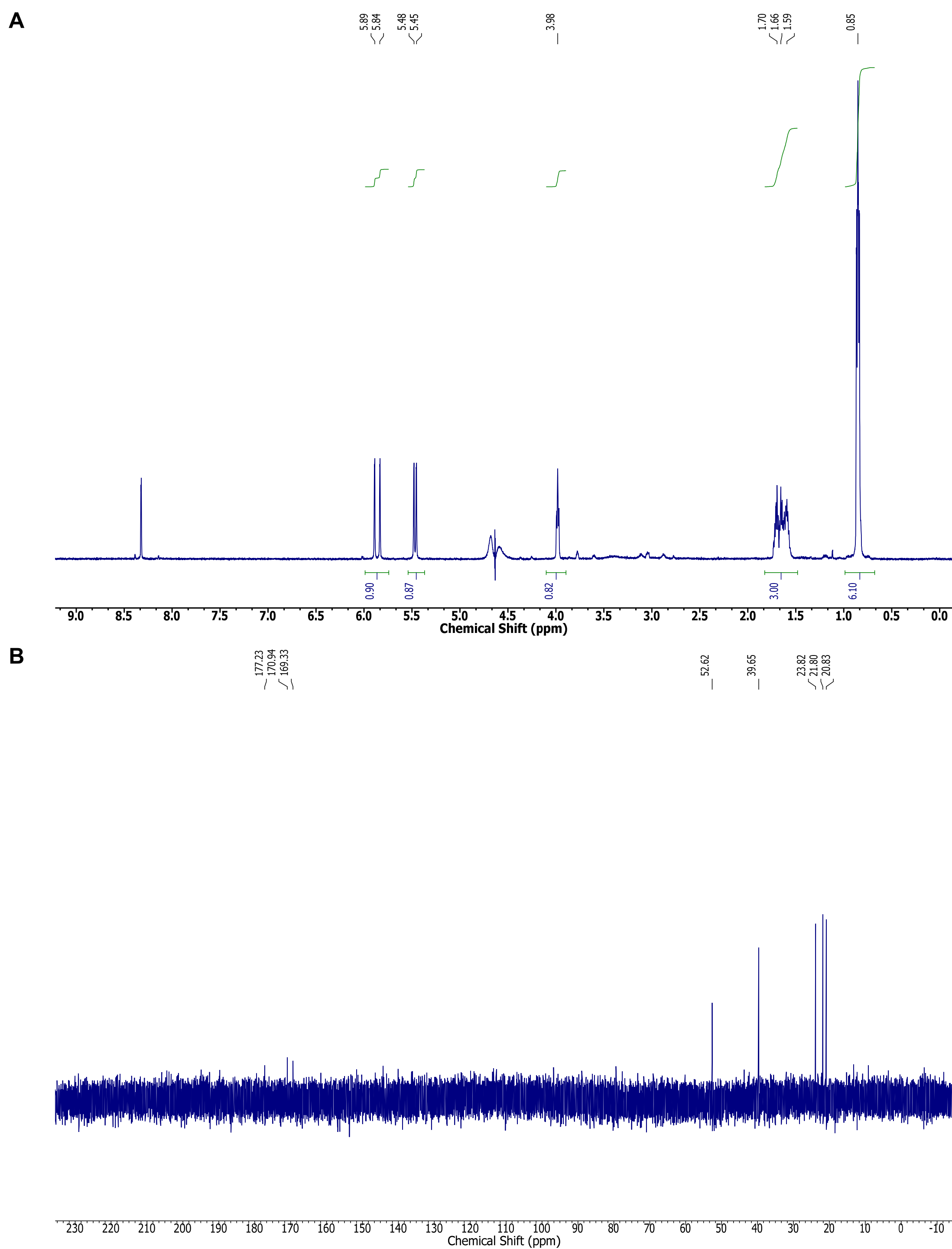


Fig. S23. NMR analysis of HPLC-purified major product of DhpJ reaction with L-Leu-L-Ala(P). A. ^{1}H NMR spectrum of L-Leu-ΔAla(P). **B.** ^{13}C NMR spectrum of L-Leu-ΔAla(P). Both spectra taken in D_2O.

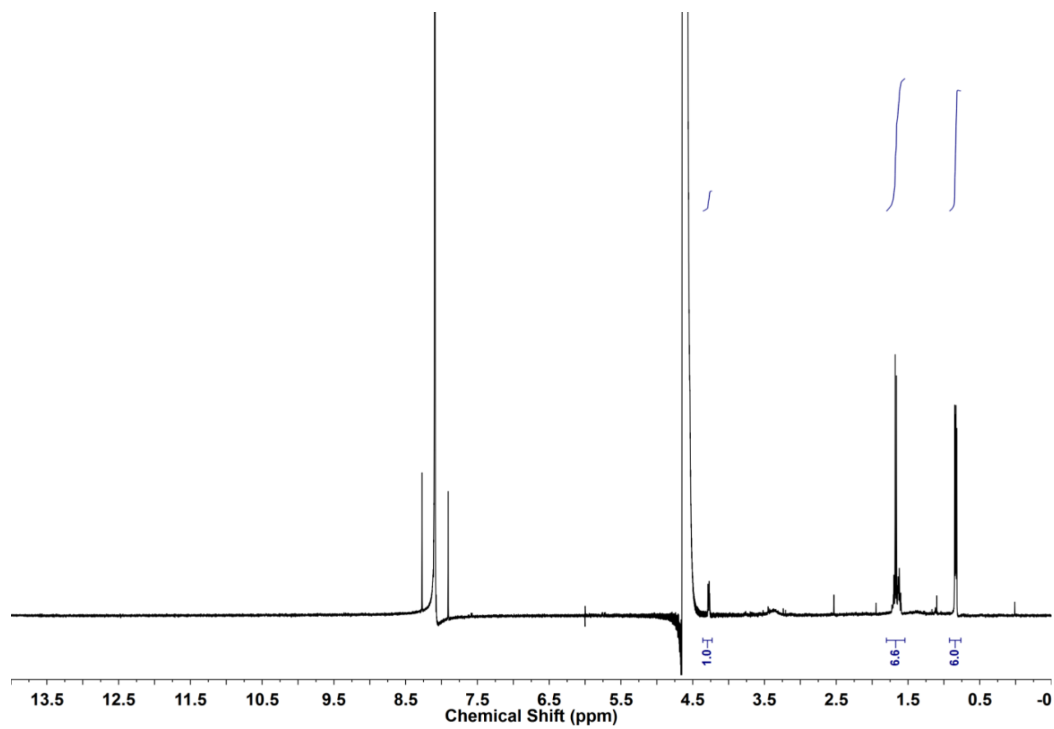


Fig. S24. ^{1}H NMR spectrum of HPLC-purified side-product of DhpJ reaction with L-Leu-L-Ala(P). Peak at 8.1 ppm belongs to formic acid. Attempts to confirm that this product is hydroxylated at the alpha carbon by mass spectrometry were unsuccessful. The mass of this compound observed by electrospray ionization mass spectrometry was that of L-Leu-Δ-Ala(P), which we assign to loss of water in the mass spectrometer from the hemiaminal group in the proposed structure of the side product.

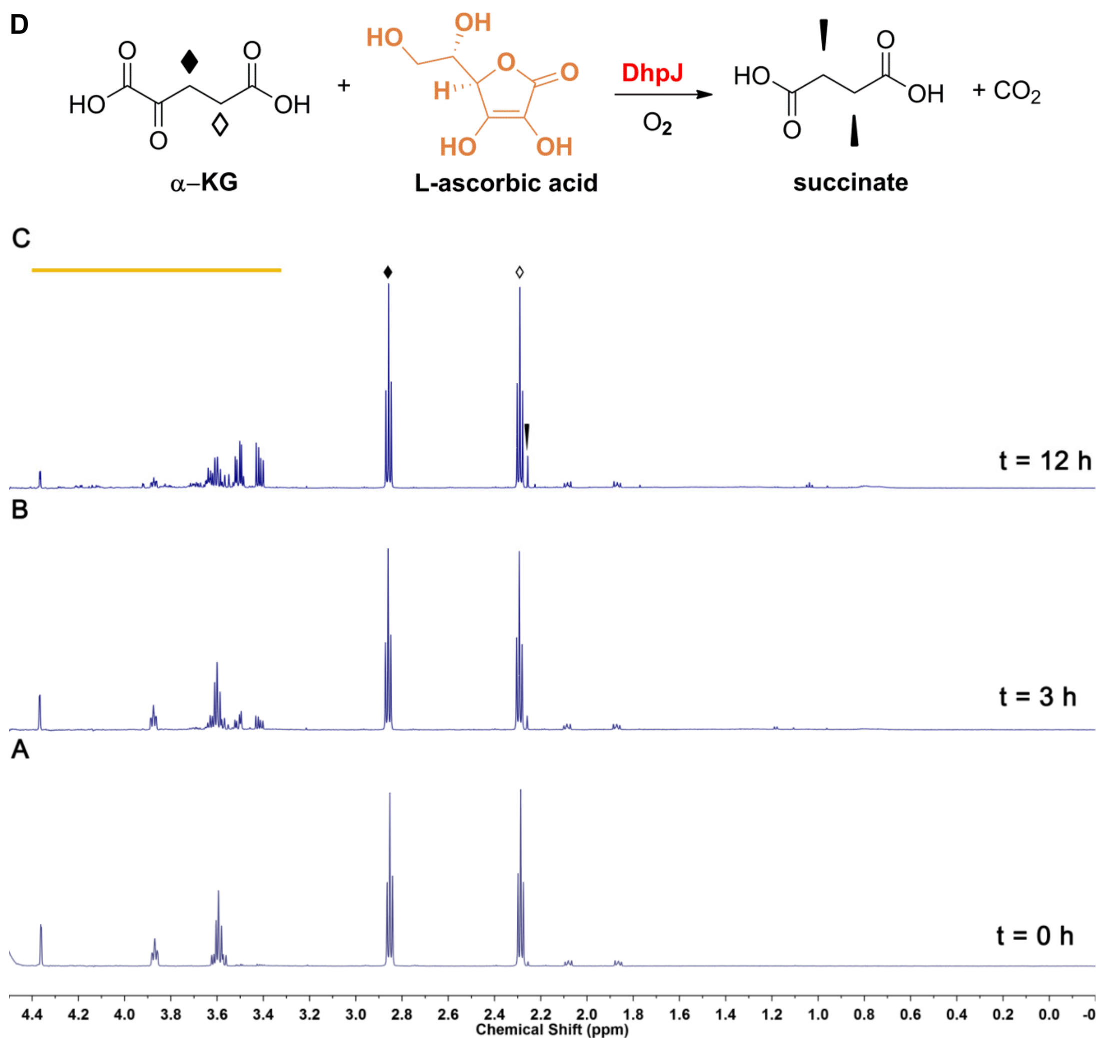


Fig. S25. ^{1}H NMR spectra of control experiments in which Fe(II)-reconstituted DhpJ was incubated with α-KG/O_2 and L-ascorbate in the absence of substrate. Reaction (1 mL final volume) was carried out in 25 mM NaPi, pH 7.5 and contained 5 mM α-KG, 2 mM L-ascorbic acid, 60 μM DhpJ previously reconstituted anaerobically with 1.2 equiv. of Fe(II). Aliquots of ca. 300 μL were removed at different time points, treated with Chelex resin and passed through an Amicon spin column (30 kDa MWCO) before the addition of D_2O and NMR analysis. Orange bar indicates the region of the spectra in which signals from protons belonging to ascorbate appeared. Diamonds denote signals of α-KG protons. Triangle denotes signals of succinate protons.

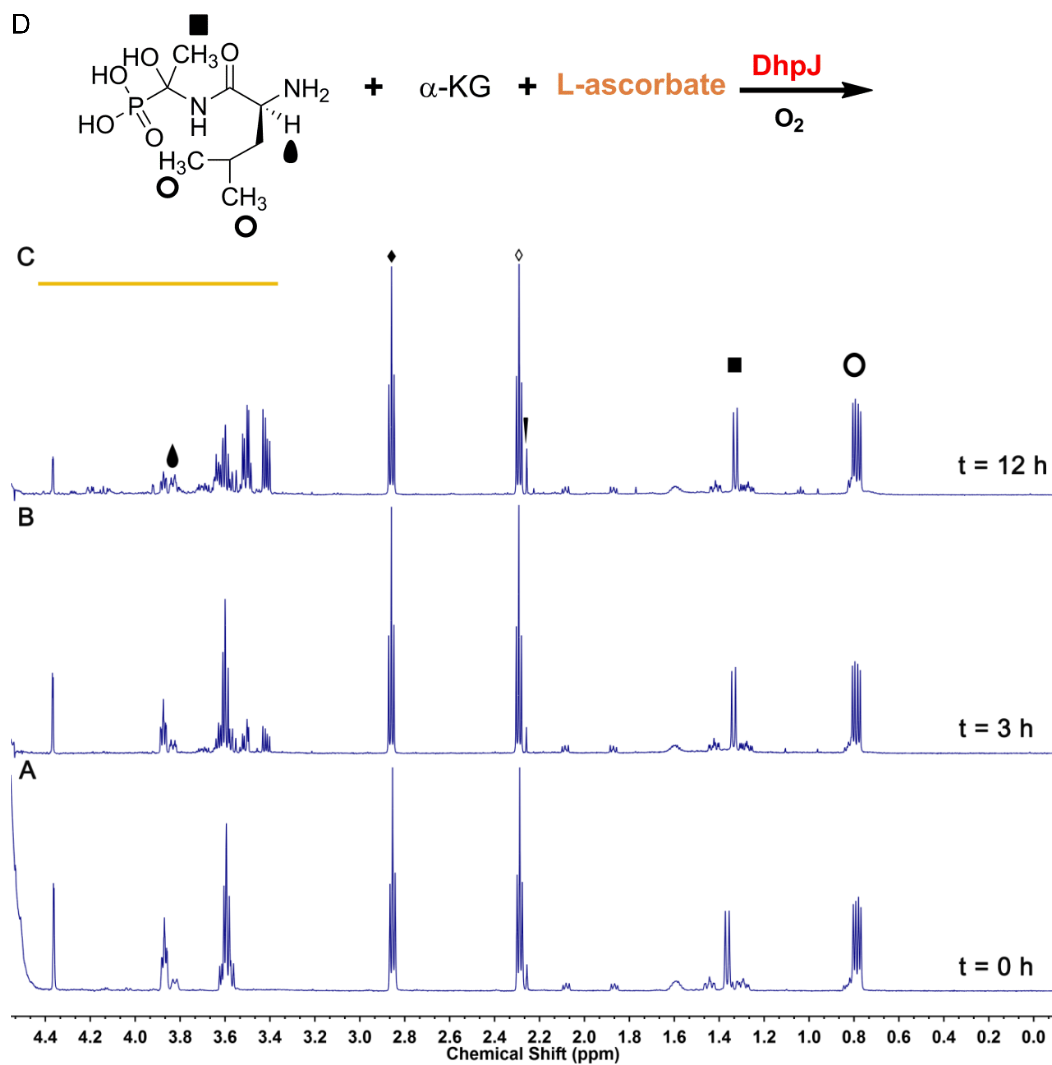


Fig. S26. ^{1}H NMR analysis of DhpJ incubation with the putative hydroxylated side-product. Reaction (1 mL final volume) was carried out in 25 mM NaPi pH 7.5 and contained ca. 1.5 mM of side-product, 3 mM α-KG, 2 mM L-ascorbic acid, 60 μM DhpJ previously reconstituted anaerobically with 1.2 equiv. of Fe(II). Aliquots of ca. 300 μL were removed at different time points, treated with Chelex resin and passed through an Amicon spin column (30 kDa MWCO) before the addition of D_2O and NMR analysis. Orange bar indicates the region of NMR spectra in which signals from protons belonging to ascorbate appeared. Diamonds denote signals of α-KG protons. Triangle denotes signals of succinate protons.

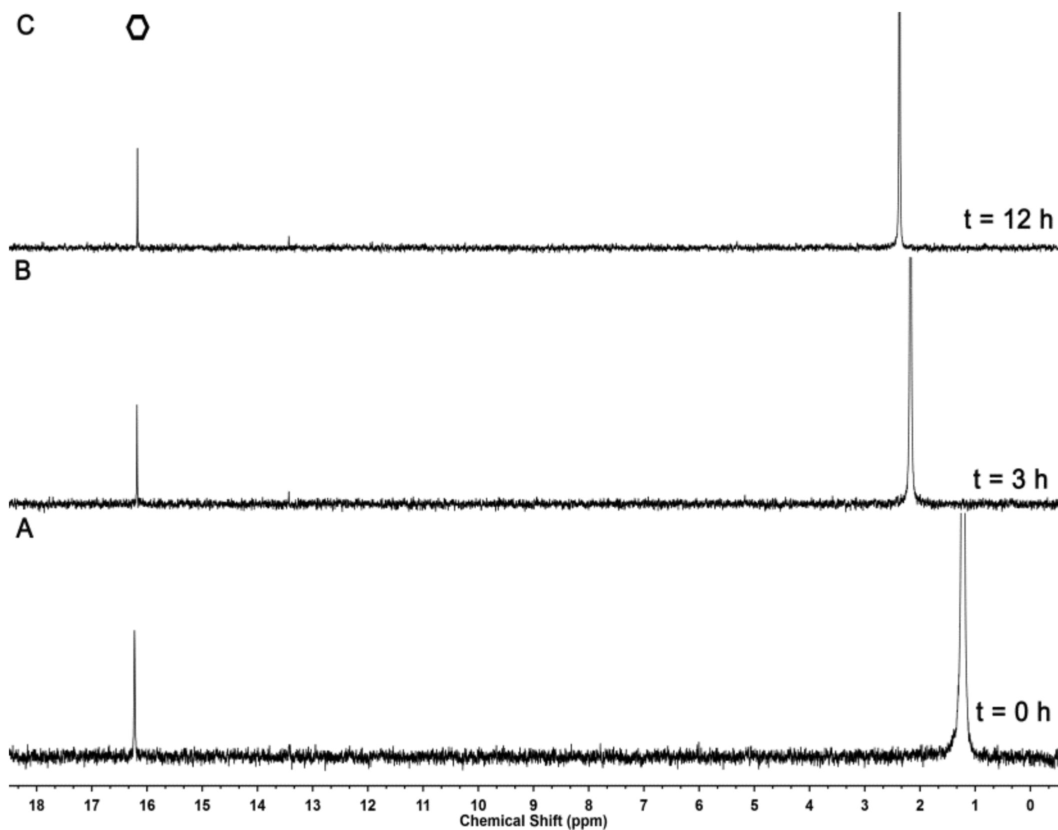


Fig. S27. ^{31}P NMR analysis of DhpJ incubation with the putative hydroxylated side-product. See Fig. S26 for experimental details. The unlabeled peak around 1.2 ppm in panel A and at 2.3 ppm in panels B, and 2.5 ppm in panel C belongs to Pi. The differences in chemical shifts is the result of small changes in pH that have a relatively large effect on chemical shift of these compounds as they have pK_a values near 7.

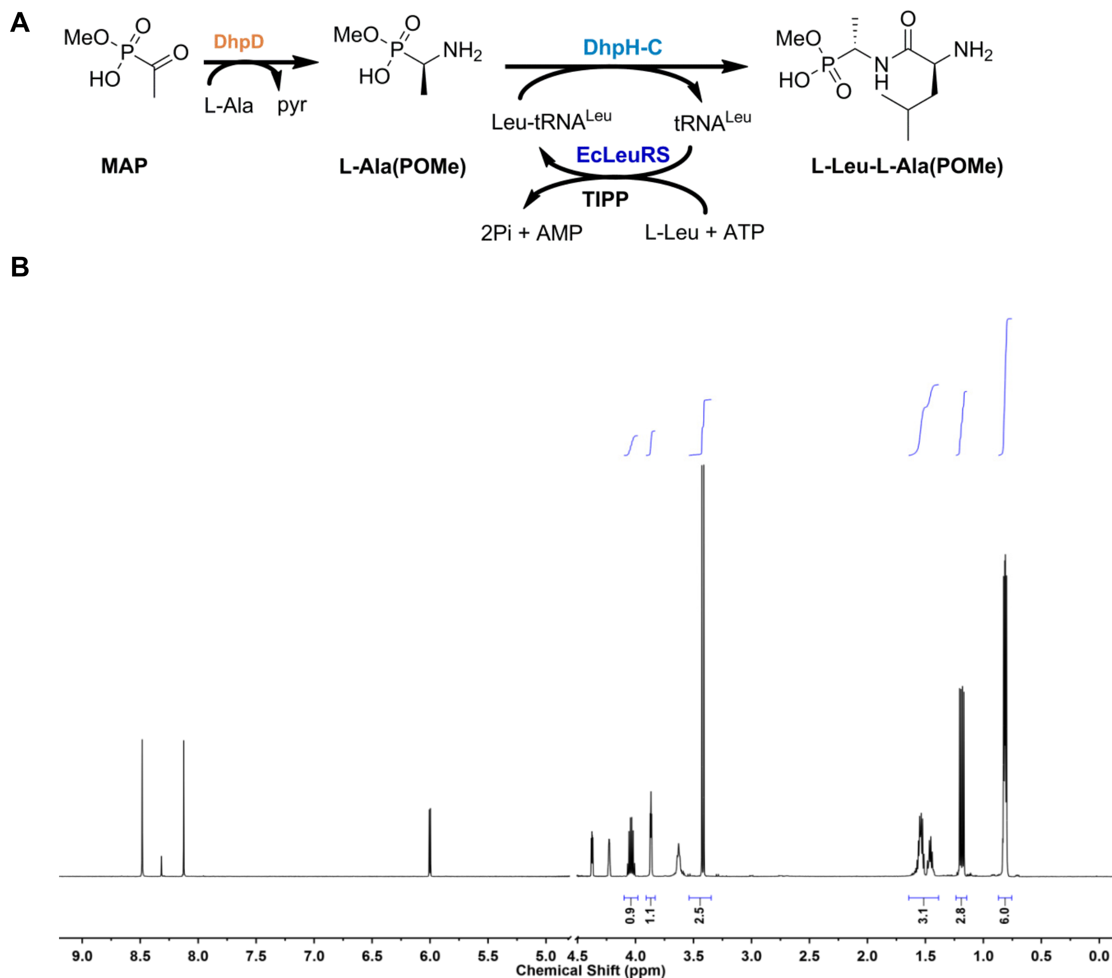


Fig. S28. Enzymatic preparation of L-Leu-L-Ala(POMe). A. Reaction scheme. The reaction mixture (5.5 mL final volume) contained: 10 mg bulk tRNA (*E. coli*), 10 mM MAP, 12 mM L-Ala, 12 mM L-Leu, 12 mM ATP, 20 μM PLP, 50 μM DhpD, 40 μM DhpH-C, 15 μM EcLeuRS, 20 U TIPP in 50 mM Na-HEPES, 20 mM KCl, 20 mM $MgCl_2$, pH 7.5. **B.** ^1H NMR spectrum of enzymatically prepared L-Leu-L-Ala(POMe) after cation-exchange column and HPLC purification. Signals that are not intergrated belong to AMP, which co-eluted with the dipeptide.

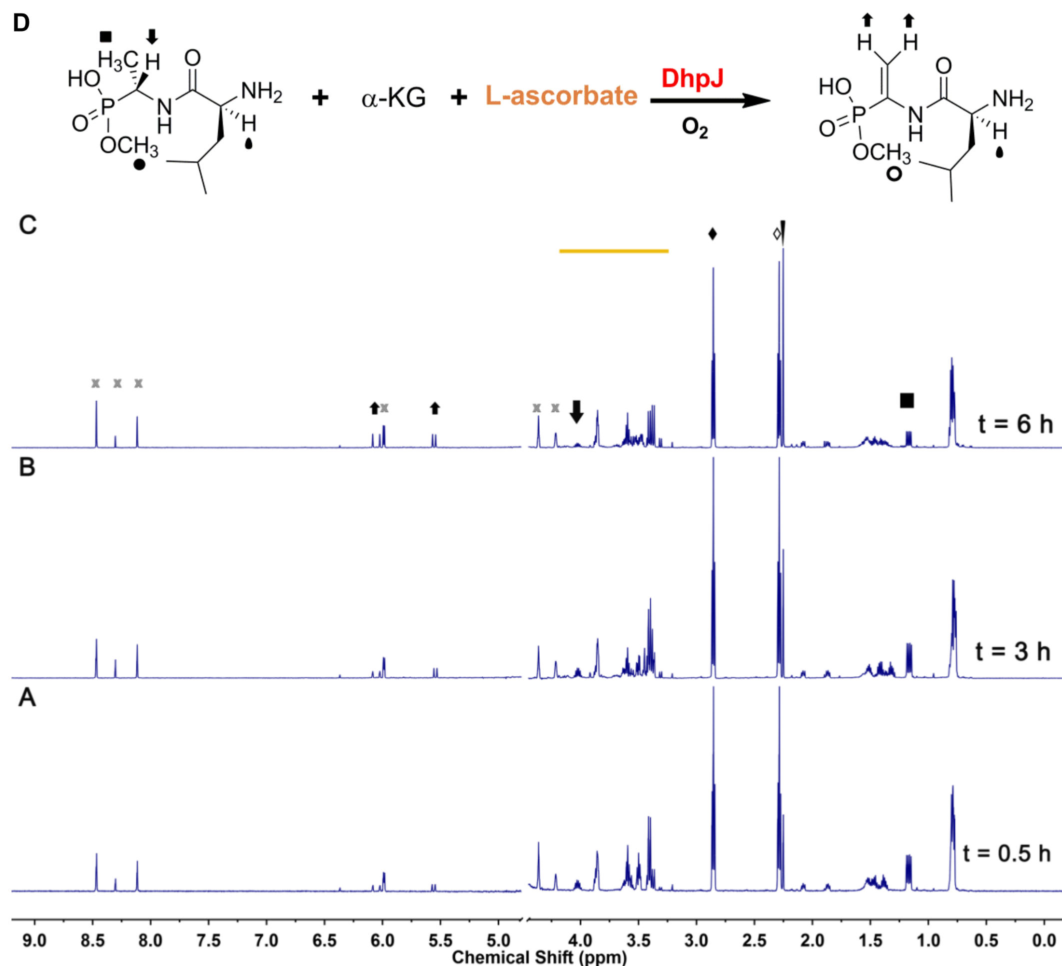


Fig. S29. ^{1}H NMR analysis of the DhpJ activity in the presence of L-Leu-L-Ala(POMe). Reaction (2 mL final volume) was carried out in 25 mM NaPi, pH 7.5 and contained 2 mM of L-Leu-L-Ala(POMe), 4 mM α-KG, 2 mM L-ascorbic acid, 60 μM DhpJ previously reconstituted anaerobically with 1.2 equiv. of Fe(II). Aliquots of ca. 300 μL were removed at different time points, treated with Chelex resin and passed through an Amicon spin column (30 kDa MWCO) before the addition of D_2O and NMR analysis. Orange bar indicates the region of NMR spectra in which signals from protons belonging to ascorbate appeared. Diamonds denote signals of α-KG protons. Triangle denotes signals of succinate protons. Signals marked with × belong to AMP which is an impurity of the enzymatically prepared starting material. The water peak is omitted for clarity.

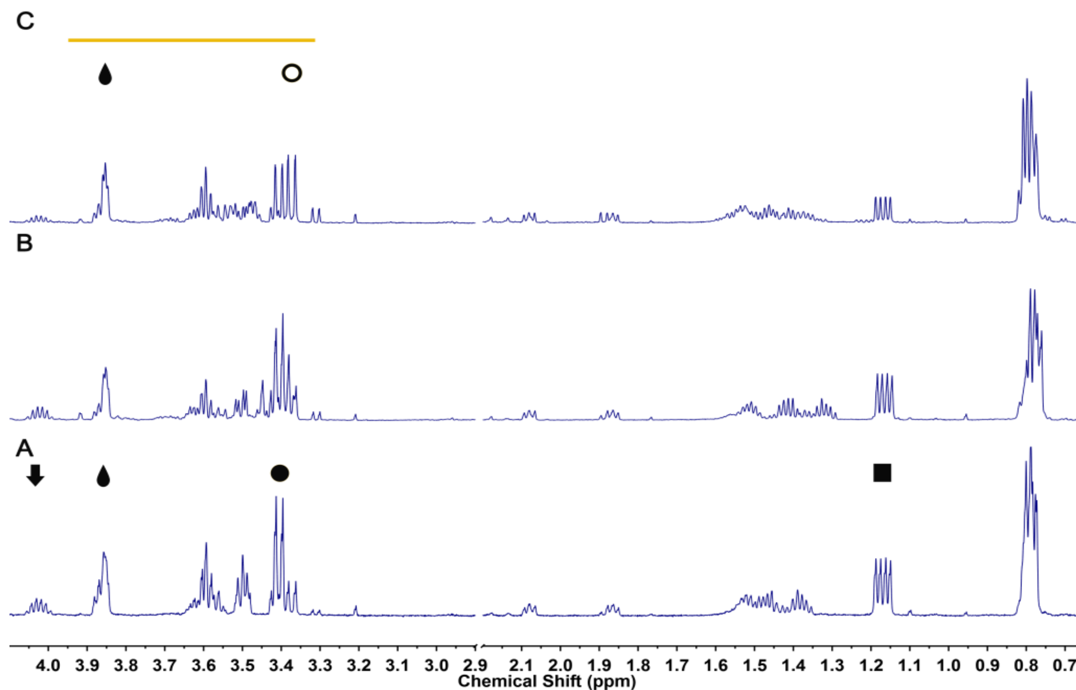


Fig. S30. Expanded region of the ^{1}H NMR spectrum of the DhpJ activity with L-Leu-L-Ala(POMe). See Fig. S29 for experimental details and annotation of signals.

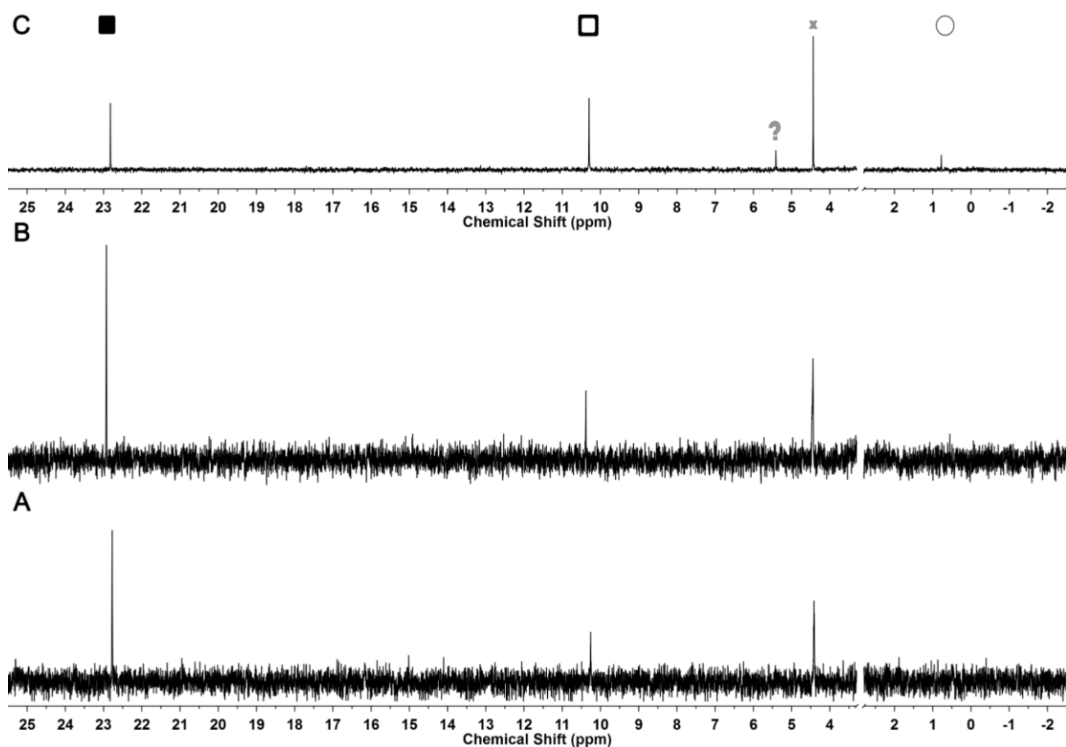


Fig. S31. ^{31}P NMR analysis of the DhpJ activity with L-Leu-L-Ala(POMe). See Fig. S29 for experimental details. Closed square denotes L-Leu-L-Ala(POMe), open square denotes L-Leu-L-ΔAla(POMe), × denotes AMP present in the starting material, open circle denotes MAP, presumably due to hydrolysis of L-Leu-ΔAla(P). Phosphate peak was omitted for clarity.

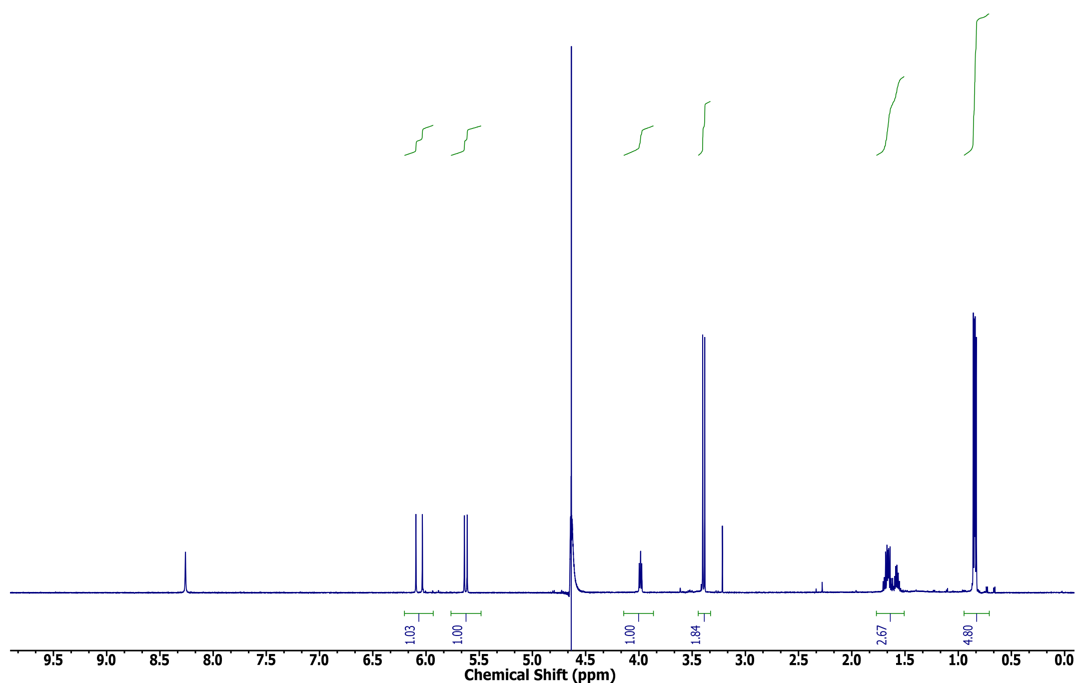


Fig. S32. ^{1}H NMR analysis of the HPLC purified product L-Leu-ΔAla(POMe) after incubation of DhpJ with L-Leu-L-Ala(OMe).

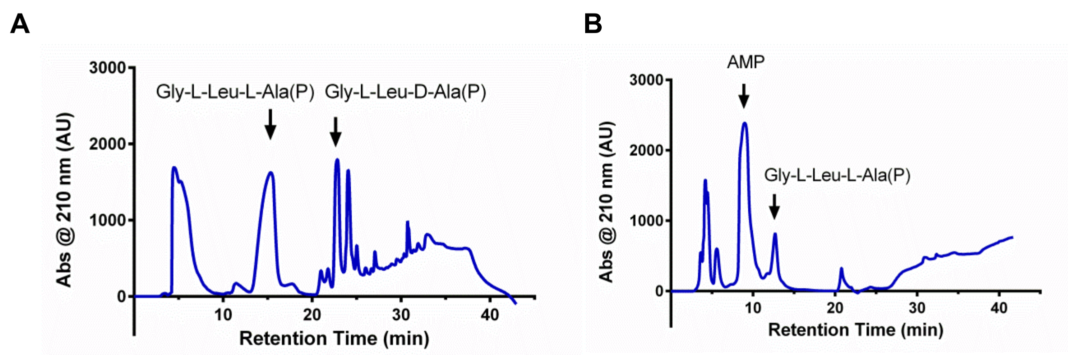


Fig. S33. HPLC traces of Gly-L-Leu-L-Ala(P). A. Synthetically prepared diastereoisomeric mixture of tripeptides. **B.** Enzymatically prepared Gly-L-Leu-L-Ala(P).

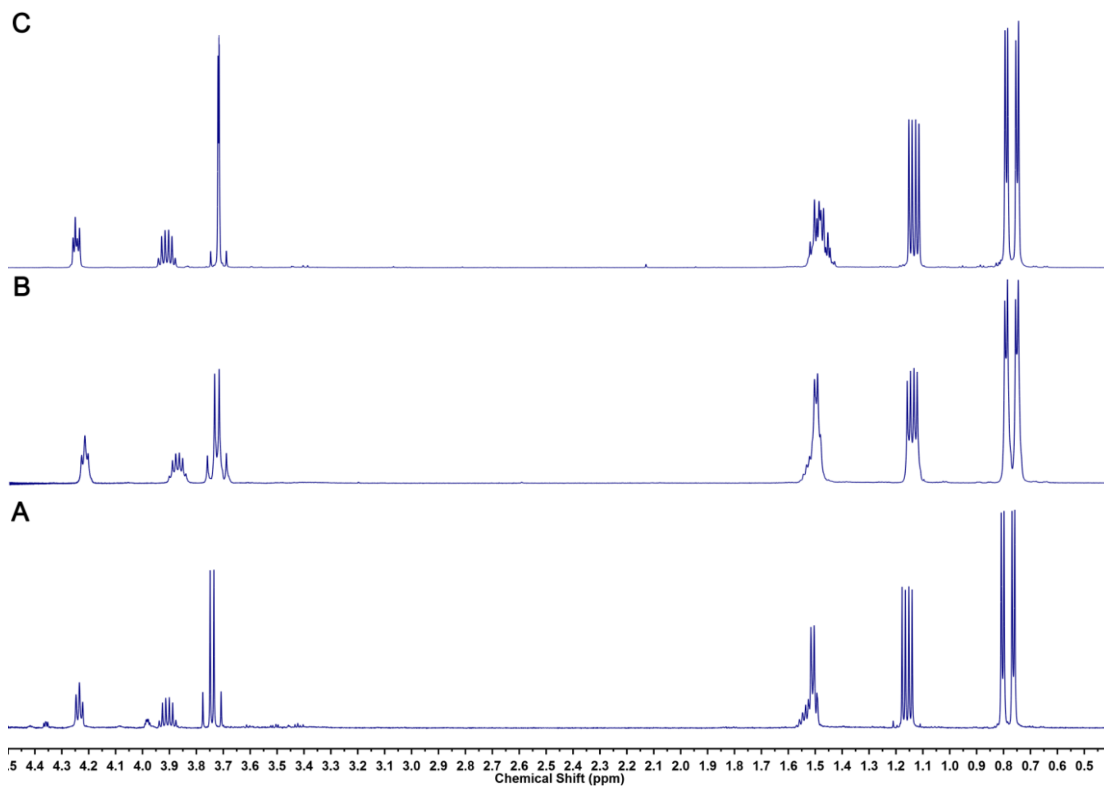


Fig. S34. ^{1}H NMR spectra of enzymatically (DhpK) and chemically prepared Gly-Leu-Ala(P) tripeptides. **A.** Enzymatically prepared Gly-L-Leu-L-Ala(P)**.** **B.** Gly-L-Leu-L-Ala(P) (synthetic)**.** **C.** Gly-L-Leu-D-Ala(P) (synthetic).

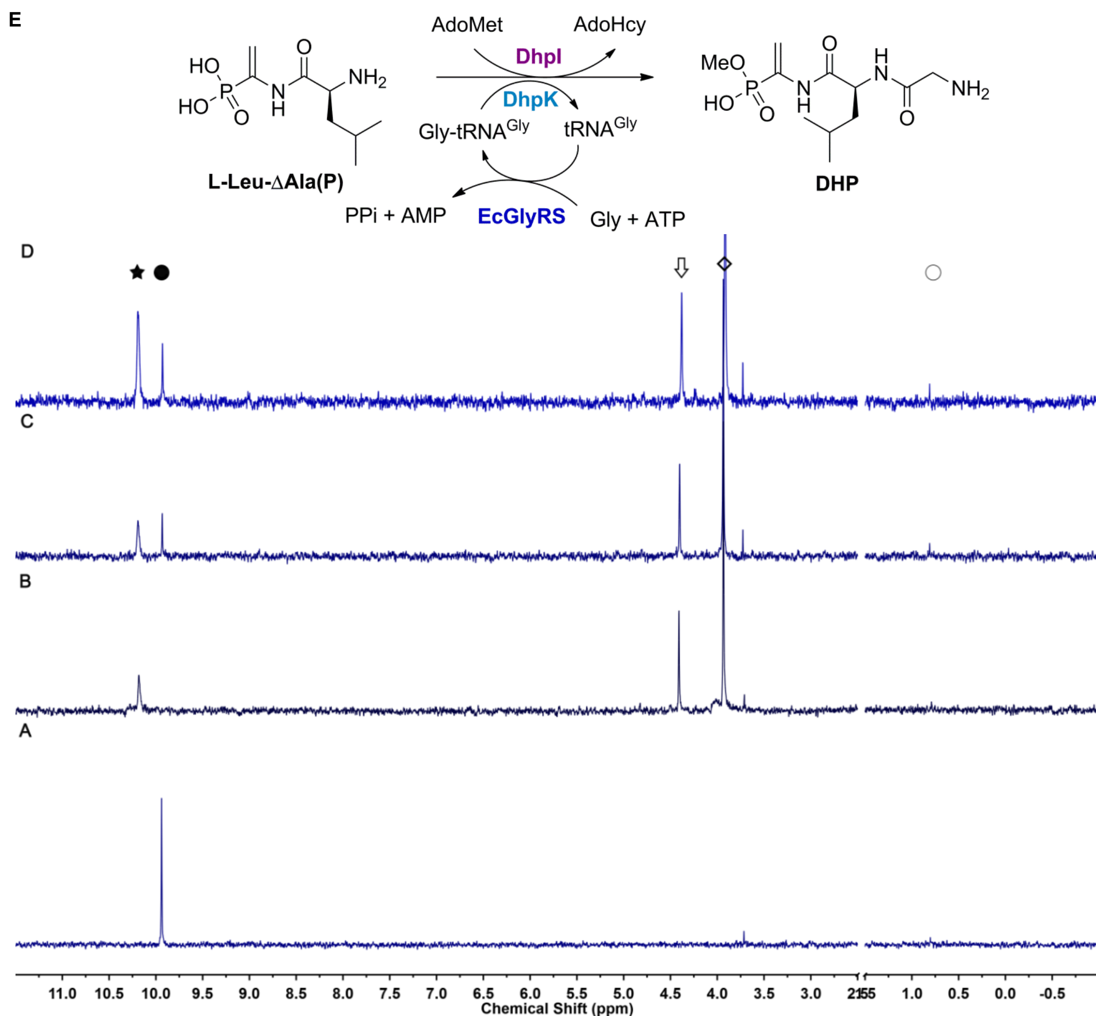


Fig. S35. Enzymatic formation of DHP from L-Leu-ΔAla(P) in one-pot cascade reaction with DhpK and DhpI. A. L-Leu-ΔAla(P) incubated with DhpI. The reaction was carried out in 50 mM Na-HEPES, pH 7.5 (500 μL total volume) and contained L-Leu-ΔAla(P) (ca. 2 mM), SAM (8 mM), S-adenosylhomocysteine (AdoHcy) nucleosidase (10 μM), and DhpI (40 μM). **B.** L-Leu-ΔAla(P) incubated with DhpI and DhpK. The reaction was carried out in 50 mM Na-HEPES, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$ (500 μL final volume) and contained: 2 mM L-Leu-ΔAla(P) , 10 mM glycine, 0.5 mg tRNA, 5 mM ATP, 8 mM SAM, 10 μM S-adenosylhomocysteine (AdoHcy) nucleosidase, 20 μL EcGlyRS, 40 μM DhpI, 50 μM DhpK, and 2 U TIPP. Phosphate peak was omitted for clarity. Star denotes dehydrophos, closed circle denotes Leu-Ala(POMe), arrow denotes uncharacterized phosphorylated compound, diamond denotes AMP, and open circle denotes MAP (presumably due to hydrolysis of DHP). **C.** ^{31}P NMR spectrum of reaction mixture B after spiking with the solution from the reaction A. **D**. ^{31}P NMR spectrum of panel C spiked with synthetic standard of DHP.

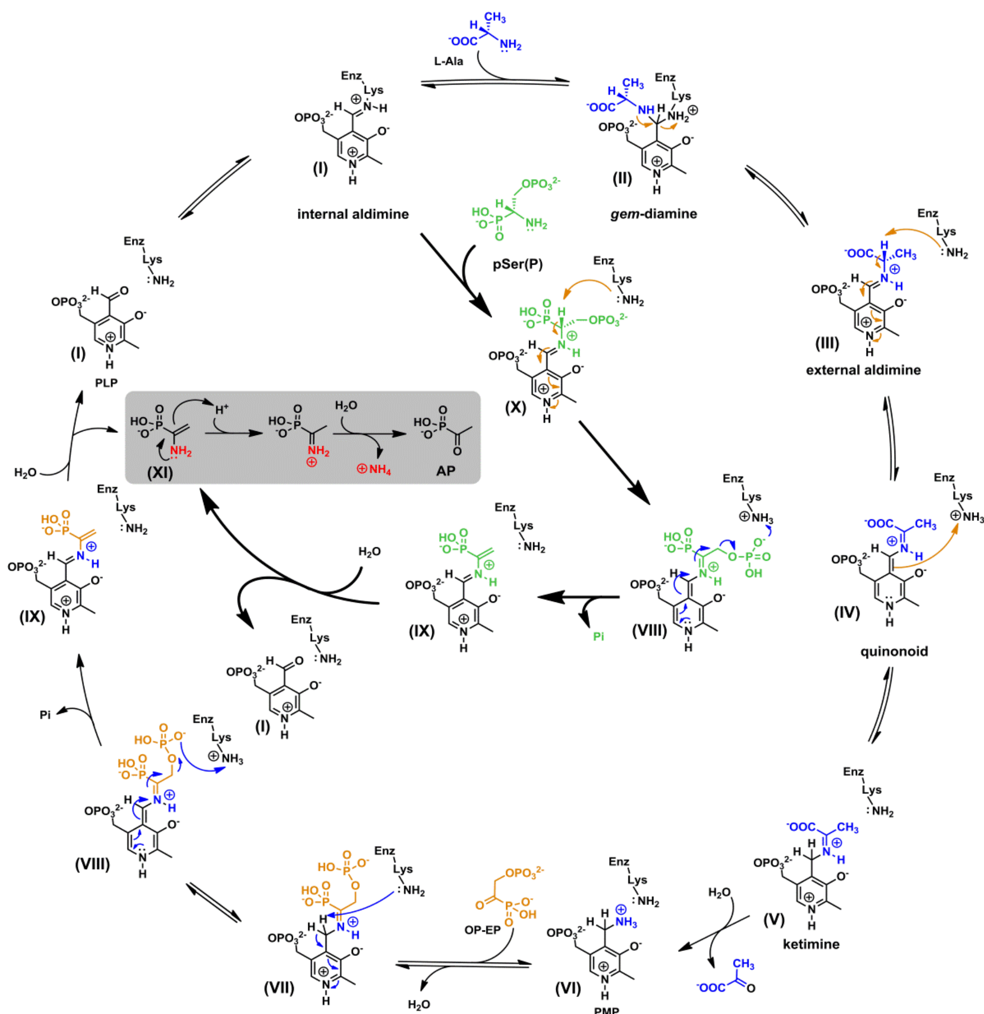


Fig. S36. Proposed catalytic cycle for DhpH-N. On the top of the figure ("12 o'clock", outside catalytic cycle), Ala is used as amine donor to convert the internal aldimine of PLP to the PMP form at 6 o'clock. PMP reacts with OP-EP to initiate phosphate elimination and generate the external aldimine IX, which upon hydrolysis first forms enamine XI that subsequently hydrolyzes to AP. The cycle shown in the inside is if pSer(P) instead of OP-EP is the substrate for DphH-N.

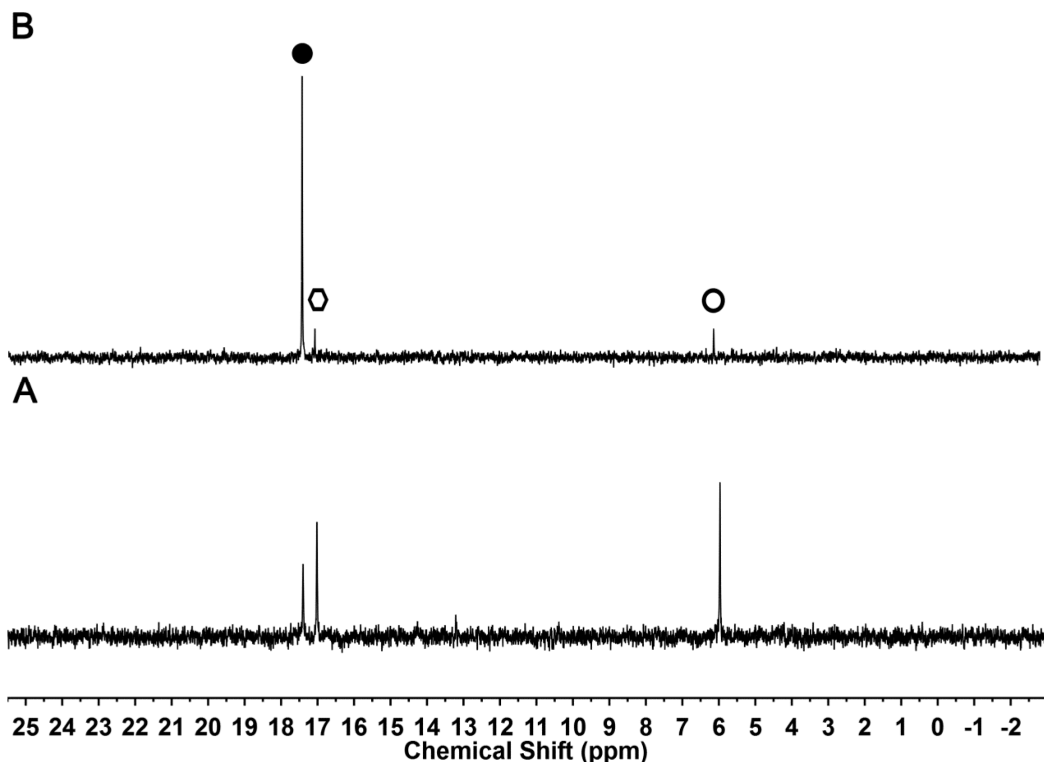


Fig. S37. ^{31}P NMR analysis of the DhpJ activity on L-Leu-L-Ala(P) in the presence or absence of L-ascorbic acid. A. Reaction assay (500 µL final volume) containing 50 mM HEPES, pH 7.5, 3 mM L-Leu-L-Ala(P) (●), 6 mM α-KG, 0.20 mM Fe(II), 3 mM L-ascorbic acid and 60 µM DhpJ (reconstituted anaerobically with 1.2 equiv. of Fe(II) prior to its use). **B.** Reaction assay identical to (A) but without L-ascorbic acid. L-Leu-ΔAla(P) is marked with an open circle, the hydroxylated side-product is marked with a hexagon.

Supplementary Tables

Table S1. List of strains and plasmids used in this study.

Name	Features	Source
Rosetta 2(DE3)pLysS	CAM^R; provides seven rare, in *E. coli*, tRNAs for the codons CGG, AUA, AGG, AGA, CUA, CCC, and GGA in the same plasmid that habors the T7 lysozyme	Novagen
BL21(DE3)	F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
***E. coli* DH5α λpir**	sup E44, ΔlacU169 (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen.	in house
pET15b	AMP^R; carries an N terminal 6xHis-tag® of the protein of interest, contains a thrombin recognition sequence	Novagen
pMAL-c2X	AMP^R; provides maltose binding protein (MBP) fusion constructs at the N terminal of the protein of interest, contains protease factor Xa recognition sequence	a gift from DrTobias J. Erb
Fosmid 17E11-4	CAM^R, AMP^R ; carries dehydrophos biosynthetic cluster from *S. luridus*	in house[a]
pET15b-dhpD		this study

Table S1. Continued.

Name	Features	Source
pET15b-dhpH		in house[a]
pET15b-dhpH-N		this study
pET15b-dhpH-C		this study
pET15b-dhpI		in house[a]
pET15b-dhpK		this study
pET15b-dhpJ		in house[a]
pET15b-GlyRS		this study
pET14b-LeuRS		a gift from Prof. Susan A. Martinis
pMAL-dhpK		this study
pMAL-dhpJ		this study

[a] B.T. Circhello, PhD Thesis, UIUC, 2010

Table S2. List of oligonucleotides used in this study.

Name	Sequence (5'→3')[b]	Scope
pET15-XhoI_fw	ctcgaggatccggctgctaacaaagcccgaaagg	Amplification of pET15b vector Gibson cloning
pET15-NdeI_rc	catatggctgccgcgcggcaccaggccgctg	Amplification of pET15b vector
pMAL-HindIII_fw	aagcttggcactggccgtcgttttacaacgtcg	Amplification of pMAL-c2x vector
pMAL-EcoRI_rc	gaattctgaaatccttccctcgatcccgagg	Amplification of pMAL-c2x vector
NdeI-dhpK_fw	gcagcggcctggtgccgcgcggcagccatatggtgacagaagggatgccggtgtcggagg	Amplification of *dhpK* for Gibson cloning into pET15b vector
XhoI-dhpK_rc	Cctttcgggctttgttagcagccggatcctcgagtcactgcgagaaccggcccaggtacg	Amplification of *dhpK* for Gibson cloning into pET15b vector
pMAL-sec-fw	ggtcgtcagactgtcgatgaagcc	Sequencing genes inserted into pMAL-c2x vector
pMaL-sec-rc	aacgccagggttttcccagtcac	Sequencing genes inserted into pMAL-c2x vector
NdeI-dhpH_ATG_fw	gcagcggcctggtgccgcgcggcagccatatgcgcaactacagggtgacggggccggtcg	Amplification of *dhpH* gene (first 1071 bases; PLP domain) for Gibson cloning into pET15b vector
XhoI-dhpH_N_term	ctttcgggctttgttagcagccggatcctcgagtcaggccgaaccgtccaggcaggccgc	Amplification of *dhpH* gene (first 1071 bases; PLP domain) for Gibson cloning into pET15b vector
NdeI-dhpH_C353	gcagcggcctggtgccgcgcggcagccatatgctggacggttcggccggcggccccgc	Amplification of *dhpH* gene (1057-2097 bases; nucleic acids binding domain) for Gibson cloning into pET15b vector
XhoI-dhpH_rc	Ctttcgggctttgttagcagccggatcctcgagtcagagcgaagggccgagcatcgcc	Amplification of *dhpH* gene (1057-2097 bases; nucleic acids binding domain) for Gibson cloning into pET15b vector
EcoRI-DhpK_fw	Cctcgggatcgagggaaggatttcagaattcgtgacagaagggatgccggtgtcggag	Amplification of *dhpK* for Gibson cloning into pMAL vector

[b] Underlined sequence indicates homology to expression vector.

Table S2. Continued.

Name	Sequence (5'→3')[b]	Scope
HINDIII-DhpK_rc	Cgttgtaaaacgacggccagtgccaagctttcactgcgagaa ccggcccaggtacg	Amplification of *dhpK* for Gibson cloning into pMaL vector
NdeI-dhpD_fw	Gcagcggcctggtgccgcgcggcagccatgtgaccggctcc gcgcgcggctcctcg	Amplification of *dhpD* for Gibson cloning into pET15b vector
XhoI-dhpD_rc	cctttcgggctttgttagcagccggatcctcgagtcagtgatggg tgacctcgcgcgccgcagcc	Amplification of *dhpD* for Gibson cloning into pET15b vector
EcoRI-dhpJ_fw	cctcgggatcgagggaaggatttcagaattcgtgaacggttcc ctcagcatcaaccagc	Amplification of *dhpJ* for Gibson cloning into pMAL vector
HINDIII-dhpJ_rc	cgttgtaaaacgacggccagtgccaagctttcagatgtcgccg cccgattccttgatgaactgg	Amplification of *dhpJ* for Gibson cloning into pMAL vector
NdeI-EcGlyRS_fw	gcagcggcctggtgccgcgcggcagccatatgcaaaagtttg ataccaggaccttccagg	Amplification of *glyQ* and *glyS* for Gibson cloning into pET15b vector
XhoI-EcGlyRS_rc	cctttcgggctttgttagcagccggatcctcgagttattgcaaca gcgaaatatccgcaacg	Amplification of *glyQ* and *glyS* for Gibson cloning into pET15b vector

[b]Underlined sequence indicates homology to expression vector.

References

1. Sambrook JF & Russell DW (2006) *The condensed protocols from molecular cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press, New York) 1st Ed.
2. Circello BT, Eliot AC, Lee J-H, van der Donk WA, & Metcalf WW (2010) Molecular cloning and heterologous expression of the dehydrophos biosynthetic gene cluster. *Chem Biol* 17(4):402-411.
3. Gibson DG*, et al.* (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6(5):343-345.
4. Kuemin M & van der Donk WA (2010) Structure-activity relationships of the phosphonate antibiotic dehydrophos. *Chem Commun* 46(41):7694-7696.
5. Mortier J, Gridnev ID, & Guénot P (2000) Reactions of phosphonates with organohaloboranes: New route to molecular borophosphonates. *Organometallics* 19(21):4266-4275.
6. Lee J-H*, et al.* (2010) Characterization and structure of DhpI, a phosphonate O-methyltransferase involved in dehydrophos biosynthesis. *Proc Natl Acad Sci USA* 107(41):17557-17562.
7. Circello BT (2010) Investigations into the biosynthesis and mode of action of the phosphonate antibiotic dehydrophos. PhD (University of Illinois at Urbana-Champaign).
8. Zhao G & Winkler ME (1996) A novel alpha-ketoglutarate reductase activity of the serA-encoded 3-phosphoglycerate dehydrogenase of *Escherichia coli* K-12 and its possible implications for human 2-hydroxyglutaric aciduria. *J Bacteriol* 178(1):232-239.
9. Whitteck JT, Cicchillo RM, & van der Donk WA (2009) Hydroperoxylation by Hydroxyethylphosphonate Dioxygenase. *J Am Chem Soc* 131(44):16225-16232.