- Supplementary Information -

Mining the cellular inventory of pyridoxal phosphate-dependent enzymes with functionalized cofactor mimics

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1. Supplementary Schemes

Supplementary Scheme 1 | Synthesis of PL1 and Ctrl. Pyridoxine hydrochloride was protected with three equivalents of TBS-Cl to yield 3. N-oxidation using mCPBA followed by Boekelheide rearrangement with trifluoroacetic anhydride and quenching with MeOH served to install the 2'-alcohol of intermediate 4 and resulted in loss of the phenolic TBS group. The primary alcohol was oxidized to the corresponding aldehyde (5) with MnO₂ and the phenol was re-protected using MOM-Cl. Reaction of 6 with the Ohira-Bestmann reagent (dimethyl-1-diazo-2-oxopropylphosphonate) in the presence of K₂CO₃ gave terminal alkyne 7. Upon removal of silane protecting groups, oxidation of 8 with MnO₂ in neutral methanol gave a 2.5:1 mixture of isomers, 9a and 9b, which were separated by preparative HPLC and assigned by 2D NMR spectroscopy. Hydrolysis under acidic conditions yielded PL1 and Ctrl probes.
Supplementary Scheme 2 | Synthesis of PL3. The 3 and 4’ alcohols of pyridoxine hydrochloride were protected as the cyclic acetal 10 by reacting with acetone under acidic conditions. Protection of the 5’ alcohol using PMBCl (para-methoxybenzyl chloride) yielded 11. N-oxidation with mCPBA was followed by Boekelheide rearrangement\(^1\) using trifluoroacetic anhydride and quenching with MeOH in order to generate alcohol 12. Reaction with SOCl\(_2\) installed a chloride at the 2’-position which was subsequently displaced by sodium azide. Upon removal of the acetal protecting group of 13, the primary alcohol was oxidized to the corresponding aldehyde 14 using MnO\(_2\) and final deprotection under acidic conditions afforded the PL3 probe.
Supplementary Scheme 3 | Synthesis of PL4 and PL5. Extended linkers of PL4 and PL5 were installed by alkylation of 1 using LDA and corresponding alkyl halides. Two-step deprotection using $K_2CO_3$ followed by dilute aqueous $H_2SO_4$ yielded the respective probes.
2. Supplementary Figures

**Supplementary Figure 1 | PLK phosphorylation.** MS/MS spectra of phosphorylated probes PL1P, PL2P and PL3P upon incubation with PLK. HRMS: PL1P (found: 258.01666 [M+H]+ calc.: 258.01620), PL2P (found: 286.04819 [M+H]+ calc.: 286.04750), PL3P (found: 289.03403 [M+H]+ calc.: 289.03325).
Supplementary Figure 2 | Additional representations of X-ray crystal structures of Alr bound to PL1P and PL2P. (a) Surface representation of the *S. aureus* Alr dimer (blue and grey) in complex with PL2P (gold, stick representation); the two active sites are indicated by red arrows. (b) Zoom onto the PL2P binding site. (c) The electrostatic potential of the amino acid side chains of the Alr active site is mapped onto the surface (positive = blue, negative = red) with the PL2P co-factor (gold, stick representation). (d) Two-dimensional LIGPLOT1-representation of the interactions between *S. aureus* Alr and PL2P. (e) Superposition of the active sites of the apo- (green, PDB 6G56), PL1P- (grey, PDB 6G58) and PL2P- (blue, PDB 6G59) complex structures.
**Supplementary Figure 3** | Modeling of cofactor derivatives in the active sites of PLK and PLP-DES. (a) *S. aureus* PLK (PDB 4C5L) modeled with PL2 and PL5 probes. (b) PL2P modeled into the active site of the *S. aureus* class I aminotransferase (PDB 2O1B). (c) PL2P modeled into the active site of the *Mycobacterium smegmatis* class IV aminotransferase (PDB 3JZ6).

**Supplementary Figure 4** | Additional gels for Figure 2f. Full fluorescence gels and Coomassie stained gels of Alr labeling experiments using PL-probes.
Supplementary Figure 5 | PL2 labeling optimizations. (a) Fluorescence SDS-PAGE showing concentration-dependent labeling of PL2 in *S. aureus* USA300 TnPdxS and corresponding Coomassie gel staining below. (b) Fluorescence SDS-PAGE showing time-dependent labeling of PL2 in *S. aureus* USA300 TnPdxS and corresponding Coomassie gel staining below.

Supplementary Figure 6 | Chemical proteomics using PL2 in *S. aureus* USA300 TnPdxS (2h labeling protocol). (a) (left) Alternative representation of the volcano plot in Fig. 3b showing enrichment of proteins with 100 µM PL2 without coloured confidence classification (*n* = 9 biological replicates). Known PLP-DEs are highlighted with blue dots and are numerically annotated according to Table 1. Remaining proteins significantly enriched are listed in Supplementary Table 1. (right) Volcano plot showing enrichment of proteins with 10 µM PL2 (*n* = 8 biological replicates). (b) Fluorescence SDS-PAGE showing PL2-labeling during proteomic experiments upon CuAAC to trifunctional rhodamine-biotin-azide tag and avidin bead enrichment.
| Fold-change | Significance | Name                                                                 | Uniprot ID            | Cluster |
|----------------|--------------|----------------------------------------------------------------------|-----------------------|---------|
| 33.            | 4.18         | Dihydrorotate dehydrase (quinone) (EC 1.3.5.2)                        | A0A0H2XFV6            | 3       |
| 34.            | 4.10         | Signal peptide I (EC 3.4.21.89)                                      | A0A0H2XEA7            | 2       |
| 35.            | 4.03         | Glutaryl-aminopeptidase                                               | A0A0H2XPF6            | 3       |
| 36.            | 2.75         | Methicillin resistance protein FemB                                  | A0A0H2XHGG6           | 2       |
| 37.            | 2.75         | GTPase (EC 3.6.5.-)                                                  | Q2FGE3                | 2       |
| 38.            | 2.51         | Phosphoglucomutase (EC 5.4.2.10)                                     | Q2FEX1                | 2       |
| 39.            | 2.38         | Oxidoreductase, aldo/keto reductase family                           | A0A0H2XG47            | 2       |
| 40.            | 2.30         | Uncharacterized protein                                               | A0A0H2XUQ7            | 5       |
| 41.            | 2.27         | 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19) (EPSPS)   | Q2FEX1                | 2       |
| 42.            | 2.06         | Isocitrate dehydrogenase (NADP) (EC 1.1.1.124)                        | A0A0H2XHS2            | 3       |
| 43.            | 1.98         | Elongation factor P (EF-P)                                           | Q2FGJ3                | 3       |
| 44.            | 1.97         | Putative NAD(P)H reductase                                           | Q2FDY2                | 2       |
| 45.            | 1.73         | Uncharacterized protein                                               | A0A0H2XGG0            | 3       |
| 46.            | 1.70         | Uncharacterized protein                                               | A0A0H2XJ48            | 4       |
| 47.            | 1.42         | Deoxyribose-phosphate aldolase (DERA) (EC 4.1.2.4)                   | A0A0H2XF3             | 1       |
| 48.            | 1.35         | Lipote–protein ligase (EC 2.7.7.63)                                   | A0A0H2XFJ1            | 3       |
| 49.            | 1.34         | Bifunctional protein FoD (EC 1.5.1.5); (EC 3.5.4.9)                   | Q2FIF5                | 2       |
| 50.            | 1.26         | Uncharacterized protein                                               | A0A0H2XEV2            | 2       |
| 51.            | 1.13         | Uracil phosphoribosyltransferase (EC 2.4.2.9) (UMP pyrophosphorylase)| Q2FEX1                | 4       |
| 52.            | 0.94         | Putative universal stress protein                                    | A0A0H2XE6             | 1       |
| 53.            | 0.82         | Single-stranded DNA-binding protein (SSB)                             | A0A0H2XJY5;A0A0H2XG16 | 3       |

**Table 2.1 continued**

| Fold-change | Significance | Name                                                                 | Uniprot ID            | Cluster |
|----------------|--------------|----------------------------------------------------------------------|-----------------------|---------|
| 73.            | 0.97         | Chorismate mutase/phospho-2-dehydro-3-deoxyheptanolate aldolase (EC 5.4.9.9) | A0A0H2XIB5            | 2       |

**Table 3 continued**

| Fold-change | Significance | Name                                                                 | Uniprot ID            | Cluster |
|----------------|--------------|----------------------------------------------------------------------|-----------------------|---------|
| 74.            | 2.23         | Inositol monophosphatase family protein (EC 3.1.3.25)                 | A0A0H2XH13            | 3       |
| 75.            | 2.11         | Serine acetyltransferase (EC 2.3.1.30)                                | A0A0H2XKQ4             | 2       |
| 76.            | 1.89         | Phosphate acetyltransferase (EC 2.3.1.88)                             | A0A0H2XHL4             | 2       |
| 77.            | 2.23         | Inositol monophosphatase family protein (EC 3.1.3.25)                 | A0A0H2XH13            | 3       |
| 78.            | 2.11         | Serine acetyltransferase (EC 2.3.1.30)                                | A0A0H2XKQ4             | 2       |
| 79.            | 1.89         | Phosphate acetyltransferase (EC 2.3.1.88)                             | A0A0H2XHL4             | 2       |
| 80.            | 1.66         | UDP-N-acetylenolopyruvoglutamin reductase (EC 1.3.1.98) (UDP- N-acetylmalamate dehydrase) | Q2FJQ3                | 4       |
| 81.            | 1.53         | Ribulokinase (EC 2.7.1.16)                                            | Q2FG8                 | 5       |
| 82.            | 1.47         | 30S ribosomal protein S20                                              | Q2FDQ8                | 5       |
| 83.            | 1.42         | Pyridine nucleotide-disulfide oxidoreductase (EC 1.8.1.9)             | A0A0H2XJR2             | 2       |
| 84.            | 1.39         | 30S ribosomal protein L25 (General stress protein CTC)                | A0A0H2XUFO             | 4       |
| 85.            | 1.37         | Orotate phosphoribosyltransferase (OPRTase) (EC 2.4.2.10)             | A0A0H2XH75            | 5       |
| 86.            | 1.36         | SIF system FeS assembly protein, NIF family                           | A0A0H2XJC0             | 2       |
| 87.            | 1.30         | Uncharacterized protein                                               | A0A0H2XJG9             | 5       |
| 88.            | 1.24         | UFP0457 protein SAUSA300_2132                                        | Q2FDV9                | 3       |
| 89.            | 1.21         | Tryptophan-IRNA ligase (EC 6.1.1.2) (Tryptophan-IRNA synthetase)       | A0A0H2XG6             | 3       |
| 90.            | 1.17         | Uncharacterized (EC 2.3.1.10)                                         | A0A0H2XJU3             | 5       |
| 91.            | 1.14         | S1 RNA binding domain protein                                         | A0A0H2XUFJ             | 5       |
| 92.            | 1.13         | Protein RecA (Recombinase A)                                          | A0A0H2XFW9             | 4       |
| 93.            | 1.12         | Uncharacterized protein                                               | A0A0H2XHS2             | 3       |
| 94.            | 1.10         | 30S ribosomal protein L6                                               | Q2FEG4                | 3       |
| 95.            | 1.09         | Enoyl-[acyl-carrier-protein] reductase [NADPH] (ENR) (EC 1.3.1.39)     | A0A0H2XJU1             | 5       |
| 96.            | 1.09         | Uncharacterized protein                                               | A0A0H2XFW2             | 3       |
| 97.            | 1.05         | Staphylococcal accessory regulator                                    | A0A0H2XHA2             | 5       |
| 98.            | 1.03         | Bifunctional protein PyPR [Includes: Pyrimidine operon regulatory protein, Uracil phosphoribosyltransferase (URPase) (EC 2.4.2.9)]| Q2FPH0                | 1       |
| 100.           | 1.00         | UTP-glucos-1-phosphate uridyltransferase (EC 2.7.7.9)                 | Q2FEO5                | 3       |
| 101.           | 0.99         | 30S ribosomal protein L17                                             | Q2FER6                | 3       |
| 102.           | 0.98         | Uncharacterized protein                                               | A0A0H2XH92             | 5       |
| 103.           | 0.97         | 30S ribosomal protein L24                                             | Q2FEXF6               | 5       |
| 104.           | 0.94         | Polyribonucleotide nucleotidytransferase (EC 2.7.7.8)                 | A0A0H2XEG5             | 5       |
| 105.           | 0.94         | Ribosome-binding factor A                                            | Q2FHG8                | 4       |
| 106.           | 0.92         | Uncharacterized protein                                               | A0A0H2XH40             | 2       |
| 107.           | 0.92         | Alkaline shock protein                                                | Q2FXE4                | 4       |
| 108.           | 0.88         | Phosphoglyceraldehyde kinase                                          | Q2FM04                | 4       |
Supplementary Figure 7 | Chemical proteomics with PL1 and Ctrl in *S. aureus* USA300 TnPdxS (2h labeling protocol). (a) Volcano plots showing enrichment of proteins with 100 μM PL1 (left) or Ctrl (right) (*n* = 3 biological replicates). Known PLP-DEs are highlighted with blue dots and are numerically annotated according to Supplementary Table 2. (b) Fluorescence SDS-PAGE showing PL1 and Ctrl labeling during proteomic experiments upon CuAAC to trifunctional rhodamine-biotin-azide tag 3 and avidin bead enrichment.

Supplementary Table 2. Proteins significantly enriched using 100 μM PL1 or Ctrl corresponding to Supplementary Fig. 7.

|   | Fold-change | Significance | Name                                          | Uniprot ID   |
|---|-------------|--------------|-----------------------------------------------|--------------|
| PL1 | 1.        | 4.69         | Putative pyridoxal phosphate-dependent acyltransferase | A0A0H2XJW8  |
|    | 2.        | 4.13         | Cysteine synthase (EC 2.5.1.47)                | A0A0H2XFQ3  |
|    | 3.        | 2.39         | Cysteine desulphurase (EC 2.8.1.7)             | A0A0H2XHJ5  |
|    | 4.        | 2.18         | Uncharacterized protein                        | A0A0H2XHH8  |
|    | 5.        | 1.44         | Aminotransferase                              | A0A0H2XJK0  |
|    | 6.        | 1.76         | UPF0340 protein SAUSA300_2068                 | Q2FF14      |
|    | 7.        | 1.37         | Putative heme-dependent peroxidase (EC 1.11.1.7) | Q2FJ50      |
| Ctrl | 8.        | 2.46         | Putative pyridoxal phosphate-dependent acyltransferase | A0A0H2XJW8  |
|    | 9.        | 0.78         | Cysteine synthase (EC 2.5.1.47)                | A0A0H2XFQ3  |
|    | 10.       | 2.27         | Protein-tyrosine-phosphatase PtpA (EC 3.1.3.48) | Q2FFL4      |
|    | 11.       | 1.56         | Putative thioredoxin                          | A0A0H2XHS5  |
|    | 12.       | 1.42         | Lipoate–protein ligase (EC 6.3.1.20)           | A0A0H2XFJ1  |
|    | 13.       | 1.36         | ATP-dependent protease ATPase subunit HsIJ (Unfoldase HsIJ) | Q2FHJ4      |
|    | 14.       | 0.98         | Transferrin receptor                          | A0A0H2XKM7  |
Supplementary Figure 8 | Chemical proteomics with PL3 in *S. aureus* USA300 TnPdxS (2h labeling protocol). (a) Volcano plot showing enrichment of proteins with 100 µM PL3 (*n* = 6 biological replicates). Known PLP-DEs are highlighted with blue dots and are numerically annotated according to Supplementary Table 3. (b) Commercially available phosphine-PEG3-biotin reagent used for Staudinger ligation of PL3-labeled proteins.4

**Supplementary Table 3.** Proteins significantly enriched using 100 µM PL3 corresponding to Supplementary Fig. 8.

| No. | Fold-change | Significance | Name | Uniprot ID |
|-----|-------------|--------------|------|------------|
| 1.  | 4.56        | 7.58         | Cysteine synthase (EC 2.5.1.47) | A0A0H2XFQ3 |
| 2.  | 4.28        | 7.12         | Cysteine desulfurase (EC 2.8.1.7) | A0A0H2XHU5 |
| 3.  | 4.17        | 5.00         | Aminotransferase, class V | A0A0H2XI8 |
| 4.  | 3.98        | 4.29         | Orn/Lys/Arg decarboxylase (EC 4.1.1.18) | A0A0H2XI6 |
| 5.  | 1.89        | 3.47         | Threonine synthase (EC 4.2.3.1) | A0A0H2XH24 |
| 6.  | 1.58        | 6.27         | Putative pyridoxal phosphate-dependent acyltransferase | A0A0H2XJW8 |
| 7.  | 1.44        | 4.84         | Branched-chain-amino-acid aminotransferase (EC 2.6.1.42) | A0A0H2XIS2 |
| 8.  | 1.43        | 2.55         | Cysteine synthase/cystathionine beta-synthase (EC 2.5.1.47) | A0A0H2XG73 |
| 9.  | 1.29        | 5.16         | Alanine racemase (EC 5.1.1.1) | Q2FF55 |
| 10. | 1.01        | 4.43         | L-threonine dehydratase catabolic TdcB (EC 4.3.1.19) (Threonine deaminase) | Q2FH01 |
| 11. | 4.55        | 8.09         | Pseudouridine-5’-phosphate glycosidase (PsiMP glycosidase) (EC 4.2.1.70) | A0A0H2XJC6 |
| 12. | 3.38        | 3.99         | Signal peptidase I (EC 3.4.21.89) | A0A0H2XEA7 |
| 13. | 2.12        | 2.61         | UPF0340 protein SAUSA300_2068 | Q2FF14 |
| 14. | 0.79        | 6.34         | Putative heme-dependent peroxidase SAUSA300_0569 (EC 1.11.1.-) | Q2FJ68 |
Supplementary Figure 9 | Concentration-dependent chemical proteomics of PL2 in S. aureus USA300 TnPdxS. (a) LFQ intensity profile clusters (1-5) of proteins significantly enriched from Fig. 3b (104 proteins total). Data represent n = 3 biological replicates and mean LFQ intensities were normalized (DMSO = 0; maximum LFQ intensity = 1; negative enrichment = 0). Clustering was performed using Perseus (hierarchical clustering, Pearson correlation, 5 groups). (b) Fluorescence SDS-PAGE showing concentration-dependent PL2 labeling during proteomic experiments upon CuAAC to trifunctional rhodamine-biotin-azide tag and avidin bead enrichment. (c) LFQ intensity profiles of uncharacterized proteins selected for validation (left), alternative enzymes selected for validation (center) and all PLP-DEs identified from proteomic analysis using PL2 (right).
### Supplementary Table 4. Proteins significantly enriched using PL1 (25 µM) growth medium supplementation, corresponding to Fig. 4b.

| No. | Fold-change | Significance | Name                                                                 | Uniprot ID  |
|-----|-------------|---------------|----------------------------------------------------------------------|-------------|
| 1.  | 7.78        | 3.23          | Aminotransferase, class V                                            | A0A0H2XKJ8  |
| 2.  | 6.65        | 6.85          | Putative pyridoxal phosphate-dependent acyltransferase               | A0A0H2XWJ8  |
| 3.  | 5.15        | 5.45          | Alanine racemase (EC 5.1.1.1)                                        | Q2FF65      |
| 4.  | 5.13        | 2.44          | Tryptophan synthase beta chain (EC 4.2.1.20)                         | Q2FH64      |
| 5.  | 4.85        | 4.69          | L-threonine dehydratase biosynthetic IlvA (EC 4.3.1.19) (Threonine deaminase) | Q2FF63      |
| 6.  | 4.83        | 3.87          | Uncharacterized protein                                               | A0A0H2XH68  |
| 7.  | 3.57        | 5.50          | Cysteine desulfurase (EC 2.8.1.7)                                    | A0A0H2XHJ5  |
| 8.  | 3.38        | 5.32          | Cysteine synthase (EC 2.5.1.47)                                      | A0A0H2XFQ3  |
| 9.  | 2.83        | 3.58          | Aminotransferase, class V                                            | A0A0H2XG27  |
| 10. | 2.01        | 4.09          | Trans-sulfuration enzyme family protein                               | A0A0H2XG37  |
| 11. | 1.88        | 5.12          | Serine hydroxymethyltransferase (SHMT) (Serine methylase) (EC 2.1.2.1) | Q2FF15      |
| 12. | 1.82        | 3.96          | Alanine racemase (EC 5.1.1.1)                                        | A0A0H2X95   |
| 13. | 1.52        | 3.77          | Cys/Met metabolism PLP-dependent enzyme (EC 4.4.1.8)                 | A0A0H2XFH9  |
| 14. | 1.51        | 5.48          | Threonine synthase (EC 4.2.3.1)                                      | A0A0H2XH24  |
| 15. | 1.44        | 2.13          | Transcriptional regulator, grfr family protein                        | A0A0H2XF80  |
| 16. | 0.82        | 3.59          | Aminotransferase, class I (EC 2.6.1.-)                                | A0A0H2XF99  |
| 17. | 4.27        | 3.47          | UPF0340 protein SAUSA300_2068                                        | Q2FF14      |
| 18. | 2.58        | 2.62          | Bacterial luciferase family protein                                   | A0A0H2XF54  |
| 19. | 2.46        | 1.89          | NADPH-dependent oxidoreductase (EC 1.6.-.-)                           | Q2FJN3      |
| 20. | 2.37        | 1.48          | UPF0348 protein SAUSA300_1025                                        | Q2FHV5      |
| 21. | 2.05        | 3.99          | Transcriptional repressor, ArsR family                                | A0A0H2XG84  |
| 22. | 1.75        | 1.67          | Glyoxalase family protein (EC 1.13.11.39)                             | A0A0H2X94   |
| 23. | 1.54        | 1.54          | Peptide methionine sulfoxide reductase MsrA (Protein-methionine-S-oxide reductase) (EC 1.8.4.11) (Peptide-methionine (S)-S-oxide reductase) | A0A0H2XEQ6 |
| 24. | 1.36        | 4.03          | Bifunctional purine biosynthesis protein PurH [Includes: Phosphoribosylaminomimidazolecarboxamide formyltransferase (EC 2.1.2.3) (AICAR transformylase);IMP cyclohydrolase (EC 3.5.4.10) (ATIC) (IMP synthase) (inosicinase)];] | Q2FI05      |
| 25. | 1.26        | 3.22          | Organic hydroperoxide resistance protein-like                         | Q2FJ2       |
| 26. | 1.18        | 2.74          | Lantibiotic epidermin immunity protein F                              | A0A0H2XGC6  |
| 27. | 1.12        | 3.00          | Phosphoribosylaminomimidazole-succinocarboxamide synthase (EC 6.3.2.6) | Q2FI12      |
| 28. | 1.11        | 2.09          | Uncharacterized oxidoreductase SAUSA300_2422 (EC 1.1.-.-)              | Q2FE21      |
| 29. | 1.03        | 3.34          | Putative heme-dependent peroxidase SAUSA300_0569 (EC 1.11.1.-)         | Q2FJ56      |
| 30. | 0.93        | 3.86          | UPF0173 metal-dependent hydrolase SAUSA300_1653                       | Q2FG31      |
Table 5. Proteins significantly enriched using PL3 (25 μM) growth medium supplementation, corresponding to Fig. 4c.

| No. | Fold-change | Significance | Name                                                                 | Uniprot ID |
|-----|-------------|--------------|----------------------------------------------------------------------|------------|
| 1.  | 7.90        | 11.82        | Glutamate-1-semialdehyde 2,1-aminomutase 2                           | Q2FFN1     |
| 2.  | 7.41        | 9.86         | Aminotransferase, class V                                            | A0A0H2XKB4|
| 3.  | 6.78        | 7.53         | Aminotransferase, class V                                            | A0A0H2XK08|
| 4.  | 5.91        | 10.94        | D-alanine aminotransferase (EC 2.6.1.21)                             | A0A0H2XU66|
| 5.  | 5.88        | 12.54        | Putative pyridoxal phosphate-dependent acyltransferase               | A0A0H2XJW8|
| 6.  | 5.08        | 7.64         | Trans-sulfuration enzyme family protein                              | A0A0H2XG37|
| 7.  | 5.07        | 10.23        | Cysteine desulfurase (EC 2.8.1.7)                                    | A0A0H2XJL5|
| 8.  | 4.94        | 12.04        | Orotate aminotransferase (OAT) (EC 2.6.1.13)                         | A0A0H2XK4K|
| 9.  | 4.34        | 5.60         | Cystathionine gamma-synthase (EC 4.4.1.8)                            | A0A0H2XFF8|
| 10. | 4.15        | 9.95         | Probable glycine dehydrogenase (decarboxylating) subunit 2 (EC 1.4.4.2) | Q2FF17     |
| 11. | 3.91        | 7.93         | Serine hydroxymethyltransferase (SHMT) (Serine methylase) (EC 2.1.2.1) | Q2FF15     |
| 12. | 3.49        | 6.77         | Cysteine synthase (EC 2.5.1.47)                                      | A0A0H2XQ32|
| 13. | 3.47        | 9.72         | Cys/Met metabolism PLP-dependent enzyme (EC 4.4.1.8)                 | A0A0H2XFH9|
| 14. | 3.46        | 9.67         | Branched-chain-aminooxy-acid aminotransferase (EC 2.6.1.42)          | A0A0H2XIS2|
| 15. | 3.27        | 11.73        | Glutamate-1-semialdehyde 2,1-aminomutase 1                           | Q2FG69     |
| 16. | 3.19        | 6.61         | Cysteine synthase/cystathionine beta-synthase (EC 2.5.1.47)          | A0A0H2XG73|
| 17. | 2.96        | 4.45         | Orotate aminotransferase (OAT) (EC 2.6.1.13)                         | A0A0H2XH00|
| 18. | 2.65        | 5.23         | Aminotransferase, class V                                            | A0A0H2XG7Z|
| 19. | 2.28        | 4.59         | Om/Lys/Arg decarboxylase (EC 4.1.1.18)                               | A0A0H2XJL6|
| 20. | 2.16        | 4.45         | Aminotransferase, class V                                            | A0A0H2XJX6|
| 21. | 2.08        | 4.42         | Diaminopimelate decarboxylase (DAP decarboxylase) (DAPDC) (EC 4.1.1.20)| A0A0H2XFDF9|
| 22. | 1.93        | 10.19        | Alanine racemase (EC 5.1.1.1)                                        | Q2FFS5     |
| 23. | 1.75        | 4.26         | Uncharacterized protein                                              | A0A0H2XOH8|
| 24. | 1.37        | 4.06         | Histidinol-phosphate aminotransferase (EC 2.6.1.9)                   | Q2FIR7     |
| 25. | 1.24        | 4.99         | Aminotransferase                                                     | A0A0H2XJQ0|
| 26. | 0.71        | 4.44         | Aminotransferase, class I (EC 2.6.1.-)                               | A0A0H2XF08|
| 27. | 6.05        | 6.22         | Uncharacterized protein                                              | A0A0H2XIC2|
| 28. | 4.13        | 4.85         | Uncharacterized protein                                              | A0A0H2XIU9|
| 29. | 2.09        | 1.93         | PTS system, glucose-specific IIA component (EC 2.7.1.69)             | A0A0H2XJ98|
| 30. | 1.84        | 3.43         | Iron-sulfur cluster repair protein ScS                               | Q2FK11     |
| 31. | 1.76        | 8.70         | Bifunctional purine biosynthesis protein PurH [includes: Phosphoribosylaminomimidazolecarboxamide formyltransferase (EC 2.1.2.3); IMP cyclohydrolase (EC 3.5.4.10)] | Q2FI05 |
| 32. | 1.28        | 4.96         | Methylene-tetrahydrofolate–5′-RNA (uracil-5′-)–methyltransferase TrmFO (EC 2.1.1.74) | Q2FHI7 |
| 33. | 1.21        | 0.90         | Histidine biosynthesis bifunctional protein HisIIE                     | A0A0H2XGN5|
| 34. | 1.20        | 3.49         | Putative thioredoxin                                                 | A0A0H2XH55|
| 35. | 1.09        | 1.36         | D-lactate dehydrogenase (EC 1.1.1.28)                                | A0A0H2XH62|
| 36. | 0.87        | 7.15         | Phosphoribosylamine–glycine ligase (EC 6.3.4.13) (Glycinamide ribonucleotide synthetase) | A0A0H2XF4S|
| 37. | 0.87        | 5.03         | Uncharacterized protein                                              | A0A0H2XJES|
| 38. | 0.86        | 3.93         | Uncharacterized protein                                              | A0A0H2XDW9|
| 39. | 0.86        | 2.24         | Uncharacterized protein                                              | A0A0H2XFG6|
| 40. | 0.84        | 3.08         | Phosphoribosylaminomimidazole-succinocarboxamide synthase (EC 6.3.2.6) (SAICAR synthetase) | Q2F112 |
| 41. | 0.83        | 2.05         | Deoxyribose-phosphate aldolase (DERA) (EC 4.1.2.4) (2-deoxy-D-ribose 5-phosphate aldolase) (Phosphodeoxyriboaldolase) | A0A0H2XF03|
| 42. | 0.82        | 2.47         | Probable succinyl-diaminopimelate desuccinylase                      | A0A0H2XKKA|
| 43. | 0.81        | 3.08         | Phosphoribosylformylglycinamide synthase subunit PurQ (EC 6.3.5.3) (Glutaminase PurQ) (EC 3.5.1.2) (Phosphoribosylformylglycinamide synthase subunit I) | Q2FI010 |
| 44. | 0.78        | 7.10         | S-adenosylmethionine synthase (AdoMet synthase) (EC 2.5.1.6) (MAT) (Methionine adenosyltransferase) | Q2FV06 |
| 45. | 0.77        | 5.48         | N5-carboxyribosylaminomimidazole ribonucleotide synthase (N5-CAIR synthase) (EC 6.3.4.18) (5-carboxyamino)imidazole ribonucleotide synthetase) | A0A0H2XEX9|
| 46. | 0.76        | 3.37         | Phosphoribosylformyltransferase (EC 2.1.2.2) (5′-phosphoribosylglycinamide transferase) | A0A0H2XHN0|
| 47. | 0.74        | 6.05         | SUF system FeS assembly protein, NifU family                         | A0A0H2XC02|
| 48. | 0.70        | 5.76         | Bacillioregulatin consorlatory protein (EC 1.11.1.-)                  | A0A0H2XHS7|
Supplementary Figure 10 | Analysis of PLP-binding and substrate scope of aminotransferases. (a) UV-Vis spectra of two additional PLP-dependent aminotransferases, AT1 and AT2, and formation of characteristic PMP intermediate upon incubation with amino acid substrates (10 equivalents). Curves were normalized to absorbance at 300 nm. (b) Heatmap representation of amino acid substrate preferences for aminotransferases AT1, AT2 (L-amino acids tested) and DAT (D-amino acids tested) as analyzed by UV-Vis. (c) Percent of enzyme bound upon incubation with PLP or cofactor analogues (4 equivalents), NaBH₄-reduction and measurement by intact protein MS (n = 3, error bars: mean ± standard deviation).

Supplementary Table 6. Analysis of cofactor binding by MS (full data). Percent of enzyme bound by cofactor upon incubation with PL/PLP, PL2/PL2P, PL3/PL3P (4 equivalents), NaBH₄-reduction and intact-protein MS.

| Enzyme | % Enzyme bound upon incubation with PL (5 eq) | % Enzyme bound upon incubation with PLP (5 eq) | % Enzyme bound upon incubation with PL2/PL2P (5 eq) | % Enzyme bound upon incubation with PL3/PL3P (5 eq) |
|--------|-----------------------------------------------|-----------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| AT1    | 0 (PL) 47.6 ± 7.2 (PLP) 100 (PLP) 0 (PL2) 44.3 ± 20.4 (PLP) 37.0 ± 14 (PL2P) 50.8 ± 9.1 (PLP) |
| AT2    | 0 (PL) 0 (PLP) 100 (PLP) 0 (PL2) 0 (PLP) 100 (PL2P) 0 (PLP) |
| CS     | 5.9 ± 1.0 (PL) 53.3 ± 8.3 (PLP) 100 (PLP) 0.7 ± 0.7 (PL2) 50.3 ± 4.6 (PLP) 100 (PL2P) 0 (PLP) |
| DAT    | 0 (PL) 0 (PLP) 97.1 ± 1.3 (PLP) 0 (PL2) 0 (PLP) 97.0 ± 1.7 (PL2P) 0 (PLP) |
| GP0    | 0 (PL) 87.2 ± 4.9 (PLP) 94.8 ± 5.7 (PLP) 0 (PL2) 90.6 ± 3.9 (PLP) 17.6 ± 3.9 (PL2P) 71.1 ± 6.0 (PLP) |
| HH8    | 0 (PL) 75.2 ± 3.5 (PLP) 97.1 ± 1.3 (PLP) 0 (PL2) 64.9 ± 8.2 (PLP) 55.5 ± 1.6 (PL2P) 43.8 ± 1.5 (PLP) |
| IC2    | 0 (PL) 6.8 ± 11.4 (PLP) 30.1 ± 3.9 (PLP) 0 (PL3) 0 (PLP) 0 (PL3P) 18.1 ± 9.7 (PLP) |
| IU9    | 0.6 ± 1.7 (PL) 99.4 ± 0.7 (PLP) 96.0 ± 1.3 (PLP) 4.0 ± 1.3 (2x PLP) 0 (PL3) 0 (PL3P) 0.1 ± 0.1 (PL3P) 99.9 ± 0.1 (PLP) |
| ODC    | 0 (PL) 0 (PLP) 100 (PLP) 0 (PL2) 0 (PLP) 100 (PL2P) 0 (PLP) |
| Q2FF14 | 0 (PL) 0 (PLP) 20.5 ± 4.1 (PLP) 0 (PL2) 0 (PLP) 5.1 ± 4.1 (PL2P) 0 (PLP) |
Supplementary Figure 11 | Additional gels for Figure 5d. (a) Full fluorescence gels and Coomassie stained gels of known PLP-DE labeling experiments using PL2 and PL2P, upon NaBH₄ reduction and CuAAC with rhodamine azide. (b) Full fluorescence gels and Coomassie stained gels of uncharacterized protein labeling experiments using PL2/PL3 and PL2P/PL3P upon NaBH₄ reduction and CuAAC with rhodamine azide (PL2-based samples) or rhodamine alkyne (PL3-based samples).
Supplementary Figure 12 | Analysis of PLP-binding for proteins with alternative, non-PLP-related functions. (a) Full fluorescence gels and Coomassie stained gels of select alternative function proteins labeled with PL2 (5 equivalents) or PL2P (0.5, 1, 5 equivalents). HemQ (coprohaem decarboxylase, Q2FJ56), PsiMP (pseudouridine-5’-phosphate glycosidase, A0A0H2XJC6), HemH (ferrochelatase, AOA0H2XGS5). (b) UV-Vis spectra of alternative function proteins. Curves were normalized to absorbance at 300 nm. (c) Percent of alternative function proteins bound upon incubation with PL, PLP, PL2 or PL2P (4 equivalents), NaBH₄-reduction and measurement by intact protein MS (n = 3, error bars: mean ± standard deviation).
Supplementary Figure 13 | Characterization of JW8 as a glycine acetyltransferase. (a) Full fluorescence (left) and Coomassie stained gels (right) of JW8 protein labeling using PL2 and PL2P. (b) UV-Vis spectrum of JW8; curve normalized to absorbance at 300 nm. (c) Percent of JW8 bound to PLP upon incubation with extra PLP (2 equivalents), NaBH₄-reduction and measurement by intact protein MS (n = 3, error bars: mean ± standard deviation). Approximately 7% of protein was found to have 2-fold PLP addition in the presence of excess PLP. (d) Specific N-acetylation of glycine (2.5 mM; Ctrl = no amino acid substrate) by JW8 (1 μM) using acetyl-CoA (0.5 mM) as measured by absorbance of 2-nitro-5-thiobenzoate at 415 nm upon treatment of reaction mixtures with Ellman’s reagent. Inactivated JW8 (upon NaBH₄ reduction of PLP internal aldimine) is unable to catalyze the conversion. Fold changes were calculated relative to the reduced enzyme control (n = 3, error bars: mean ± standard deviation; significance calculated using a one-way ANOVA test). (e) Extracted ion chromatogram at 118.05 m/z. (Blue) formation of N-acetylglycine upon incubation of JW8 with acetyl-CoA and glycine. (Orange) negative control assay mix without the addition of glycine.
PLP binding site for AT1 (A0A0H2XFY9), K233

PLP binding site for AT2 (A0A0H2XFA8), K232
PLP binding site for CS (A0A0H2XFQ3), K46

PLP binding site for DAT (A0A0H2XHU6), K157
PLP binding site for GP0 (A0A0H2XGP0), K172

PLP binding site for HH8 (A0A0H2XHH8), K32
PLP binding site for IC2 (A0A0H2XIC2), K195

PLP binding site for IU9 (A0A0H2XIU9), K100
PLP binding site for JW8 (A0A0H2XJW8), K243

PLP binding site for ODC (A0A0H2XII6), K210
PLP binding site for Q2FF14, K58

Supplementary Figure 14 | Additional information for PLP binding-site identification. MS/MS spectra of binding site peptides as exported from MaxQuant Viewer.

Supplementary Figure 15 | PLK phosphorylation of probes PL4 and PL5. (a) Kinetics of probe phosphorylation by PLK. (n = 4, error bars: mean ± standard deviation). (b) MS/MS spectra of phosphorylated probes PL4P and PL5P upon incubation with PLK. HRMS: PL4P (found: 328.09518 [M+H]+ calc.: 328.09445), PL5P (found: 362.07956 [M+H]+ calc.: 362.07880).
Supplementary Figure 16 | Chemical proteomics with PL2, PL4 and PL5 in *S. aureus* USA300 TnpxS (2h labeling protocol). Supplementary volcano plots for Fig. 6c showing enrichment of proteins with 100 µM PL2 (left), PL4 (center) or PL5 (right) (*n* = 6 biological replicates). Known PLP-DEs are marked with blue dots and are listed in Supplementary Table 7.

Supplementary Table 7. Comparison of PLP-DEs significantly enriched by chemical proteomics using PL2, PL4, or PL5, corresponding to Supplementary Fig. 16.

| PL2 (sig.) | PL4 (sig.) | PL5 (sig.) | Name | Uniprot ID |
|------------|------------|------------|------|------------|
| +          | +          |            | Alanine racemase (EC 5.1.1.1) | Q2FF55 |
| +          | +          |            | Aminotransferase, class I (EC 2.6.1.-) | A0A0H2XF9Y |
| +          |            |            | Aminotransferase, class V | A0A0H2XFA8 |
| +          | +          | +          | Branched-chain-amino-acid aminotransferase (EC 2.6.1.42) | A0A0H2XES2 |
| +          | +          |            | Cystathionine gamma-synthase (EC 4.4.1.8) | A0A0H2XF8 |
| +          | +          |            | Cysteine desulfurase (EC 2.8.1.7) | A0A0H2XHJ5 |
| +          | +          |            | Cysteine synthase (EC 2.5.1.47) | A0A0H2XFQ3 |
| +          | +          |            | Cysteine synthase/cystathionine beta-synthase (EC 2.5.1.47) | A0A0H2XG73 |
| +          | +          |            | D-alanine aminotransferase (EC 2.6.1.21) | A0A0H2XHU6 |
| +          | +          | +          | Diaminopimelate decarboxylase (DAP decarboxylase) (DAPDC) (EC 4.1.1.20) | A0A0H2XFD9 |
| +          | +          | +          | D-threonine dehydratase (TdcB) (EC 4.3.1.19) (Threonine deaminase) | Q2FFH1 |
| +          | +          | +          | Orn/Lys/Arg decarboxylase (EC 4.1.1.18) | A0A0H2XIL6 |
| +          | +          |            | Threonine synthase (EC 4.2.3.1) | A0A0H2XH24 |
| +          | +          | +          | Uncharacterized protein | A0A0H2XGP0 |
| +          | +          | +          | Uncharacterized protein | A0A0H2XHH8 |
| +          | +          | +          | Uncharacterized protein | A0A0H2XH8 |
| +          | +          |            | UPF0340 protein SAUSA300_2068 | Q2FF14 |
| +          |            |            | Probable glycine dehydrogenase (decarboxylating) subunit 2 (EC 1.4.4.2) | Q2FG17 |
| +          |            |            | Putative aluminium resistance protein | A0A0H2XHV8 |
| +          |            |            | Putative pyridoxal phosphate-dependent acyltransferase | A0A0H2XJW8 |
| +          |            |            | Aminotransferase | A0A0H2XJK0 |
| +          |            |            | Glutamate-1-semialdehyde 2,1-aminomutase 2 (GSA 2) (EC 5.4.3.8) | Q2FFN1 |
| +          |            |            | Ornithine aminotransferase (OAT) (EC 2.6.1.13) | A0A0H2XIK4 |
| +          |            |            | Probable glycine dehydrogenase (decarboxylating) subunit 1 (EC 1.4.4.2) | Q2FG16 |
| +          |            |            | Serine hydroxymethyltransferase (SHMT) (Serine methylase) (EC 2.1.2.1) | Q2FF15 |
**Supplementary Table 8.** Proteins significantly depleted by DCS treatment, corresponding to Fig. 6e. PLP-DEs are coloured in blue.

| Rank | Fold-change | Significance | Name                                                                                          | Uniprot ID   |
|------|-------------|--------------|------------------------------------------------------------------------------------------------|--------------|
| 1.   | 1.95        | 4.17         | Diaminopimelate decarboxylase (EC 4.1.1.20)                                                   | A0A0H2XFD9   |
| 2.   | 2.60        | 4.12         | Orn/Lys/Arg decarboxylase (EC 4.1.1.18)                                                       | A0A0H2XI6    |
| 3.   | 2.16        | 4.10         | D-alanine aminotransferase (EC 2.6.1.21)                                                      | A0A0H2XHU6   |
| 4.   | 0.24        | 0.65         | Alanine racemase (EC 5.1.1.1)                                                                | Q2FFS5       |
| 5.   | 1.51        | 4.29         | D-alanine-D-alanine ligase (EC 6.3.2.4)                                                       | Q2FF43       |
| 6.   | 3.85        | 3.95         | Extracellular matrix-binding protein ebh (ECM-binding protein homolog)                        | Q2FH04       |
| 7.   | 3.28        | 1.01         | Uncharacterized protein                                                                         | A0A0H2XH10   |
| 8.   | 3.19        | 3.78         | Uncharacterized protein                                                                         | A0A0H2XD3    |
| 9.   | 3.10        | 1.02         | PTS system, glucose-specific IIA component (EC 2.7.1.69)                                      | A0A0H2XJ98   |
| 10.  | 2.90        | 1.43         | Octanoyltransferase LpiM (EC 2.3.1.181)                                                       | A0A0H2XF3    |
| 11.  | 2.24        | 1.62         | Uncharacterized protein                                                                         | A0A0H2XE84   |
| 12.  | 2.09        | 1.61         | Uncharacterized protein                                                                         | A0A0H2XF3    |
| 13.  | 1.92        | 3.26         | Uncharacterized protein                                                                         | A0A0H2XD3    |
| 14.  | 1.91        | 1.37         | Deoxynucleoside kinase family protein (EC 2.7.1.113)                                           | A0A0H2XGW9   |
| 15.  | 1.86        | 2.74         | Glycosyl transferase, group 1 family protein (EC 2.4.1.-)                                     | A0A0H2XIE0   |
| 16.  | 1.83        | 1.55         | Hydrolase, alpha/beta hydrolase fold family (EC 3.4.11.5)                                     | A0A0H2XC7    |
| 17.  | 1.67        | 1.77         | Uncharacterized protein                                                                         | A0A0H2XI9    |
| 18.  | 1.61        | 2.29         | Uncharacterized protein                                                                         | A0A0H2XEN1   |
| 19.  | 1.55        | 1.98         | Carboxylesterase (EC 3.1.1.1)                                                                | A0A0H2XLJ0   |

**Supplementary Figure 17 |** Inhibition of Alr activity by DCS in vitro ($n = 3$ replicates, mean ± standard deviation).
Methods and Protocols

1. Chemical Synthesis

**General Methods.** Reagents were purchased from Sigma-Aldrich, Thermo Fisher Scientific, Merck, TCI Europe, VWR International, and Alfa Aesar, and were used without further purification. All reactions involving air or water sensitive chemicals were carried out in oven-dried flasks under argon atmosphere. Dry solvents were purchased from Sigma-Aldrich and stored over molecular sieves under argon atmosphere. Flash chromatography and TLC (F254) analysis were performed using 60 Å silica gel from Merck (Darmstadt, Germany). TLC plates were visualized by staining with ceric ammonium molybdate (CAM) and phosphomolybdic acid (PMA) stains, or UV-absorption. HRMS spectra were acquired by ESI using a LTQ-FT Ultra mass spectrometer (Thermo Scientific). NMR spectra were recorded on Bruker AV-250, AV-360, AV-500, AVHD-300, AVHD-400, and AVHD-500 instruments, or an AVHD-500 spectrometer coupled to a cryo platform. The chemical shifts (δ) are reported in parts per million (ppm) and spectra are referenced to residual proton and carbon signals of the deuterated solvent. The following abbreviations are used to describe NMR coupling patterns: s – singlet; d – doublet; t – triplet; q – quartet; p – pentet; m – multiplet. The coupling constants, J, are reported in Hertz (Hz).

**HPLC Purification.** Compounds were purified by preparative, reversed-phase HPLC using a Waters 2545 quaternary gradient module equipped with a Waters 2998 photodiode array detector and fraction collector on a YMC Triart C18 column (250 × 10 mm, 5 µm). Gradients are listed in Supplementary Table 9, using ddH2O and HPLC-grade acetonitrile (no TFA) as the mobile phase.

**Supplementary Table 9: HPLC gradients**

| Method A | Method B | Method C |
|----------|----------|----------|
| Time (min) | % H2O | % ACN | % H2O | % ACN | Time (min) | % H2O | % ACN |
| 0          | 98      | 2       | 98      | 2       | 0          | 98      | 2       |
| 1          | 98      | 2       | 98      | 2       | 12         | 2       | 98      |
| 30         | 50      | 50      | 80      | 20      | 14         | 2       | 98      |
| 35         | 2       | 98      | 2       | 98      | 15         | 98      | 2       |
| 37         | 2       | 98      | 2       | 98      | 17         | 98      | 2       |
| 40         | 98      | 2       | 98      | 2       | 45         | 98      | 2       |
| 45         | 98      | 2       | 98      | 2       |            |         |         |
**General alkylation protocol.** LDA was freshly prepared by adding diisopropylamine (1.5 ml, 10.7 mmol) and n-butyllithium (2.5 M in hexanes; 3.9 ml, 9.8 mmol) to a dry flask containing THF (30 ml) under argon at 0°C, and stirring for 15 min. After cooling to -78°C, starting material 1 (1.0 g, 4.4 mmol) dissolved in dry THF (5 ml) was added dropwise. The reaction was stirred for 1 h and then re-cooled to -78°C prior to the addition of alkyl halide (17.8 mmol). Upon stirring the reaction for 1 h at -78°C, the ice bath was removed and the reaction was allowed to warm to room temperature for 1 h. The reaction was quenched by adding satd. NH₄Cl (5 ml) at -78°C, and extra H₂O (30 ml) was added prior to separating the phases. The aqueous layer was extracted with EtOAc (3 x 50 ml), and the combined organic phases were washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The product was purified by flash chromatography (5% MeOH/DCM).

**Synthesis of PL2**

1-methoxy-7-(methoxymethoxy)-6-methyl-1,3-dihydrofuro[3,4-c]pyridine

The monomethoxy acetal intermediate was synthesized according to a previously reported protocol by Kim & Jacobson.⁵ A suspension of pyridoxal hydrochloride (8 g, 39.3 mmol) in MeOH (50 ml) was refluxed for 1 h. Upon cooling to room temperature, solid NaHCO₃ (3.3 g, 39.3 mmol) was added and the reaction was refluxed overnight. The mixture was filtered to remove precipitated NaCl and concentrated to yield crude intermediate acetal, which was used in the following reaction without further purification.

DIPEA (17.0 ml, 98.3 mmol) and MOMCl (4.5 ml, 59.0 mmol) were sequentially added to a solution of crude acetal (39.3 mmol) dissolved in anhydrous DCM (50 ml), and the reaction was stirred for 2 h at room temperature. Upon completion, the solution was concentrated under reduced pressure and the residue was purified by flash chromatography to yield 1 (5.94 g, 26.4 mmol, 67% over 2 steps) as a clear oil. Rᵢ = 0.50 (5% MeOH/DCM). ¹H NMR (300 MHz, CDCl₃) δ 8.08 (s, 1H), 6.19 (d, J = 1.7 Hz, 1H), 5.36 (d, J = 6.5 Hz, 1H), 5.12 (ddt, J = 12.7, 1.8, 0.9 Hz, 1H), 5.02 (d, J = 6.5 Hz, 1H), 4.95 (dt, J = 12.6, 0.8 Hz, 1H), 3.45 (s, 3H), 3.39 (s, 3H), 2.48 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 150.2, 146.2, 135.6, 135.4, 133.6, 106.1, 96.1, 70.0, 56.7, 54.7, 19.6; HRMS for C₁₁H₁₅NO₄ [M+H]⁺ calcd. 226.1079, obtained 226.1072.
6-(but-3-yn-1-yl)-1-methoxy-7-(methoxymethoxy)-1,3-dihydrofuro[3,4-c]pyridine

The general alkylation protocol was used to react 1 (1.0 g, 4.4 mmol) with propargyl bromide (80% in toluene; 2.0 ml, 17.8 mmol) in order to synthesize the alkylated product 2 (560 mg, 2.2 mmol, 50%) as a yellow oil. Rf = 0.66 (5% MeOH/DCM). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.19 (s, 1H), 6.26 (d, $J$ = 1.7 Hz, 1H), 5.45 (d, $J$ = 6.6 Hz, 1H), 5.18 (ddd, $J$ = 12.8, 1.7, 0.9 Hz, 1H), 5.08 (d, $J$ = 6.6 Hz, 1H), 5.02 (d, $J$ = 12.8 Hz, 1H), 3.51 (s, 2H), 3.46 (s, 2H), 3.13 (t, $J$ = 7.7 Hz, 2H), 2.68 – 2.62 (m, 2H), 1.93 (t, $J$ = 2.7 Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 151.2, 146.3, 136.3, 135.6, 133.8, 106.3, 96.3, 84.3, 70.1, 68.5, 57.0, 54.9, 31.5, 17.5; HRMS for C$_{14}$H$_{17}$NO$_4$ [M+H]$^+$ calcd. 264.1236, obtained 264.1228.

PL2

Compound 2 (40 mg, 0.15 mmol) was dissolved in 5% H$_2$SO$_4$ (2 ml) in acetone/H$_2$O (1:1) and heated at 85°C for 1 h. The reaction was allowed to cool and the acetone was removed under reduced pressure. The product was purified by HPLC using method B ($t_R$ = 9.8 min) and lyophilized to generate PL2 as the H$_2$SO$_4$ salt (35 mg, 0.11 mmol, 76%) as a fluffy white solid. $^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.72 (s, 1H, OH), 7.98 (s, 1H), 6.67 (d, $J$ = 7.7 Hz, 1H, OH), 6.42 (dd, $J$ = 7.5, 1.8 Hz, 1H), 5.05 (ddd, $J$ = 12.9, 1.9, 0.9 Hz, 1H), 4.86 (d, $J$ = 13.1 Hz, 1H), 2.96 – 2.91 (m, 2H), 2.71 (t, $J$ = 2.6 Hz, 1H), 2.57 – 2.50 (m, 1H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 146.6, 145.7, 135.5, 133.8, 132.5, 98.4, 84.5, 71.0, 68.8, 30.5, 16.5; HRMS for C$_{14}$H$_{11}$NO$_3$ [M+H]$^+$ calcd. 206.0817, obtained 206.0810.
Synthesis of PL1 and Ctrl

3-((tert-butyldimethylsilyl)oxy)-4,5-bis(((tert-butyldimethylsilyl)oxy)methyl)-2-methylpyridine

Pyridoxine hydrochloride (2.0 g, 9.7 mmol), imidazole (2.9 g, 43.6 mmol) and TBDMS-Cl (5.11 g, 34.0 mmol) were slurried in DCM (30 ml) and the resulting suspension was stirred overnight. The reaction was filtered to remove solids, washed with DCM and the filtrate was concentrated. The resulting oil was dissolved in Et₂O (50 ml) and acidified with 4 M HCl in dioxane (2.5 ml, 10 mmol) to yield the HCl salt as a white precipitate. Upon filtering and extensive washing with Et₂O, the solid was partitioned between EtOAc and satd. NaHCO₃. The organic phase was washed twice with satd. NaHCO₃, dried over Na₂SO₄ and concentrated under reduced pressure to yield 3 (3.0 g, 5.9 mmol, 61% yield) as a colourless oil. Rf = 0.50 (10% EtOAc/Hex). ¹H NMR (360 MHz, CDCl₃) δ 8.30 (s, 1H), 4.87 (s, 2H), 4.71 (s, 2H), 2.44 (s, 3H), 1.04 (s, 9H), 0.93 (s, 9H), 0.87 (s, 9H), 0.12 (s, 6H), 0.09 (s, 6H), 0.04 (s, 6H); ¹³C NMR (90 MHz, CDCl₃) δ 149.4, 146.3, 146.3, 135.7, 135.0, 61.3, 56.9, 26.1, 25.9, 25.9, 20.8, 18.6, 18.5, 18.3, -3.6, -5.2, -5.3; HRMS for C₂₆H₅₃NO₃Si₃ [M+H]⁺ calcd. 512.3412, obtained 512.3404.

4,5-bis(((tert-butyldimethylsilyl)oxy)methyl)-2-(hydroxymethyl)pyridin-3-ol

Following a modified procedure by Korytnyk et al.,⁶ mCPBA (1.0 g, 77%, 4.5 mmol) was added to a solution of 3 (2.3 g, 4.5 mmol) in DCM (50 ml) at 0°C, and the reaction was stirred until complete consumption of starting material was indicated by TLC (20% EtOAc/Hex). Upon removal of the solvent under reduced pressure, the resulting residue was dissolved in EtOAc and washed with satd. NaHCO₃ (3 × 50 ml). The organic phase was dried over Na₂SO₄ and concentrated to yield the intermediate N-oxide, which was used without further purification. Crude N-oxide was dissolved in DCM (50 ml) and cooled to 0°C prior to the
slow addition of trifluoroacetic anhydride (1.3 ml, 9.0 mmol) via syringe for the Boekelheide rearrangement.\(^1\) The reaction mixture was stirred overnight and allowed to warm to room temperature. Upon cooling to 0°C, MeOH (10 ml) was slowly added and the reaction was stirred at 0°C for 20 min, and then allowed to warm up to room temperature while stirring for another 20 min. Following removal of solvents under reduced pressure, the residue was dissolved in EtOAc and washed with satd. NaHCO\(_3\) (3 x 50 ml). The organic phase was dried over Na\(_2\)SO\(_4\), filtered and concentrated. The crude product was purified by flash chromatography to yield 4 (1.2 g, 2.9 mmol, 64% yield over 2 steps) as a white solid. \(R_f = 0.41\) (50% EtOAc/Hex). \(^1\)H NMR (360 MHz, CDCl\(_3\)) \(\delta 9.16\ (bs, 1H), 7.93\ (s, 1H), 5.12\ (s, 2H), 4.75\ (s, 2H), 4.59\ (s, 2H), 0.95\ (s, 9H), 0.89\ (s, 9H), 0.18\ (s, 6H), 0.07\ (s, 6H); \(^{13}\)C NMR (90 MHz, CDCl\(_3\)) \(\delta 150.1, 147.8, 138.3, 131.3, 129.2, 63.1, 61.7, 60.5, 25.8, 18.3, 18.3, -5.2, -5.5\); HRMS for C\(_{20}\)H\(_{39}\)NO\(_4\)Si\(_2\) [M+H]\(^+\) calcd. 414.2496, obtained 414.2482.

4,5-bis(((tert-butyldimethylsilyl)oxy)methyl)-3-hydroxypicolinaldehyde

To a solution of alcohol 4 (1.2 g, 2.9 mmol) in DCM (10 ml) was added MnO\(_2\) (0.7 g, 8.7 mmol), and the resulting slurry was stirred vigorously for approximately 3 h until complete consumption of starting material was indicated by TLC (20% EtOAc/Hex). If the reaction stalled, more MnO\(_2\) was added. Once the oxidation was complete, the reaction mixture was filtered through celite and concentrated under reduced pressure to yield aldehyde 5 (1.0 g, 2.4 mmol, 83%) as a colourless oil which did not require further purification. \(R_f = 0.72\) (20% EtOAc/Hex). \(^1\)H NMR (360 MHz, CDCl\(_3\)) \(\delta 11.03\ (s, 1H), 10.04\ (s, 1H), 8.59\ (s, 1H), 5.00\ (s, 2H), 4.81\ (s, 2H), 0.96\ (s, 9H), 0.89\ (s, 9H), 0.13\ (s, 6H), 0.09\ (s, 6H); \(^{13}\)C NMR (90 MHz, CDCl\(_3\)) \(\delta 198.7, 155.6, 143.9, 141.6, 135.5, 133.6, 61.0, 54.6, 26.0, 18.5, 18.4, -5.3, -5.3\); HRMS for C\(_{20}\)H\(_{37}\)NO\(_4\)Si\(_2\) [M+H]\(^+\) calcd. 412.2339, obtained 412.2326.
4,5-bis(((tert-butyldimethylsilyl)oxy)methyl)-3-(methoxymethoxy)picolinaldehyde

DIPEA (145 µl, 0.83 mmol) and MOM-Cl (86 µl, 1.13 mmol) were sequentially added to a solution of 5 (310 mg, 0.75 mmol) in DCM (5 ml) and the reaction mixture was stirred at room temperature for 2 h. Upon removal of the solvents under reduced pressure, the crude product was purified by flash chromatography to yield 6 (209 mg, 0.46 mmol, 76% yield) as a colourless oil which crystallizes upon freezing. R_f = 0.30 (20% EtOAc/Hex). ^1H NMR (360 MHz, CDCl_3) δ 10.17 (s, 1H), 8.82 (s, 1H), 5.10 (s, 2H), 4.99 (s, 2H), 4.84 (s, 2H), 3.57 (s, 3H), 0.95 (s, 9H), 0.89 (s, 9H), 0.12 (s, 6H), 0.10 (s, 6H); ^13C NMR (90 MHz, CDCl_3) δ 191.7, 152.4, 144.8, 143.9, 142.6, 140.3, 102.4, 61.0, 58.1, 56.1, 26.0, 25.9, 18.5, 18.3, -5.3, -5.4; HRMS for C_{22}H_{41}NO_5Si_2 [M+H]^+ calcd. 456.2602, obtained 456.2601.

4,5-bis(((tert-butyldimethylsilyl)oxy)methyl)-2-ethynyl-3-(methoxymethoxy)pyridine

To a solution of 6 (209 mg, 0.46 mmol) in MeOH (5 ml) were added the Ohira-Bestmann reagent^2 (dimethyl-1-diazo-2-oxopropylphosphonate) (131 mg, 0.68 mmol) and solid K_2CO_3 (124 mg, 0.90 mmol), and the reaction was rapidly stirred for 16 h. EtOAc (20 ml) was then added to the reaction mixture and the organic phase was washed with H_2O (2 × 10 ml), dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography to yield 7 (120 mg, 0.27 mmol, 58% yield) as a yellow solid. R_f = 0.47 (20% EtOAc/Hex). ^1H NMR (360 MHz, CDCl_3) δ 8.55 (s, 1H), 5.20 (s, 2H), 4.92 (s, 2H), 4.79 (s, 2H), 3.58 (s, 3H), 3.31 (s, 1H), 0.93 (s, 9H), 0.88 (s, 9H), 0.11 (s, 6H), 0.09 (s, 6H); ^13C NMR (90 MHz, CDCl_3) δ 153.1, 144.7, 138.8, 137.9, 135.3, 100.7, 80.8, 60.9, 58.1, 56.3, 26.0, 25.9, 18.4, 18.3, -5.3, -5.4; HRMS for C_{23}H_{41}NO_5Si_2 [M+H]^+ calcd. 452.2652, obtained 452.2635.
(6-ethynyl-5-(methoxymethoxy)pyridine-3,4-diyl)dimethanol

TBAF (514 µl, 1 M in THF, 514 mmol) was added to a solution of 7 (116 mg, 0.257 mmol) in THF (5 ml). The reaction was stirred for 10 min at room temperature and following removal of solvents under reduced pressure, the crude product was purified by flash chromatography to yield 8 (55 mg, 0.246 mmol, 96% yield) as a tan solid. \( R_f = 0.48 \) (10% MeOH/DCM). \(^1\)H NMR (360 MHz, MeOD) \( \delta \) 8.38 (s, 1H), 5.28 (s, 2H), 4.85 (s, 2H), 4.80 (s, 2H), 4.01 (s, 1H), 3.63 (s, 3H); \(^{13}\)C NMR (90 MHz, MeOD) \( \delta \) 155.5, 145.8, 142.8, 139.4, 137.0, 101.7, 84.1, 80.6, 60.4, 58.3, 55.7; HRMS for C\(_{11}\)H\(_{13}\)NO\(_4\) [M+H]+ calcd. 224.0923, obtained 224.0916.

2-ethynyl-5-(hydroxymethyl)-3-(methoxymethoxy)isonicotinaldehyde and 6-ethynyl-4-(hydroxymethyl)-5-(methoxymethoxy)nicotinaldehyde

To a solution of 8 (30 mg, 0.13 mmol) in MeOH (3 ml) was added MnO\(_2\) (34 mg, 0.39 mg), and the resulting slurry was stirred vigorously for 1 h. The reaction mixture was subsequently filtered through a 0.4 µM syringe filter and concentrated under reduced pressure. The product was dissolved in H\(_2\)O and the two isomers, 9a and 9b, were separated by HPLC purification using method A (9a \( t_R = 13.4 \) min, 9b \( t_R = 14.0 \) min). \( R_f = 0.53 \) (10% MeOH/DCM). The product distribution was 2.5:1 and the respective final yields were 9a (15 mg, 0.067 mmol, 52%) and 9b (6 mg, 21%). 9a \(^1\)H NMR (500 MHz, MeOD) \( \delta \) 8.22 (s, 1H), 6.56 (s, 1H), 5.59 (d, \( J = 6.4 \) Hz, 1H), 5.28 (d, \( J = 6.5 \) Hz, 1H), 5.23 (dd, \( J = 13.8 \) Hz, \( J = 1.2 \) Hz, 1H), 5.05 (d, \( J = 13.8 \) Hz, 1H), 3.92 (s, 1H), 3.55 (s, 3H); \(^{13}\)C NMR (125 MHz, MeOD) \( \delta \) 151.8, 140.0, 139.4, 138.3, 135.2, 101.1, 98.1, 83.6, 80.2, 70.6, 57.5; HRMS for C\(_{11}\)H\(_{11}\)NO\(_4\) [M+H]+ calcd. 222.0766, obtained 222.0759. 9b \(^1\)H NMR (500 MHz, MeOD) \( \delta \) 8.27 (s, 1H), 6.48 (d, \( J = 2.1 \) Hz, 1H), 5.33 (dd, \( J = 14.2, 2.2 \) Hz, 1H), 5.23 (s, 2H), 5.16 (d, \( J = 14.2 \) Hz, 1H), 3.96 (s, 1H), 3.53 (s, 3H); \(^{13}\)C NMR (125 MHz, MeOD) \( \delta \) 151.4, 140.9, 140.0, 139.8, 135.7, 101.1, 98.1, 84.1, 80.0, 71.0, 57.4; HRMS for C\(_{11}\)H\(_{11}\)NO\(_4\) [M+H]+ calcd. 222.0766, obtained 222.0759.
Synthesis of PL1

9a (20.7 mg, 0.094 mmol) was dissolved in 0.5 M HCl and stirred at rt for 15 min. The solution was diluted by half with H2O and purified by HPLC using method B (tR = 8.6 min). Upon lyophilization, the PL1 hydrochloride salt (17.5 mg, 0.082 mmol, 88% yield) was obtained as a light yellow, fluffy solid. 1H NMR (500 MHz, DMSO-d6) δ 8.05 (s, 1H), 6.43 (s, 1H), 5.08 (d, J = 13.7 Hz, 1H), 4.91 (d, J = 13.7 Hz, 1H), 4.29 (s, 1H); 13C NMR (125 MHz, DMSO-d6) δ 150.6, 137.8, 134.5, 134.5, 129.5, 98.5, 83.7, 80.4, 69.2; HRMS for C9H7NO3 [M+H]+ calcd. 178.0504, obtained 178.0498.

Synthesis of Ctrl

9b (12.3 mg, 0.056 mmol) was dissolved in 0.5 M HCl and stirred at room temperature for 15 min. The solution was diluted by half with H2O and purified by HPLC using method B (tR = 9.1 min). Upon lyophilization, the Ctrl hydrochloride salt (10.9 mg, 0.051 mmol, 92% yield) was obtained as a light yellow, fluffy solid. 1H NMR (500 MHz, MeOD) δ 8.09 (s, 1H), 6.49 (d, J = 2.3 Hz, 1H), 5.18 (dd, J = 14.3, 2.3 Hz, 1H), 5.01 (d, J = 14.3 Hz, 1H), 3.94 (s, 1H); 13C NMR (126 MHz, MeOD) δ 152.1, 139.5, 137.8, 134.5, 129.5, 98.5, 84.7, 79.3, 70.4; HRMS for C9H7NO3 [M+H]+ calcd. 178.0504, obtained 178.0498.
Synthesis of PL3

(2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)methanol

Acetal protection of pyridoxine was performed using a previously described protocol by Yang et al.\textsuperscript{7} To a suspension of pyridoxine hydrochloride (5.0 g, 24.3 mmol) in dry acetone (100 ml) were added 2,2-dimethoxypropane (75 ml) and $p$-toluenesulfonic acid (18.5 g, 97.2 mmol). The mixture was stirred overnight upon which the dark-brown solution was neutralized with satd. NaHCO\textsubscript{3} until light yellow. The aqueous layer was extracted with EtOAc (3 × 100 ml) and the combined organic layers were washed with brine and concentrated under reduced pressure until crystalline. The crude product was recrystallized from EtOH/Et\textsubscript{2}O (40:60) to yield pure 10 (2.8 g, 13.4 mmol, 56% yield) as a white crystalline solid. $R_f$ = 0.29 (10% MeOH/DCM). $^1$H NMR (400 MHz, CDCl\textsubscript{3}) $\delta$ 8.00 (s, 1H), 4.95 (s, 2H), 4.62 (s, 2H), 2.45 (s, 3H), 1.57 (s, 6H). $^{13}$C NMR (91 MHz, CDCl\textsubscript{3}) $\delta$ 147.6, 146.2, 138.6, 129.8, 126.1, 99.9, 60.0, 58.7, 24.9, 18.2. HRMS for C\textsubscript{11}H\textsubscript{15}NO\textsubscript{3} [M+H]\textsuperscript{+} calcd. 210.1130, obtained 210.1123.

5-(((4-methoxybenzyl)oxy)methyl)-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridine

To a dry flask under argon was added NaH (60% dispersion in mineral oil; 1.9 g, 47.8 mmol) followed by dry DMF (20 ml). The suspension was heated to 70°C in an oil bath and 10 (2.0 g, 9.6 mmol) dissolved in DMF was slowly added. Once addition was complete, the reaction was left at 70°C for 5 min and then allowed to cool to room temperature. The reaction was cooled to 0°C for the slow addition of PMBCl (1.6 ml, 11.5 mmol) and was allowed to warm to room temperature while stirring overnight. Upon cooling on ice, the reaction was slowly quenched with H\textsubscript{2}O (50 ml), extracted with EtOAc (3 × 50 ml), dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated under reduced pressure. The crude product was purified by flash chromatography to yield 11 (2.2 g, 6.7 mmol, 70%) as a clear oil. $R_f$ = 0.43 (10% MeOH/DCM). $^1$H NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 7.94 (s, 1H), 7.24 (d, $J$ = 8.6 Hz, 2H), 6.88 (d, $J$ = 8.7 Hz, 2H), 4.85 (s, 2H), 4.42 (s, 2H),
4.39 (s, 2H), 3.80 (s, 3H), 2.40 (s, 3H), 1.54 (s, 6H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 159.4, 148.2, 146.1, 139.8, 129.6, 126.4, 126.1, 113.9, 99.7, 71.8, 67.1, 58.8, 55.3, 24.8, 18.6. HRMS for C$_{39}$H$_{23}$NO$_4$ [M+H]$^+$ calcd. 330.1705, obtained 330.1699.

(5-(((4-methoxybenzyl)oxy)methyl)-2,2-dimethyl-4H-[1,3]dioxino[4,5-c]pyridin-8-yl)methanol

Following a modified procedure by Korytnyk et al.,$^5$ mCPBA (0.8 g, 77%, 3.7 mmol) was added to a solution of 11 (1.0 g, 3.0 mmol) in DCM (50 ml) and the reaction was stirred overnight at room temperature. Upon removal of the solvent under reduced pressure, the resulting residue was dissolved in EtOAc and washed with satd. NaHCO$_3$ (3 × 30 ml). The organic phase was dried over Na$_2$SO$_4$ and concentrated to yield the intermediate N-oxide, which was used directly in the next step. Crude N-oxide was dissolved in DCM (50 ml) and cooled to 0°C prior to the slow addition of trifluoroacetic anhydride (0.86 ml, 6.0 mmol) via syringe for the Boekelheide rearrangement.$^1$ The ice bath was then removed and the reaction was stirred for 4 h. Upon re-cooling to 0°C, MeOH (10 ml) was slowly added and the reaction was stirred at 0°C for 20 min and then allowed to warm up to room temperature while stirring for another 20 min. Following removal of solvents under reduced pressure, the residue was dissolved in EtOAc and washed with satd. NaHCO$_3$ (3 × 50 ml). The organic phase was dried over Na$_2$SO$_4$, filtered and concentrated. The crude product was purified by flash chromatography to yield 12 (941 mg, 2.73 mmol, 90% yield over 2 steps) as an off-white crystalline solid. R$_t$ = 0.24 (50% EtOAc/Hex). $^1$H NMR (300 MHz, CDCl$_3$) δ 8.02 (s, 1H), 7.24 (d, $J$ = 8.6 Hz, 2H), 6.89 (d, $J$ = 8.7 Hz, 2H), 4.87 (s, 2H), 4.69 (s, 2H), 4.44 (s, 2H), 4.42 (s, 2H), 3.80 (s, 3H), 1.53 (s, 6H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 159.5, 147.7, 144.8, 139.1, 129.7, 129.5, 127.8, 126.6, 114.0, 100.2, 72.1, 67.1, 59.8, 58.8, 55.4, 24.8. HRMS for C$_{19}$H$_{23}$NO$_5$ [M+H]$^+$ calcd. 346.1655, obtained 346.1649.
8-[(azidomethyl)-5-(((4-methoxybenzyl)oxy)methyl)-2,2-dimethyl-4H-[1,3]dioxino[4,5-c]pyridine

A solution of 12 (0.6 g, 1.7 mmol) in dry DCM (20 ml) was cooled to 0°C prior to the slow addition of SOCl₂ (1.3 ml, 17.4 mmol). Upon stirring at 0°C for 20 min and then at room temperature for 20 min, the reaction was quenched by adding satd. NaHCO₃ at 0°C until basic. The phases were separated and the organic layer was washed with satd. NaHCO₃ (20 ml) and brine prior to concentration under reduced pressure. The resulting chloride was used directly in the next reaction.

To a solution of crude intermediate chloride in ACN (20 ml) was added NaN₃ (108 mg, 1.66 mmol) and the reaction was refluxed for 16 h. The reaction was washed with brine, concentrated under reduced pressure and the crude product was purified by flash chromatography to yield 13 (430 mg, 1.16 mmol, 67%) as a clear oil. Rf = 0.20 (5% MeOH/DCM). ¹H NMR (300 MHz, CDCl₃) δ 8.05 (s, 1H), 7.25 (d, J = 8.7 Hz, 2H), 6.90 (d, J = 8.7 Hz, 2H), 4.88 (s, 2H), 4.46 (s, 2H), 4.43 (s, 2H), 4.42 (s, 2H), 3.80 (s, 3H), 1.57 (s, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 159.4, 146.4, 144.3, 140.2, 129.6, 129.4, 129.03, 127.4, 114.1, 113.9, 100.5, 72.2, 66.9, 58.7, 55.3, 50.8, 24.7. HRMS for C₁₉H₂₂N₄O₄ [M+H]⁺ calcd. 371.1719, obtained 371.1711.

2-[(azidomethyl)-3-hydroxy-5-(((4-methoxybenzyl)oxy)methyl)isonicotinaldehyde

A solution of 13 (350 mg, 0.95 mmol) and PPTS (950 mg, 3.8 mmol) in EtOH (20 ml) was refluxed for 16 h. Upon completion, the reaction was cooled and the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (20 ml) and washed with satd. NaHCO₃ (3 × 10 ml). The organic phase was concentrated and the crude diol was used directly in the next step.

To a solution of the intermediate alcohol in DCM (5 ml) was added MnO₂ (739 mg, 8.5 mmol). The reaction was stirred vigorously for 2 h until complete and was subsequently filtered through celite. The filtrate was
evaporated and the resulting residue was purified flash chromatography to yield 14 (127 mg, 0.39 mmol, 41% over 2 steps) as a pale yellow oil. Rf = 0.52 (5% MeOH/DCM). 1H NMR (400 MHz, CDCl3) δ 11.49 (s, 1H), 10.40 (s, 1H), 8.17 (s, 1H), 7.25 (td, J = 8.7, 2.1 Hz, 2H), 6.90 (td, J = 8.7, 2.1 Hz, 2H), 4.75 (s, 2H), 4.56 (s, 2H), 4.54 (s, 2H), 3.82 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 197.0, 159.7, 153.7, 148.4, 140.4, 132.5, 129.7, 128.9, 121.7, 114.1, 72.6, 66.1, 55.3, 50.2. HRMS for C16H16N4O4 [M+H]+ calcd. 329.1250, obtained 329.1244.

Synthesis of PL3

Aldehyde 14 (64 mg, 0.19 mmol) was dissolved in a mixture of 0.5 N HCl and acetone (1:1, 6 ml) and refluxed for 2 h. Upon completion, the reaction was cooled and the acetone was removed under reduced pressure. After washing the remaining solution with Et2O (3 × 2 ml), the product was purified from the aqueous phase using HPLC method B (tR = 12.0 min) and was lyophilized to generate the HCl salt of PL3 (19 mg, 0.078 mmol, 40%) as a fluffy yellow solid. 1H NMR (500 MHz, MeOD) δ 8.03 (s, 1H), 6.53 (d, J = 2.0 Hz, 1H), 5.22 (dd, J = 13.2, 2.0 Hz, 1H), 5.01 (d, J = 13.1 Hz, 1H), 4.53 (d, J = 13.3 Hz, 1H), 4.48 (d, J = 13.3 Hz, 1H). 13C NMR (75 MHz, MeOD) δ 149.0, 144.2, 139.6, 136.0, 133.9, 100.4, 70.8, 51.3. HRMS for C8H8N4O3 [M+H]+ calcd. 209.0675, obtained 209.0668.

Synthesis of PL4

1-Methoxy-7-(methoxymethoxy)-6-(7-(trimethylsilyl)hept-6-in-1-yl)-1,3-dihydrofuro[3,4-c]pyridine

The general alkylation protocol was used to react 1 (200 mg, 0.89 mmol) with TMS-protected 7-bromohex-1-yn (620 mg, 2.67 mmol) in order to synthesize the alkylated product 15 (132 mg, 0.35 mmol, 40%) as
a yellow oil. Rf = 0.67 (50% EtOAc/hexanes). 1H NMR (400 MHz, CDCl3) δ 8.17 (s, 1H), 6.25 (s, 1H), 5.42 (d, J = 6.5 Hz, 1H), 5.17 (d, J = 12.7 Hz, 1H), 5.06 (d, J = 6.5 Hz, 1H), 5.01 (d, J = 12.8 Hz, 1H), 3.51 (s, 3H), 3.45 (s, 3H), 2.89 (t, J = 7.8 Hz, 2H), 2.21 (t, J = 7.0 Hz, 2H), 1.71 (p, J = 7.6 Hz, 2H), 1.60-1.52 (m, 2H), 1.50-1.44 (m, 2H), 0.13 (s, 9H).

13C NMR (100 MHz, CDCl3) δ 153.8, 146.2, 135.8, 135.4, 134.0, 107.7, 106.3, 96.4, 84.4, 70.1, 57.0, 54.9, 32.5, 29.0, 28.7, 28.4, 19.9, 0.3. HRMS for C20H31NO4Si [M+H]+ calcd. 378.2101, obtained 378.2094.

6-(hept-6-yn-1-yl)-1-methoxy-7-(methoxymethoxy)-1,3-dihydrofuro[3,4-c]pyridine

To a solution of 15 (241 mg, 0.63 mmol) dissolved in dry MeOH (10 ml) was added K2CO3 (441 mg, 3.2 mmol) and the reaction was stirred overnight at room temperature. Upon completion, Et2O and H2O were added (each 20 ml) and the aqueous layer was extracted with Et2O (3 × 20 ml). The organic phase was concentrated under reduced pressure and the resulting residue was purified by flash chromatography to yield 16 (176 mg, 0.58 mmol, 91%) as a yellow oil. Rf = 0.62 (50% EtOAc/hexane). 1H NMR (400 MHz, CDCl3) δ 8.16 (s, 1H), 6.24 (s, 1H), 5.41 (d, J = 6.5 Hz, 1H), 5.16 (d, J = 13.0 Hz, 1H), 5.05 (d, J = 6.5 Hz, 1H), 5.00 (d, J = 13.0 Hz, 1H), 3.50 (s, 3H), 3.45 (s, 3H), 2.87 (t, J = 7.8 Hz, 2H), 2.18 (td, J = 7.0, 2.6 Hz, 2H), 1.91 (t, J = 2.7 Hz, 1H), 1.77-1.65 (m, 2H), 1.63-1.52 (m, 2H), 1.52-1.44 (m, 2H). 13C NMR (100 MHz, CDCl3) δ 153.8, 146.1, 135.7, 135.7, 133.8, 106.3, 96.4, 84.8, 70.1, 68.3, 56.9, 54.9, 32.6, 28.9, 28.5, 28.4, 18.4. HRMS for C17H23NO4 [M+H]+ calcd. 306.1705, obtained 306.1701.

Synthesis of PL4.

Compound 16 (100 mg, 0.372 mmol) was dissolved in 5% H2SO4 (2 ml) in acetone/H2O (1:1) and heated at 85°C for 1 h. The reaction was allowed to cool and the acetone was removed under reduced pressure. The product was purified by HPLC using method A (tR = 7.5 min) and lyophilized to generate PL4 as the H2SO4
salt (80 mg, 0.232 mmol, 63%) as a fluffy white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.49 (s, 1H, OH), 7.94 (s, 1H), 6.63 (d, $J = 7.1$ Hz, 1H, 0H), 6.41 (d, $J = 7.1$ Hz, 1H), 5.04 (d, $J = 12.8$ Hz, 1H), 4.85 (d, $J = 12.8$ Hz, 1H), 2.77 – 2.65 (m, 3H), 2.14 (td, $J = 6.9$, 2.6 Hz, 2H), 1.62 (p, $J = 7.6$ Hz, 2H), 1.55-1.43 (m, 2H), 1.43-1.34 (m, 2H).

Synthesis of PL5

1-Methoxy-7-(methoxymethoxy)-6-(4-((trimethylsilyl)ethinyl)phenethyl)-1,3-dihydrofuro[3,4-c]pyridine

The general alkylation protocol was used to react 1 (200 mg, 0.89 mmol) with TMS-protected 1-ethynyl-4-(iodomethyl)benzene (839 mg, 2.67 mmol) in order to synthesize the alkylated product 17 (106 mg, 0.26 mmol, 29%) as a yellow oil. R$_f$ = 0.63 (50% EtOAc/hexanes). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.20 (s, 1H), 7.37 (d, $J = 8.3$ Hz, 2H), 7.16 (d, $J = 8.3$ Hz, 2H), 6.26 (s, 1H), 5.39 (d, $J = 6.6$ Hz, 1H), 5.19 (d, $J = 13.0$ Hz, 1H), 5.03 (d, $J = 13.0$ Hz, 1H), 5.01 (d, $J = 6.6$ Hz, 1H), 3.47 (s, 3H), 3.46 (s, 3H), 3.22-3.11 (m, 2H), 3.07-2.99 (m, 2H), 0.23 (s, 9H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 152.3, 146.5, 142.7, 136.4, 132.1, 128.5, 120.8, 106.3, 96.4, 93.7, 70.1, 57.0, 57.0, 55.0, 34.9, 33.9, 0.17. HRMS for C$_{23}$H$_{29}$NO$_3$Si [M+H]$^+$ calcd. 412.1944, obtained 412.1937.

6-(4-ethynlphenethyl)-1-methoxy-7-(methoxymethoxy)-1,3-dihydrofuro-[3,4-c]pyridine

To a solution of 17 (48 mg, 0.17 mmol) dissolved in dry MeOH (10 ml) was added K$_2$CO$_3$ (23 mg, 0.17 mmol) and the reaction was stirred overnight at room temperature. Upon completion, Et$_2$O and H$_2$O were added (each 10 ml) and the aqueous layer was extracted with Et$_2$O (3 × 10 ml). The organic phase
was concentrated under reduced pressure and the resulting residue was purified by flash chromatography to yield 18 (30 mg, 0.09 mmol, 54%) as a yellow oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.20 (s, 1H), 7.41 (d, \(J = 8.2\) Hz, 2H), 7.19 (d, \(J = 8.2\) Hz, 2H), 6.26 (s, 1H), 5.41 (d, \(J = 6.6\) Hz, 1H), 5.20 (d, \(J = 12.9\) Hz, 1H), 5.05 (d, \(J = 12.9\) Hz, 1H), 5.02 (d, \(J = 6.6\) Hz, 1H), 3.46 (s, 3H), 3.6 (s, 3H), 3.30-3.16 (m, 2H), 3.11-3.03 (m, 3H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 152.3, 146.3, 143.2, 136.1, 135.6, 133.9, 132.2, 128.6, 119.7, 106.3, 96.3, 83.9, 76.7, 70.1, 56.9, 54.9, 34.8, 34.1. HRMS for C\(_{20}\)H\(_{21}\)NO\(_4\) [M+H]\(^+\) calcd. 340.15499, obtained 340.1546.

Synthesis of PL5.

Compound 18 (30 mg, 0.088 mmol) was dissolved in 5% H\(_2\)SO\(_4\) (2 ml) in acetone/H\(_2\)O (1:1) and heated at 85°C for 1 h. The reaction was allowed to cool and was concentrated under reduced pressure to remove acetone. The product was purified by HPLC using method C (\(t_R = 8.5\) min) and lyophilized to generate PL5 as the H\(_2\)SO\(_4\) salt (21 mg, 0.0554 mmol, 63%) as a fluffy white solid. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 9.65 (s, 1H, OH), 7.98 (s, 1H), 7.38 (d, \(J = 8.2\) Hz, 2H), 7.26 (d, \(J = 8.2\) Hz, 2H), 6.66 (d, \(J = 7.6\) Hz, 1H, OH), 6.42 (d, \(J = 7.6\) Hz, 1H), 5.05 (d, \(J = 12.9\) Hz, 1H), 4.87 (d, \(J = 12.9\) Hz, 1H), 4.10 (s, 1H), 3.08-2.91 (m, 4H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 147.6, 145.7, 143.2, 135.2, 133.8, 132.5, 131.6, 128.6, 119.1, 98.5, 83.6, 68.8, 33.5, 32.7. HRMS for C\(_{17}\)H\(_{15}\)NO\(_3\) [M+H]\(^+\) calcd. 282.1130, obtained 282.1123.
2. Biological Methods

Cloning and overexpression of recombinant PLK. The N-terminally STREP-II tagged *S. aureus* Mu50 pyridoxal kinase (PLK) was prepared as previously described.8

Cloning and overexpression of recombinant proteins. N-terminally STREP-II tagged *S. aureus* (Mu50 for Alr and PLK; USA300 for all others) proteins were cloned and expressed using the primers and conditions listed in Supplementary Table 10. Cloning was performed using the Invitrogen Gateway cloning system with pDONR201kan as the donor vector and pET55destAmp as the destination vector. Proteins were expressed in *E. coli* BL21(DE3) grown in LB-media containing 0.1 mg/ml Ampicillin at 37°C to an OD600 of 0.5-0.6 through the addition of 1 mM isopropyl-1-thio-β-galactopyranoside (IPTG). Bacteria were harvested and washed with PBS prior to cell lysis and protein purification.

Supplementary Table 10. PCR primers and overexpression conditions for recombinant proteins. MW of proteins was confirmed by intact-protein MS.

| No | Protein Name | Uniprot ID, Gene name | Primers | Overexpression | MW found (Da) |
|----|--------------|-----------------------|---------|----------------|---------------|
| 1. | (Alr) Alanine racemase 1 | P63479, *Alr1* | (fwd) gggacaagtttgacaaaaaagcaggttt tggataaatatatatag (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 30°C, o/n | m_{det} = 45,099.1 |
| 2. | (AT1) Aminotransferase, Class I | A0A0H2XFY9, USA300_2497 | (fwd) gggacaagtgcacaaaaagcaggttt atcttccccaaatagcataa (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 25°C, 6 h | m_{det} = 45,024.0 |
| 3. | (AT2) Aminotransferase, Class I | A0A0H2XFA8, USA300_0952 | (fwd) gggacaagtttgacaaaaaagcaggttt aaactttctttaaattctaatt (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 25°C, o/n | m_{det} = 45,433.4 |
| 4. | (DAT) D-alanine aminotransferase | A0A0H2XHU6, *dat* | (fwd) gggacaagtttgacaaaaaagcaggttt aaactttctttaaattctaatt (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 25°C, o/n | m_{det} = 34,184.5 |
| 5. | (CS) Cysteine synthase | A0A0H2XFO3, *cysK* | (fwd) gggacaagtttgacaaaaaagcaggttt gcacaaaaaccagagatataatta (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 37°C, 4 h | m_{det} = 35,265.5 |
| 6. | (ODC) Ornithine/Lysine/Arginine decarboxylase | A0A0H2XI16, USA300_0458 | (fwd) gggacaagtttgacaaaaaagcaggttt aagacccatatatttaaatattag (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 25°C, o/n | m_{det} = 53,372.2 |
| 7. | (HemQ) coprohaem decarboxylase | Q2FJ56, USA300_0569 | (fwd) gggacaagtttgacaaaaaagcaggttt agtcagcgcagcccacacatta (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 25°C, o/n | m_{det} = 31,679.7 |
| 8. | (PsiMP) pseudouridine-5'-phosphate glycosidase | A0A0H2XC6, USA300_0312 | (fwd) gggacaagtttgacaaaaaagcaggttt aacatattttaaaatatttgaag (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 25°C, o/n | m_{det} = 35,154.5 |
| 9. | (HemH) Ferrochelatase | A0A0H2XGSS, USA300_1782 | (fwd) gggacaagtttgacaaaaaagcaggttt aacatattttaaaatatttgaag (rev) ggggaccttttgacaaagaagtgtggtt attaatcaagttcatat | 25°C, o/n | m_{det} = 37,346.6 |
|   | Description                                      | Accession Numbers | Forward Primer | Reverse Primer | Temperature | Concentration | MW Confirmation |
|---|-------------------------------------------------|-------------------|----------------|----------------|--------------|---------------|-----------------|
|10 | (Q2FF14) Uncharacterized protein Q2FF14, SAUSA300_2068 | (fwd) ggggacaagtttgacaaaaagcaggcttt aaagatttgacaaagtttaggtcatgctggtttctgtactttgacgctt | ggacacagttgacaaaaagcaggcttt gacatggttcgattgtcctggtt | 25 °C, o/n | m_Met = 21,184.9 |
|11 | (GP0) Uncharacterized protein A0AH2XP0, SAUSA300_2209 | (fwd) ggggacaagtttgacaaaaagcaggcttt aaagatttgacaaagtttaggtcatgctggtttctgtactttgacgctt | ggacacagttgacaaaaagcaggcttt gacatggttcgattgtcctggtt | 20 °C, o/n | m_Met = 37,052.9 |
|12 | (HH8) Uncharacterized protein A0AH2XHH8, SAUSA300_1082 | (fwd) ggggacaagtttgacaaaaagcaggcttt aaagatttgacaaagtttaggtcatgctggtttctgtactttgacgctt | ggacacagttgacaaaaagcaggcttt gacatggttcgattgtcctggtt | 25 °C, o/n | m_Met = 27,636.3 |
|13 | (PabB) Aminodeoxychorismate synthase component 1 PO5041, pabB (Escherichia coli K12) | (fwd) ggggacaagtttgacaaaaagcaggcttt aaagatttgacaaagtttaggtcatgctggtttctgtactttgacgctt | ggacacagttgacaaaaagcaggcttt gacatggttcgattgtcctggtt | 25 °C, o/n | m_Met = 53,257.9 |
|14 | (IU9) Uncharacterized protein A0AH2XIU9, SAUSA300_0700 | (fwd) ggggacaagtttgacaaaaagcaggcttt aaagatttgacaaagtttaggtcatgctggtttctgtactttgacgctt | ggacacagttgacaaaaagcaggcttt gacatggttcgattgtcctggtt | 25 °C, o/n | m_Met = 26,130.5 |
|15 | (IC2) Uncharacterized protein A0AH2XIC2, SAUSA300_1214 | (fwd) ggggacaagtttgacaaaaagcaggcttt aaagatttgacaaagtttaggtcatgctggtttctgtactttgacgctt | ggacacagttgacaaaaagcaggcttt gacatggttcgattgtcctggtt | 25 °C, o/n | m_Met = 40,966.7 |
|16 | (JW8) put. PLP-dependent acyltransferase A0AH2XJW8, SAUSA300_0535 | (fwd) ggggacaagtttgacaaaaagcaggcttt aaagatttgacaaagtttaggtcatgctggtttctgtactttgacgctt | ggacacagttgacaaaaagcaggcttt gacatggttcgattgtcctggtt | 25 °C, 2 h | m_Met = 45,180.0 |

**Small-scale protein purification (AT1, AT2, DAT, CS, ODC, HH8, HemQ, PsiMP, HemH, Q2FF14, GP0, IU9, IC2).** Bacterial cell pellets from overexpression cultures (50 ml) were resuspended in 1 ml phosphate buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). Cells were lysed by sonication and the lysate was clarified by centrifugation (21,000 x g, 30 min, 4°C). Strep-Tactin® spin columns (Iba Lifesciences) were equilibrated with phosphate buffer according to the user manual (2 × 500 µl, 700 x g, 30 sec, 4°C) and the lysate was loaded onto the columns (700 x g, 30 sec, 4°C). Columns were washed with phosphate buffer (4 x 100 µl, 16,200 x g, 30 sec, 4°C) prior to eluting the protein in buffer containing D-biotin (5 mM) (2 × 100 µl, 700 x g, 30 sec, 4°C). Protein concentrations were measured on a Tecan infinite M200Pro plate reader by absorption at 280 nm (Nanoquant plate). A yield of approximately 3 mg/ml (≈ 100 µM) was obtained for each protein and the MW of each protein was confirmed by intact-protein MS. Proteins were found to be sufficiently pure by SDS-PAGE and were stored at -80°C.

**Large-scale protein purification (Alr, Apo-Alr).** The bacterial overexpression culture (2 L) cell pellet was resuspended in phosphate buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) and cells were lysed by sonication. The lysate was clarified by centrifugation (36,000 x g, 30 min, 4°C) before loading onto a pre-equilibrated 5 ml StrepTrap column (GE Healthcare) using an Äkta purification system (GE Healthcare). The column was washed with phosphate buffer (50 ml, flow rate = 4 ml/min) and Alr was eluted in
phosphate buffer containing desthiobiotin (2.5 mM) as the PLP-holoenzyme. In order to generate the apoenzyme (Apo-Alr), column-bound proteins were flushed with phosphate buffer containing 25 mM hydroxylamine (50 ml) prior to washing and elution. Protein-containing fractions were concentrated, desalted into phosphate buffer using a 5 ml HiTrap Desalting column (GE Healthcare) and protein aliquots were flash frozen in liquid nitrogen for storage at -80°C. Alr was found to be sufficiently pure by SDS-PAGE and its MW was confirmed by intact-protein MS. Protein concentrations were measured on a Tecan infinite M200Pro plate reader by absorption at 280 nm (Nanoquant plate).

**Large-scale protein purification of IU9, EcPabB and JW8.** Each protein was purified in phosphate buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) on a 5 ml StrepTrap column (GE Healthcare) using an Äkta purification system (GE Healthcare) as described above. For the PLP-DEs IU9 and JW8, 100 µM PLP was added to the lysate prior to purification in order to saturate cofactor binding of the enzyme. JW8 was additionally purified by size-exclusion chromatography using a Sepharose Superdex 200prep column (GE Healthcare) in phosphate buffer. The proteins were found to be sufficiently pure by SDS-PAGE and their MW was confirmed by intact-protein MS. Protein concentrations were measured on a Tecan infinite M200Pro plate reader by absorption at 280 nm (Nanoquant plate).

**PLK kinetics.** PLK activity was measured using the previously reported coupled assay.⁸

**In vitro phosphorylation of PL-probes using PLK.** A mixture containing 2 mM PL-probe, 25 µM PLK and 10 mM ATP was prepared in 1 ml kinase buffer (50 mM Tris, 50 mM KCl, 10 mM MgCl₂, pH 7.9) and incubated overnight at 4°C with gentle shaking. The solution was filtered through a 10 kDa MWCO centrifugal filter (Sartorius Stedim Biotech) to remove PLK, and phosphorylated probes were used in subsequent experiments without further purification, assuming full conversion (800 µM final concentration). Phosphorylation of PL probes could be confirmed by LC-MS/MS analysis using the H-ESI-II probe and LC conditions as described in the metabolomics section with slight modifications. A positive multiple reaction monitoring (MRM) was used as the detection mode and daughter ions of the respective HRMS probe ions were recorded in a range of 50-2000 m/z. Quantification of PL2P product samples showed the presence of > 99.5% PL2P.

**Reconstitution of Apo-Alr with phosphorylated probes.** Apo-Alr (50 µM in phosphate buffer) was incubated with phosphorylated probes (5 equivalents) overnight at 4°C with gentle shaking. Residual
probe was removed by desalting into phosphate buffer using a 5 ml HiTrap Desalting column (GE Healthcare) on an Äkta purification system.

**UV-Vis measurements.** UV-Vis spectra of protein samples (100 µM) were recorded in phosphate buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8.0) at 25°C on a Tecan infinite M200Pro plate reader (300-600 nm, 5 nm increments). For substrate studies, proteins were incubated with 10 equivalents of substrate for 15 min prior to measurement.

**Alr kinetics.** Racemization of D-Ala to L-Ala was measured using a coupled assay that monitors NADH formation ($\lambda_{abs} = 340$ nm) by L-alanine dehydrogenase.$^9$ A premix containing 2 units/ml L-alanine dehydrogenase (Roche), 10 mM NAD$^+$ and 10 mM Alr was prepared in CHES buffer (100 mM CHES, 100 mM KCl, pH 8.8). 100 µl of the premix was pipetted into flat-bottom 96-well plates (Nunclon™ Delta Surface, Thermo Scientific) containing 5 µl D-Ala (1, 2, 10, 20, 50, 100, 200 and 300 mM). The reactions were incubated at 37°C and NADH formation was measured at 340 nm in 1 min kinetic intervals using a Tecan infinite M200Pro plate reader. Enzymatic activity was assayed in three replicates. Initial rates of NADH formation (µM/min) were determined for the different substrate concentrations and the data (mean ± standard deviation) was fitted to the Michaelis-Menten equation (non-linear regression) using GraphPad Prism-v5.0 software in order to calculate $k_{cat}$, $K_m$ (mM) and $V_{max}$ (µM/min).

**NaBH$_4$-reduction of proteins for intact-protein MS.** 10 µM protein samples (25 µl) were incubated with 4 equivalents of cofactor derivative (1 µl of 500 µM stock PL, PLP, PL2/PL3 or PL2P/PL3P) in PBS for 30 min and subsequently treated with 10 mM NaBH$_4$ (2 µl of 250 mM stock prepared fresh in 0.1 M NaOH) at room temperature for 30 min. For Alr holoenzymes substituted with phosphorylated cofactor probes, samples were directly reduced without prior incubation. The NaBH$_4$ was quenched by acidification to pH 5-6 with HCl (5-10 µl of 0.5% FA) and neutralized to pH 7 with NaOH (5-10 µl of 0.1 M NaOH). Samples were diluted to 50 µl with PBS (5 µM final enzyme concentration) for measurement by intact-protein MS.

**Intact-protein mass spectrometry.** Samples were desalted and measured using a MassPREP On-Line Desalting Cartridge (Waters) on an Ultimate 3000 HPLC system (Dionex) coupled to a Finnigan LTQ-FT Ultra mass spectrometer (Thermo Scientific) with electrospray ionization (spray voltage 4.0 kV, tube lens 110 V, capillary voltage 48 V, sheath gas 60 arb, aux gas 10 arb, sweep gas 0.2 arb). Xcalibur Xtract Software (Thermo Scientific) was used for data analysis and deconvolution.
Gel-based labeling of recombinant proteins. 10 µM protein samples (50 µl in PBS) were labeled with PL(P)-probes (2 µl of 25× stock) at room temperature for 30 min. Upon reduction with 10 mM NaBH₄ (2 µl of 250 mM prepared fresh in 0.1 M NaOH) at room temperature for 30 min, proteins were precipitated by adding ice-cold acetone (4× volume) and incubating at -20°C for at least 1 h. Precipitated proteins were pelleted by centrifugation (18,000 x g, 15 min, 4°C) and washed with ice-cold MeOH (2 × 0.2 ml), using sonication to resuspend the pellets between washes. Pelletized proteins were resuspended in 50 µl PBS containing 0.4% (w/v) SDS and CuAAC to rhodamine-azole was performed by adding 0.1 mM Rh-N₃ (1 µl of 5 mM stock in DMSO), 0.5 mM BTTAA ligand¹⁰ (2.5 µl of 10 mM stock in ddH₂O), 1 mM CuSO₄ (1 µl of 50 mM stock in ddH₂O), 2 mM NaAsc (1 µl of 100 mM prepared fresh in ddH₂O) and incubating at room temperature for 1 h in the dark. For PL3(P)-labeled proteins, CuAAC was performed using 0.1 mM Rh-alkyne (1 µl of 5 mM stock in DMSO) instead of Rh-azole. Samples were quenched with 2× gel loading buffer and analyzed by SDS-PAGE (12.5% polyacrylamide gels) with fluorescence scanning.

*S. aureus* USA300 TnPdxS growth curves. The *S. aureus* USA300 transposon mutant TnPdxS (gene: SAUSA300_0504; pyridoxal biosynthesis lyase PdxS) was obtained from the Nebraska Transposon Mutant Library within the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA).¹¹ Bacteria were kept under antibiotic pressure (erythromycin) for all stages of the experiment to select for the transposon phenotype. Chemically-defined media (CDM) free of PL was prepared according to Liebeke et al.¹² and sterilized by filtration through a 0.2 µM PES filter (VWR). Overnight cultures of *S. aureus* USA300 TnPdxS were grown in B-medium containing 5 µg/ml erythromycin (Erm). Cultures were harvested, washed twice with 5 ml CDM-Erm (5 µg/ml) and resuspended in CDM-Erm. 150 µl of bacterial culture (initial OD₆₀₀ = 0.08) was added to flat-bottomed 96-well plates (Nunclon Delta surface, Thermo Scientific) containing 1.5 µl PL or PL-probe (100× stock in DMSO), and the plates were incubated at 37°C for 24 h with shaking (220 rpm). Endpoint measurements of bacterial growth were recorded at OD₆₀₀ on a Tecan Infinite Pro platereader. Concentration of probe was plotted versus percent of maximum growth with respect to bacteria grown on PL-substituted media. Measurements were conducted in triplicate and were obtained from three individual biological experiments. Wells containing media substituted with DMSO or the Ctrl probe were used as negative controls and served to validate the transposon mutant phenotype as no bacterial growth could be detected (OD₆₀₀ < 0.1).
Assay for IU9 activity. Conversion of chorismate to PABA by IU9 in conjunction with E. coli PabB was measured using two previously reported methods with slight modification. The first included a lactate dehydrogenase-coupled assay to monitor pyruvate formation and the second used LC-MS to confirm the PABA product. In brief, the enzyme activity of IU9 was assayed by incubating 1 mM chorismate (barium salt, Sigma Aldrich), 0.25 mM NADH, 25 µg lactate dehydrogenase (Roche), 10 µM recombinant E. coli PabB and 1 µM enzyme IU9 in 100 µl buffer (50 mM Tris, 5 mM MgCl₂, 50 mM (NH₄)₂SO₄, pH 8.6). The assays were conducted in a 96-well plate (Nunclon Delta Surface, Thermo Scientific) and monitored at a wavelength of 340 nm at 37°C in 30-second kinetic intervals on a Tecan infinite M200Pro plate reader. The rates of NADH consumption (µM/min) were determined from the linear portions of the curves (n = 3 replicates, mean ± standard deviation).

The reaction product PABA was subsequently confirmed by LC-MS by preparing a 100 µl reaction mixture containing 1 mM chorismate, 10 µM recombinant E. coli PabB and 1 µM IU9 in buffer (50 mM Tris, 5 mM MgCl₂, 50 mM (NH₄)₂SO₄, pH 8.6) and incubating at 37°C for 30 min. Reactions were then acidified using 10 µl of 2 M HCl and extracted with 400 µl EtOAc (vortexing and brief centrifugation). The organic phase transferred to a fresh Eppendorf tube, evaporated in a speedvac centrifuge and the resulting residue was resuspended in 50 µl H₂O (LC-MS grade) for MS measurement. Samples (5 µl injection volume) were analyzed on an Ultimate 3000 RSLC system (Thermo Scientific) coupled to a LTQ-FT Ultra (Thermo Finnigan) operated in positive ion mode (ESI source, Thermo Scientific). Chromatographic separation was performed using a Waters XBridge C18 column (4.0 x 100 mm, 3.5 µm; A: 90% H₂O 10% ACN 0.1%FA; B: ACN 0.1% FA) at 30°C with a flow rate of 1.1 ml/min using the following described gradient. After pre-equilibrating with 5% B (2 min), samples were eluted with an isocratic gradient at 5% B (5 min) followed by a linear gradient from 5-98% B (2 min). The ion of interest (PABA 138.14 m/z) was detected in full scan mode at a resolution of 50000 in the FT-ICR cell. Retention time and exact mass were matched to a synthetic standard (Merck). The LCMS-based assay was carried out in triplicate.

Assay for JW8 activity. The PLP-dependent acetyltransferase (2-amino-3-ketobutyrate CoA lyase) activity of JW8 was measured according to a previously described assay protocol with minor modifications and product formation was subsequently confirmed by LC-MS. In brief, Ellman’s reagent (5,5'-dithio-bis-(2-nitrobenzoic acid)) was used to monitor the acetyl-coenzyme A (AcCoA)-dependent conversion of amino acids to their acetylated derivatives as catalyzed by JW8. Reaction mixtures (50 µl final volume) consisting of 1 µM JW8 (active or inactivated), 0.5 mM AcCoA and 2.5 mM substrate amino acid (or water as a
control) in 50 mM Tris-HCl (pH 8.0) were incubated at 37°C for 1 h with shaking (400 rpm) in a 96-well plate (Nunclon™ Delta Surface, Thermo Scientific). Reactions were stopped by adding 50 µl guanidine buffer (6M guanidine-HCl, 100 mM Tris-HCl, pH 8.0), upon which 100 µl Ellman’s solution (0.4 mM 5,5'-dithio-bis-(2-nitrobenzoic acid), 0.5 mM EDTA, 100 mM Tris-HCl, pH 8) was added to each well and incubated for 10 min at room temperature while shaking (400 rpm). The formation of 2-nitro-5-thiobenzoate was monitored photometrically using a Tecan Infinite M200 Pro multiplate reader at 415 nm. For JW8 inactivation, the enzyme was treated with NaBH₄ (5 mM final conc, 250 mM stock solution in 0.1 M NaOH aq) for 30 min at room temperature to irreversibly fix PLP to the enzyme and disrupt enzymatic catalysis. NaBH₄ was quenched by the addition of 0.5% FA (10 µl) and the solution was neutralized again (pH = 7-8) through the addition of 0.1M NaOH (5-10 µl) prior to conducting the assay. The assay was performed in three independent experiments comprising three technical replicates each. Data was normalized to reduced enzyme controls and Microsoft Excel was used for calculation of mean values and standard deviations. Graphpad Prism 6 was applied to plot data and calculate one-way ANOVA significance.

In order to monitor the formation of N-acetylglycine by JW8 via LC-MS, a modified version of the assay described above was carried out. Reaction mixtures (50 µl final volume) consisting of 1 µM JW8, 0.5 mM AcCoA and 1 mM glycine in 50 mM Tris-HCl (pH 8.0) were incubated at 37°C for 1 h with shaking (400 rpm). Reactions were quenched by the addition of 200 µl ice-cold MeOH and incubated for 1 h at -20°C. The precipitated protein was then pelletized by centrifugation (21000 x g, 20 min, 4°C) and the supernatant was transferred to a fresh Eppendorf tube prior to solvent evaporation using a SpeedVac centrifuge at 30°C. Samples were reconstituted in 50 µl H₂O/ACN (50:50), centrifuged (21000 x g, 10 min, 4°C) and transferred into LC-MS vials for MS measurement. Samples (5 µl injection volume) were analyzed on an Ultimate™ 3000 RSLC system (Thermo Scientific™) coupled to a LTQ-FT Ultra (Thermo Finnigan) operated in positive ion mode (ESI source, Thermo Scientific™). Chromatographic separation was performed using a Waters™ XBridge C18 column (4.0 x 100 mm, 3.5 µm; A: 90% H₂O 10% ACN 0.1%FA; B: ACN 0.1% FA) at 30°C with a flow rate of 1.1 ml/min using the following described gradient. After pre-equilibrating with 5% B (2 min), samples were eluted with an isocratic gradient at 5% B (5 min) followed by a linear gradient from 5-98% B (2 min). Ion of interest (N-acetylglycine M+H118.05 m/z) was detected in single ion monitoring (SIM) mode (SIM width 8 m/z) at a resolution of 12500 in the FT-ICR cell. Retention time and exact mass were matched to a synthetic standard (TCI Chemicals). The LCMS-based assay was carried out in triplicates.
3. Crystallography

**Crystallization and structure determination.** High-throughput crystallization screening was carried out (Phoenix, Art Robbins) and all crystals were obtained by the sitting-drop vapour diffusion method at 4°C. Apo-Alr was crystallized with 0.1 M citric acid pH 4, 1.6 M ammonium sulphate as the precipitant. Reconstituted Alr-**PL1P** and Alr-**PL2P** holoenzymes were crystallized in 0.1 M Hepes pH 7 and 2 M sodium chloride and 0.1 M sodium acetate pH 5 containing 10% 2-methyl-1,3,3-propanediol (MPD), respectively. Prior to cryo-cooling and storage in liquid nitrogen, either lithium sulphate (Apo-Alr, Alr-**PL1P** complex) or MPD (Alr-**PL2P** complex) was added to the crystallization drop for cryo-protection. Crystals were screened and diffraction data was collected at the beamlines of the European Synchrotron Radiation Facility (ESRF, ID23-2 and ID29) and the Swiss Light Source (SLS, PX I). The data were processed with XDS\(^{15}\) and the crystals belonged to the space group P2\(_1\)2\(_1\)2\(_1\). The resolution cut-offs were chosen according to the correlation coefficient of random half-data sets (1/2 CC) at about 50%.\(^{16-18}\) The structures were solved using the coordinates of the previously reported structure (PDB code 4A3Q)\(^{19}\) by molecular replacement in PHASER.\(^{20}\) This was followed by simulated annealing in PHENIX,\(^{21,22}\) prior to iterative cycles of manual model building in COOT\(^{23}\) and restraint and TLS refinement in REFMAC5.\(^{24}\) Structure optimization was carried out using the PDBredo server.\(^{25}\) For data processing and structure refinement statistics see Supplementary Table 11. All structural figures were prepared with PyMol (Delano Scientific).
**Supplementary Table 11.** Crystallographic data collection and refinement statistics. Statistics for the highest-resolution shell are shown in parentheses.

|                      | Apo                     | PL1P-complex           | PL2P-complex           |
|----------------------|-------------------------|------------------------|------------------------|
| **Wavelength (Å)**   | 0.976                   | 0.873                  | 0.979                  |
| **Resolution range** | 45.2-2.15 (2.2-2.15)    | 49.5-2.45 (2.54-2.45)  | 49.4-1.9 (1.96-1.9)    |
| **Space group**      | P 2; 2; 2_1             | P 2; 2; 2_1            | P 2; 2; 2_1            |
| **Unit cell**        | 64.7 114.5 126.1        | 85.9 106.9 131.0       | 85.5 106.8 130.8       |
| **Total reflections**| 267,266 (22,547)        | 224,459 (21,447)       | 637,180 (55,865)       |
| **Unique reflections**| 51,458 (4,390)           | 44,955 (4,399)         | 95,389 (8,791)         |
| **Multiplicity**     | 5.2 (5.1)               | 5.0 (4.9)              | 6.7 (6.4)              |
| **Completeness (%)** | 1.00 (0.99)             | 1.00 (0.99)            | 0.99 (0.92)            |
| **Mean I/sigma(I)**  | 6.0 (0.4)               | 6.6 (1.4)              | 10.7 (1.2)             |
| **Wilson B-factor**  | 34.4                    | 32.3                   | 28.7                   |
| **R-merge**          | 0.166 (1.73)            | 0.213 (1.02)           | 0.13 (1.48)            |
| **R-meas**           | 0.20 (2.26)             | 0.24 (1.14)            | 0.14 (1.61)            |
| **CC1/2**            | 0.99 (0.85)             | 0.983 (0.5)            | 1.0 (0.45)             |
| **Reflections used in refinement** | 50,710 (4,797) | 44,949 (4,399) | 95,378 (8,791) |
| **Reflections used for R-free** | 2534 (237)   | 2,247 (219)           | 4,786 (462)            |
| **R-work**           | 0.2041 (0.337)          | 0.195 (0.297)          | 0.199 (0.346)          |
| **R-free**           | 0.234 (0.37)            | 0.215 (0.33)           | 0.21 (0.359)           |
| **Number of non-hydrogen atoms** | 6,475                 | 6,341                  | 6,712                  |
| macromolecules       | 6,040                   | 6,102                  | 6,104                  |
| ligands              | 138                     | 68                     | 125                    |
| Protein residues     | 763                     | 771                    | 764                    |
| RMS(bonds)           | 0.011                   | 0.009                  | 0.012                  |
| RMS(angles)          | 1.42                    | 1.4                    | 1.5                    |
| Ramachandran favoured (%) | 97                     | 97                     | 97                     |
| Ramachandran allowed (%) | 2.9                    | 3                      | 2.7                    |
| Ramachandran outliers (%) | 0.3                    | 0                      | 0                      |
| Rotamer outliers (%) | 1.7                     | 1.2                    | 0.9                    |
| Clashscore           | 1.5                     | 2.3                    | 1.1                    |
| Average B-factor     | 34.0                    | 35.5                   | 22.0                   |
| macromolecules       | 33.7                    | 35.5                   | 20.2                   |
| ligands              | 56.0                    | 50.9                   | 46.5                   |
| solvent              | 29.0                    | 27.7                   | 39.1                   |
| Number of TLS groups | 14                      | 2                      | 14                     |
4. Metabolomics

Sample preparation. Bacteria were grown and labeled as described for the proteomic workflow (see Proteomics section). For each sample, 2.5 ml of bacterial suspension (OD$_{600} = 40$) was incubated with 25 µl probe (1000× stock in DMSO) in culture tubes for 2 h at 37°C, with shaking. Samples were then shock-cooled to 4°C in a dry ice/ethanol bath while agitating to avoid local freezing. Bacteria were harvested (6000 rpm, 5 min, 4°C), washed with 0.6% (w/v) NaCl solution (2 × 5 ml) and resuspended in 5 ml extraction buffer (100% MeOH containing 0.5 µM PL as an internal standard). Cells were lysed by sonication (3 x 20 sec, with cooling breaks on ice) and samples were stored overnight at -80°C. Cell debris and precipitated protein were pelleted by centrifugation (18,000 x g, 30 min, 4°C) and the clarified extract was evaporated under a stream of nitrogen. Metabolome samples were dissolved in 150 µl H$_2$O/ACN (50:50, incl. 1% FA), centrifuged (18,000 x g, 15 min, 4°C) and the supernatant was stored at -80°C until LC-MS analysis. For approximate quantification of PL$_2$ and PL$_{2P}$ within the cells, calibration curve samples for both analytes were prepared. Therefore, S. aureus metabolome was extracted as stated above, and PL$_2$ and PL$_{2P}$ was spiked into the samples (at least 6 calibration points per analyte) after restoration of samples in 150 µl H$_2$O/ACN (50:50, incl. 1% FA, LC-MS grade, Sigma Aldrich).

Sample measurement. Metabolic profiling and MS/MS analysis was carried out on an Ultimate 3000 RSLC system (Thermo Scientific) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Chromatographic separation was performed using a SeQuant® ZIC®-pHILIC (250 x 2.1 mm, 5 µm, 100 Å) (Merck Millipore) at 40°C. Gradient elution was carried out with 80 mM NH$_4$OAc (LC-MS grade, Fisher Analytic) in 100% H$_2$O (LC-MS grade, Fisher Analytic) pH = 6.8 (A) and 100% ACN (LC-MS grade, Honeywell) (B). After 5 min pre-equilibration with 95% B, samples were eluted with a linear gradient from 95% to 0% B over 30 min at a flow rate of 250 µl/min followed by 8 min re-equilibration with 95% B. Mass spectrometric measurements were accomplished in HESI+ mode (H-ESI-II probe, Thermo Scientific) with following source parameters: capillary voltage 4.5 kV, tube lens 40 V, vaporizer temperature 43°C, sheath gas 50 l/h, aux gas 10 l/h, capillary voltage 12 V and capillary temperature 275°C. Full scan measurements were recorded in a range of 50 – 1000 m/z in profile mode at 100,000 resolution in the orbitrap. Parent ions of interest (PL m/z = 168.06, PL$_2$ m/z = 206.08, PL$_{2P}$ m/z = 286.04) were isolated (Isolation width 1.0 m/z) subjected to collision-induced dissociation (normalized collision energy 35 V) in the MRM mode and most intense daughter ions (PL m/z = 150.05, PL$_2$/PL$_{2P}$ m/z = 188.07) isolated and used for quantification (SIM width 10 m/z) at a resolution of 100,000 in the orbitrap. Prior to measurement, 15
pooled quality control (QC) samples were injected to equilibrate the column. In order to take into account metabolic degradation over time, the sample order was randomized and each sample, as well as each calibration point, was measured three times. QC samples were injected after every 8th sample to survey instrument performance.

**Data Analysis.** Data were processed with Xcalibur 2.2 SP1.48 Quan Browser (Thermo Scientific) using the Genesis algorithm for peak detection and manual integration mode. Peak detection was carried out with the following track peak parameters: Genesis Peak Detection: Highest Peak, Minimum peak height (S/N) = 3.0. Intensities of the corresponding daughter ions were extracted and further processed using Excel. Intensities were subsequently normalized to the signal intensity of the internal standard PL and averaged over three technical replicates. Approximate concentrations for PL2 and PL2P were calculated using calibration curves illustrated below (Supplementary Fig. 18). Experiments were carried out in three biological replicates (n = 3, mean ± standard deviation).

![Calibration curves for PL2 and PL2P](image)

**Supplementary Figure 18.** Calibration curves for PL2 and PL2P (intensities normalized to internal standard PL).
5. Proteomics

**Analytical Labeling.** *S. aureus* USA300 TnPdxS was grown to early stationary phase (5 h, OD$_{600}$ = 2.2 - 2.5) in B-medium containing 5 µg/ml Erm. Bacteria were harvested (6,000 rpm, 5 min, 4°C), washed twice with CDM-Erm (5 µg/ml) and resuspended to an OD$_{600}$ = 40. Per sample, 0.2 ml of this suspension was incubated with 2 µl PL2 or DMSO (100× stock in DMSO) for 2 h at 37°C, with shaking. Bacteria were subsequently harvested, washed with PBS (2 × 0.5 ml, 4°C), resuspended in 0.2 ml PBS and transferred to 0.5 ml glass-bead lysis tubes (0.2 g of 0.5 mm glass beads self-packed into 0.5 ml bulk tubes with caps, Mo bio Laboratories). Cells were lysed mechanically using a Precellys® 24 Homogenizer (6 × 15 sec shaking at 5400 rpm) with cooling breaks on ice between cycles. The lysates were clarified by centrifugation (18,000 x g, 30 min, 4°C) and were reduced with 20 mM NaBH$_4$ (8 µl of 500 mM stock prepared fresh in 0.1 M NaOH) at room temperature for 30 min. Proteins were subsequently precipitated by adding ice-cold acetone (4× volume) and storage at -20°C overnight. Precipitated proteins were pelleted by centrifugation (18,000 x g, 15 min, 4°C) and washed with ice-cold MeOH (2 × 0.5 ml), using sonication to resuspend the pellets between washes. Proteins were solubilized in 50 µl PBS containing 0.4% (w/v) SDS and subjected to CuAAC by adding 0.1 mM Rh-N$_3$ (1 µl of 5 mM stock in DMSO), 0.5 mM BTTAA ligand (2.5 µl of 10 mM stock in ddH$_2$O), 1 mM CuSO$_4$ (1 µl of 50 mM stock in ddH$_2$O), 2 mM NaAsc (1 µl of 100 mM prepared fresh in ddH$_2$O) and incubation at room temperature for 1 h. Samples were quenched with 2× gel loading buffer and analyzed by SDS-PAGE (12.5% polyacrylamide gels) with fluorescence scanning.

**Sample preparation for MS-based proteomics.**

(a) 2h-Labeling method: Overnight cultures of *S. aureus* USA300 TnPdxS grown in B-medium containing 5 µg/ml Erm were diluted 1:100 into fresh media (approximately 100 ml for 3 samples) and grown for 5 h until early stationary phase (OD$_{600}$ = 2.2 - 2.5). Bacteria were harvested (6,000 rpm, 5 min, 4°C) washed twice with 10 ml CDM-Erm (5 µg/ml) and resuspended to an OD$_{600}$ = 40. For each sample, 1 ml of this suspension was incubated with 10 µl probe or DMSO (100× stock in DMSO) in culture tubes for 2 h at 37°C, with shaking. For samples treated with DCS, bacteria were incubated with 50 µl DCS (20× stock prepared fresh in 0.1 M sodium phosphate buffer, pH 8) for 30 min at 37°C, with shaking, prior to probe labeling. Bacteria were harvested (6,000 rpm, 5 min, 4°C) and washed twice with 1 ml PBS prior to cell lysis. (b) Chemical supplementation method: *(see also growth curve protocol above)* Overnight cultures of *S. aureus* USA300 TnPdxS grown in B-medium (20 ml) containing 5 µg/ml Erm were harvested (6,000 rpm, 5 min, 4°C), washed twice with 5 ml CDM-Erm (5 µg/ml) and resuspended in 5 ml. This suspension was used to inoculate bacterial cultures to an OD$_{600}$ = 0.1 in 100 ml CDM-Erm supplemented with either 25 µM probe (25 µl of 100 mM stock in DMSO) or
0.5 µM PL (25 µl of 2 mM stock in DMSO) in baffled flasks. The cultures were grown for 30 h at 37°C with shaking (OD₆₀₀ = 1.4 - 1.6), at which point they were harvested (6,000 rpm, 5 min, 4°C), washed twice with 1 ml PBS, resuspended and adjusted to 1 ml of OD₆₀₀ = 60 prior to cell lysis. For the remainder of the sample preparation, both methods followed the same protocol. Bacteria were resuspended in 1 ml PBS and transferred to 2 ml glass-bead lysis tubes (Precellys® Lysing Kit, soil-grinding SK38). Cells were lysed mechanically using a Precellys® 24 Homogenizer (6 × 15 sec shaking at 5400 rpm) with cooling breaks on ice between cycles. Upon centrifugation (18,000 x g, 30 min, 4°C), the clarified lysates were reduced with 20 mM NaBH₄ (40 µl of 500 mM stock prepared fresh in 0.1 M NaOH) at room temperature for 30 min. Proteins were subsequently precipitated by adding ice-cold acetone (4× volume) and storage at -20°C overnight. Precipitated proteins were pelleted by centrifugation (18,000 x g, 15 min, 4°C) and washed with ice-cold MeOH (2 × 1 ml), using sonication to resuspend the pellets between washes. Proteins were solubilized in 0.5 ml PBS containing 0.4% (w/v) SDS and protein concentrations were adjusted to 2 mg/ml (= 1 mg total per sample) for the 2h-labeling protocol or proteins were solubilized in 1 ml PBS containing 0.4% (w/v) SDS and adjusted to 2 mg/ml (= 2 mg total per sample) for the chemical supplementation protocol. Samples were subjected to CuAAC by adding 0.1 mM tri-functional rhodamine-biotin-azide³ linker (5 µl of 10 mM stock in DMSO), 0.5 mM BTTAA ligand⁴ (25 µl of 10 mM stock in ddH₂O), 1 mM CuSO₄ (10 µl of 50 mM stock in ddH₂O) and 2 mM NaAsc (10 µl of 100 mM prepared fresh in ddH₂O) to each sample and incubating for 1 h at room temperature in the dark. PL1 and Ctrl-labeled samples were incubated with 20 mM IAA (20 µl of 500 mM prepared fresh in ddH₂O) prior to click chemistry to decrease conjugate addition of cellular nucleophiles to the activated alkyne. PL3-labeled samples were incubated with 0.2 mM EZ-Link™ Phosphine-PEG₃-Biotin (Sigma Aldrich) (10 mM stock in DMSO) for 4 h at 37°C, followed by 20 h at 25°C. Upon precipitation and washing (as described above), the protein pellets were re-solubilized in 0.5 ml PBS containing 0.4% SDS (w/v) and centrifuged (18,000 x g, 5 min, rt) prior to avidin bead enrichment. Samples were transferred to Lo-bind Eppendorf tubes containing 50 µl of avidin-agarose bead slurry (Sigma Aldrich) pre-equilibrated with 0.4% SDS (w/v) in PBS (3 × 1 ml, 400 x g, 5 min, rt), and rotated for 1 h at room temperature. The beads were then washed with 0.4% (w/v) SDS in PBS (3 × 1 ml), 6 M urea (2 × 1 ml) and finally PBS (3 × 1 ml). For gel-based analysis of enrichment, 10% of the sample was removed during the last washing step. Beads were heated (10 min, 95°C) in 25 µl gel loading buffer, centrifuged (13,000 rpm, 3 min) and analyzed by fluorescence SDS-PAGE (NuPAGE 4-12% Bis-Tris gel, Thermo Fisher Scientific). The remaining samples were resuspended in 200 µl X-buffer (7 M urea, 2 M thiourea in 20mM HEPES buffer, pH 7.5). Upon reduction with 5 mM TCEP (2 µl of 500 mM stock in ddH₂O) for 1 h at 37°C, proteins were alkylated using 10 mM IAA (4 µl of 500 mM stock in ddH₂O) for 30 min at
25°C and samples were quenched with 10 mM DTT (4 µl of 500 mM stock in ddH$_2$O) for 30 min at 25°C. Enzymatic digestion using LysC (1 µl of 0.5 µg/µl, Wako, MS-grade) was first carried out for 2 h at 25°C, upon which samples were diluted with triethylammonium bicarbonate (TEAB) buffer (600 µl of 50 mM stock in ddH$_2$O) and digested with trypsin (1.5 µl of 0.5 µg/µl in 50 mM acetic acid, Promega, sequencing grade) for a further 16 h at 37°C. Samples were acidified to 1% (v/v) FA and desalted using SepPak® C18 cartridges (50 mg, Waters) with a vacuum manifold. The cartridges were first washed with ACN (2 × 1 ml) and equilibrated with 0.1% (v/v) TFA (3 × 1 ml) prior to loading the samples. After washing with 0.1% (v/v) TFA (3 × 1 ml) and 0.5% (v/v) FA (1 × 0.5 ml), peptides were eluted in 80% (v/v) ACN containing 0.5% FA (3 × 0.25 ml) and freeze-dried using a speedvac centrifuge. Samples were prepared for MS-analysis by dissolving in 25 µl of 1% (v/v) FA and filtering through 0.22 µm PVDF filters (Millipore). All samples were prepared in at least three biological replicates from individual starting cultures.

**MS measurement and analysis.** LC-MS/MS analysis was performed with an Ultimate3000 Nano-HPLC system (Thermo Scientific) coupled to an Orbitrap Fusion instrument (Thermo Scientific). Samples were loaded on a 2 cm PepMap RSLC C18 trap column (particles 2 µm, 100 Å, inner diameter 75 µm, Thermo Scientific) with 0.1% TFA and separated on a 50 cm PepMap RSLC C18 column (particles 2 µm, 100 Å, inner diameter 75 µm, Thermo Scientific) heated at 50 °C. The gradient was run from 5-32% ACN containing 0.1% formic acid during a 152 min method (7 min 5%, 105 min to 22%, 10 min to 32%, 10 min to 90%, 10 min wash at 90%, 10 min equilibration at 5%) at a flow rate of 300 nl/min. Survey scans (m/z 300-1500) were acquired in the orbitrap with a resolution of 120,000 at m/z 200 and the maximum injection time set to 50 ms (target value 2e5). The most intense ions of charge states 2-7 were selected for fragmentation with high-energy collisional dissociation at a normalized collision energy of 30%. The instrument was operated in top speed mode and spectra were acquired in the ion trap with the maximum injection time set to 50 ms (target value 1e4). The option to inject ions for all available parallelizable time was enabled. Dynamic exclusion of sequenced peptides was set to 60 s. Data were acquired using Xcalibur software version 3.0sp2 (Thermo Scientific).

MS raw files were analyzed with MaxQuant[27] software (version 1.5.3.8). MS/MS-based peptide identification was carried out using the Andromeda[28] search engine with the *S. aureus* USA300 UniProtKB database (May 2016, 2607 accessions). The following parameter settings were used: peptide and protein FDR 1%, enzyme specificity trypsin, minimal number of amino acids required for peptide identification 7, variable modification methionine oxidation, fixed modification carbamidomethylation. At least two unique peptides were required for the identification of proteins. All other parameters were used
according to the default settings. For label-free protein quantification, the MaxLFQ algorithm was used as part of the MaxQuant environment. The ‘match between runs’ option was enabled to maximize the number of quantification events across all replicates. Statistical analysis was performed in Perseus\textsuperscript{29} (version 1.5.5.3). Proteins identified only by site modification, reverse hits or contaminants were removed. Data were filtered to retain only those proteins with > 2 MS/MS counts and quantified in at least 2 out of 3 replicates (or at least 5 out of 6; 8 out of 9 replicates). Missing values were then imputed on the basis of a normal distribution (width=0.3, down-shift=1.8). Volcano plots were generated by performing a two-sample $t$-test with permutation-based statistics (FDR 0.05, $s_0 = 1$). The complete list of PLP-DEs (PLP-ome) in \textit{S. aureus} USA300 (organism ID 367830) was identified by gene ontology (GO-term: 0030170; pyridoxal-phosphate binding), EC classification (enzyme.expasy.org; cofactor search: pyridoxal 5’-phosphate), and Uniprot (www.uniprot.org, cofactor search: pyridoxal 5’-phosphate).

Overrepresentation analysis is based on gene ontology annotations and was performed with the Bingo App in the Cytoscape environment.\textsuperscript{30} Statistically significant proteins from the volcano plot were compared to all proteins present in the plot for the annotation pyridoxal phosphate binding (GO:0030170) in the category of molecular process. According to literature research (see searches described above), we additionally annotated the following proteins as pyridoxal phosphate binding and added them to the GO list: A0A0H2XFQ3, A0A0H2XG73, A0A0H2XGD2, A0A0H2XGZ7, A0A0H2XH8, A0A0H2XI6, A0A0H2XJ0, A0A0H2XIS2, A0A0H2XJX6, A0A0H2XKI8, Q2FF55, Q2FGI6, Q2FGI7, Q2FH63, Q2FH64, Q2FHT1 and Q2FKE3. Analysis was based on a hypergeometrical test with the multiple testing correction according to Benjamini-Hochberg and a significance level of 0.05.

The full mass spectrometry proteomics data (raw files and MaxQuant output tables for protein groups and peptides) for PL2 labeling (100 and 10 µM, Fig. 3b and Supplementary Fig. 6), PL2 labeling (1-100 µM, Fig. 3d), PL1 and Ctrl labeling (100 µM, Supplementary Fig. 7), PL3 labeling (100 µM, Supplementary Fig. 8), PL1 and PL3 chemical supplementation (25 µM, Fig. 4b and 4c), PL2/PL4/PL5 labeling (100 µM, Fig. 6c, Supplementary Fig. 16), PL2 labeling with DCS inhibition (Fig. 6e) in \textit{S. aureus} USA300 TnPdxS have been deposited in the ProteomeXchange Consortium\textsuperscript{31} (http://proteomecentral.proteomexchange.org) via the PRIDE\textsuperscript{32} partner repository (data set identifier: PXD006483). The processed tables for protein group analysis in Perseus have been uploaded to the supplementary information as an Excel file.

\textbf{Confidence plot analysis.} Only proteins significantly enriched (104 proteins, volcano plot Fig. 3b) were selected for analysis. Profile plots of $\log_2$ LFQ intensities of individual proteins across proteomic samples were generated using Perseus. Each of the 18 PLP-DE was individually selected as a reference profile and
the 10 or 20 most similar profiles were identified by Pearson correlation. Corresponding proteins were grouped into confidence classes as illustrated in Fig. 3b.

**Heatmap and clustering analysis for concentration-dependent probe enrichment.** The mean raw LFQ intensities from triplicate measurements were normalized to DMSO (= 0) and highest LFQ intensity (= 1) for individual proteins across eight probe concentrations. Samples negatively enriched compared to DMSO were set equal to 0 for analysis. Profile plots of the normalized mean LFQ intensities were generated across different probe concentrations using Perseus and hierarchical clustering (Pearson, max number of clusters = 5) was performed to group similarly behaving proteins. The heatmap was prepared using OriginPro (2017) graphing and analysis software.

**Heatmap analysis of PL2, PL4 and PL5.** The mean raw LFQ intensities from triplicate measurements were normalized to DMSO (= 0) and highest LFQ intensity (= 1) for individual PLP-DE within PL2-, PL4- and PL5-enriched samples. The heatmap was prepared using OriginPro (2017) graphing and analysis software.
6. PLP Binding-site Identification

**Sample preparation.** Recombinant protein samples (2 µg, 50 µl) were incubated with 5 equivalents of PLP for 30 min at room temperature, and were subsequently reduced with 10 mM NaBH₄ (2 µl of 250 mM stock prepared fresh in 0.1 M NaOH) for 30 min at room temperature. Proteins were precipitated by adding ice-cold acetone (4× volume) and incubating at -20°C for at least 1 h. Precipitated proteins were pelleted by centrifugation (18,000 x g, 15 min, 4°C) and washed with ice-cold MeOH (2 × 0.2 ml), using sonication to resuspend the pellets between washes. Samples were resuspended in 100 µl X-buffer (7 M urea, 2 M thiourea in 20 mM HEPES buffer, pH 7.5) and reduced with 5 mM TCEP (1 µl of 500 mM stock in ddH₂O) for 1 h at 37°C. Proteins were alkylated using 10 mM IAA (2 µl of 500 mM stock in ddH₂O) for 30 min at 25°C, followed by quenching with 10 mM DTT (2 µl of 500 mM stock in ddH₂O) for 30 min at 25°C. Samples were diluted with 600 µl chymotrypsin buffer (100 mM Tris-HCl, 10 mM CaCl₂, pH 8) and digested by incubating with chymotrypsin (1 µl of 0.5 µg/µl in 1 mM HCl, Promega, sequencing grade) for 16 h at 25°C. Double-digested samples (Q2FF14) were first incubated with trypsin (1 µl of 0.5 µg/µl in 50 mM acetic acid, Promega, sequencing grade) for 30 min at 37°C and were then digested with chymotrypsin as described above. Samples digested with GluC (Q2FF14) were incubated with GluC (5 µl of 0.1 µg/µl in ddH₂O, Roche, sequencing grade) for 16 h at 25°C. Enzyme reactions were quenched by acidification to 1% (v/v) FA and peptides were desalted using SepPak® C18 cartridges (50 mg, Waters) with a vacuum manifold. The cartridges were washed with ACN (2 × 1 ml) and equilibrated with 0.1% (v/v) TFA (3 × 1 ml) prior to loading the samples. After washing with 0.1% (v/v) TFA (3 × 1 ml) and 0.5% (v/v) FA (1 × 0.5 ml), peptides were eluted in 80% (v/v) ACN containing 0.5% FA (3 × 0.25 ml) and freeze-dried using a speedvac centrifuge. Peptides were prepared for MS-analysis by dissolving in 25 µl of 1% (v/v) FA and filtering through 0.22 µm PVDF filters (Millipore).

**MS measurement and analysis.** LC-MS/MS analysis was performed with an Ultimate3000 Nano-HPLC system (Thermo Scientific) coupled to an Orbitrap Fusion instrument (Thermo Scientific) as described in the proteomics section, with some modifications. For PLP binding site identification, chymotrypsin digested samples were measured with a shorter gradient during a 62 min method. Charge states 1-7 were considered for measurement and depend scans were only performed on single charge state per precursor and measured in the orbitrap (AGC target 5e4, injection time 50 ms). MS raw files were analyzed with MaxQuant software (version 1.5.3.8) as described in the proteomics section with some modifications. MS/MS-based peptide identification was carried out using the Andromeda search engine. For PLP
binding site identification, the enzyme specificity was set to chymotrypsin (with additional cleavage after leucine and methionine) for all enzymes except for Q2FF14 which was double-digested with trypsin and chymotrypsin or digested with GluC. As a variable modification, the PLP moiety (+231.02966) at lysine residues was added with neutral losses of $\text{H}_3\text{PO}_4$ and the PLP moiety implemented. As we expect one PLP modification site per protein, we selected the site with the highest confidence based on 100% localization probability, best PEP (posterior error probability), score, score difference, delta score and manual evaluation of MS/MS spectra from MaxQuant (Supplementary Fig. 14). A detailed table summarizing the binding-site experiments has been uploaded to the supplementary information as an Excel file.

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## Appendix

### Abbreviations

| Abbreviation | Full Form |
|--------------|-----------|
| ACN          | acetonitrile          |
| CDM          | chemically-defined media |
| CHES         | N-Cyclohexyl-2-aminoethanesulfonic acid |
| CuAAC        | copper-catalyzed azide-alkyne cycloaddition |
| DCM          | dichloromethane |
| DCS          | D-cycloserine |
| DIPEA        | N,N-diisopropylethylamine |
| DMF          | N,N-dimethylformamide |
| DMSO         | dimethyl sulfoxide |
| DTT          | dithiothreitol |
| Erm          | erythromycin |
| FA           | formic acid |
| IAA          | iodoacetamide |
| LDA          | lithium diisopropylamide |
| mCPBA        | meta-chloroperoxybenzoic acid |
| MOMCl        | methoxymethyl chloride |
| NaAsc        | sodium ascorbate |
| PMBCl        | para-methoxybenzyl chloride |
| PPTS         | pyridinium p-toluenesulfonate |
| PTSA         | p-toluenesulfonic acid |
| TBAF         | tetrabutylammonium fluoride |
| TBDMSCl      | tert-butyldimethylsilyl chloride |
| TCEP         | tris(2-carboxyethyl)phosphine |
| TEAB         | tetraethylammonium bromide |
| TFA          | trifluoracetic acid |
| TFAA         | trifluoracetic anhydride |
| THF          | tetrahydrofuran |
NMR spectra

$^1$H NMR of compound 1 (300 MHz, CDCl$_3$)

$^{13}$C NMR of compound 1 (75 MHz, CDCl$_3$)
$^1$H NMR of compound 2 (400 MHz, CDCl$_3$)

$^{13}$C NMR of compound 2 (100 MHz, CDCl$_3$)
$^1$H NMR of compound PL2 (400 MHz, DMSO-d$_6$)

$^{13}$C NMR of compound PL2 (100 MHz, DMSO-d$_6$)
HSQC spectrum of compound PL2

HMBC spectrum of compound PL2
COSY spectrum of compound PL2
$^1$H NMR of compound 3 (360 MHz, CDCl$_3$)

$^{13}$C NMR of compound 3 (90 MHz, CDCl$_3$)
$^1$H NMR of compound 4 (360 MHz, CDCl$_3$)

$^{13}$C NMR of compound 4 (90 MHz, CDCl$_3$)
$^1$H NMR of compound 5 (360 MHz, CDCl$_3$)

$^{13}$C NMR of compound 5 (90 MHz, CDCl$_3$)
$^1$H NMR of compound 6 (360 MHz, CDCl$_3$)

$^{13}$C NMR of compound 6 (90 MHz, CDCl$_3$)
$^1$H NMR of compound 7 (360 MHz, CDCl$_3$)

$^{13}$C NMR of compound 7 (90 MHz, CDCl$_3$)
$^1$H NMR of compound 8 (360 MHz, MeOD)

$^{13}$C NMR of compound 8 (90 MHz, MeOD)
$^1$H NMR of compound 9a (500 MHz, MeOD)

13C NMR of compound 9a (120 MHz, MeOD)
HSQC spectrum of compound 9a

HMBC spectrum of compound 9a
NOESY spectrum of compound 9a
$^1$H NMR of compound 9b (500 MHz, MeOD)

$^{13}$C NMR of compound 9b (120 MHz, MeOD)
HSQC spectrum of compound 9b

HMBC spectrum of compound 9b
NOESY spectrum of compound 9b

$^{1}$H NMR of compound PL1 (500 MHz, DMSO-d$_6$)
$^{13}$C NMR of compound PL1 (125 MHz, DMSO-d$_6$)
HSQC spectrum of compound PL1

HMBC spectrum of compound PL1
COSY spectrum of compound PL1
$^{1}H$ NMR of compound Ctrl (500 MHz, MeOD)

$^{13}C$ NMR of compound Ctrl (125 MHz, MeOD)
HSQC spectrum of compound Ctrl

HMBC spectrum of compound Ctrl
COSY spectrum of compound Ctrl
$^1$H NMR of compound 10 (400 MHz, CDCl$_3$)

$^{13}$C NMR of compound 10 (100 MHz, CDCl$_3$)
$^1$H NMR of compound 11 (300 MHz, CDCl$_3$)

$^{13}$C NMR of compound 11 (75 MHz, CDCl$_3$)
$^1$H NMR of compound 12 (300 MHz, CDCl$_3$)

$^{13}$C NMR of compound 12 (75 MHz, CDCl$_3$)
$^1$H NMR of compound 13 (300 MHz, CDCl$_3$)

$^{13}$C NMR of compound 13 (75 MHz, CDCl$_3$)
$^1$H NMR of compound 14 (400 MHz, CDCl$_3$)

$^{13}$C NMR of compound 14 (100 MHz, CDCl$_3$)
$^1$H NMR of compound PL3 (500 MHz, MeOD)

$^{13}$C NMR of compound PL3 (75 MHz, MeOD)
COSY spectrum of compound PL3
$^1$H NMR of compound 15 (400 MHz, CDCl$_3$)

$^{13}$C NMR of compound 15 (100 MHz, CDCl$_3$)
$^1$H NMR of compound 16 (400 MHz, CDCl$_3$)

$^{13}$C NMR of compound 16 (100 MHz, CDCl$_3$)
$^1$H NMR of compound PL4 (400 MHz, DMSO-$d_6$)

$^{13}$C NMR of compound PL4 (100 MHz, DMSO-$d_6$)
COSY spectrum of compound PL4
$^1$H NMR of compound **17** (400 MHz, CDCl$_3$)

$^{13}$C NMR of compound **17** (100 MHz, CDCl$_3$)
$^1$H NMR of compound 18 (400 MHz, CDCl$_3$)

$^{13}$C NMR of compound 18 (100 MHz, CDCl$_3$)
$^1$H NMR of compound **PL5** (400 MHz, DMSO-$d_6$)

$^{13}$C NMR of compound **PL5** (100 MHz, DMSO-$d_6$)
HSQC spectrum of compound PL5

HMBC spectrum of compound PL5
COSY spectrum of compound **PL5**