Experimental section:

Materials:

Plasmids and *Escherichia coli* competent cells were purchased from Novagen. All buffers and reagents including L-penicillamine, pyridoxal 5'-phosphate (PLP) and pyridoxal (PL) were from Sigma-Aldrich. Palmitoyl-CoA was from Avanti Lipids. Palmitoyl Chloride was purchased from Alfa Aesar, and coenzyme A (free hydrate) was purchased from Bosche Scientific. All other chemicals were purchased from Aldrich and used without further purification. The thioester analogue of palmitoyl-CoA, S-(2-oxoheptadecyl)-CoA, was synthesised as described [1] with modifications inspired by Gerson and Schlenk [2].

Phosphate binding cup analysis of AOS enzymes:

We analysed the X-ray crystal structures of the internal and external PLP:aldimines of the SPT, PDB codes: 2JG2, 2W8J [3, 4].

Cloning and expression of *Sphingomonas paucimobilis* SPT (*SP* SPT):

The *SP* SPT enzyme was prepared as previously described [3] using 20 mM Tris in place of potassium phosphate buffer in all steps. The apo-enzyme was prepared following previous methods [5]. The PL-reconstituted enzyme was obtained by dialysis against 1L (x3) 20 mM Tris (pH 7.5) containing 150 mM NaCl and 250 µM PL.

Spectroscopic measurements:

All UV-visible spectra were recorded on a Cary 50 UV-visible spectrophotometer (Varian) and analysed using Cary WinUV software (Varian). Prior to all UV-visible spectroscopy and assays, SPT was dialysed against fresh buffers containing 250 µM PLP or PL to ensure the enzyme was in the PLP or PL-bound, holo-form. Excess PLP or PL was removed on a PD-10 (Sephadex G-25M) desalting column (GE Healthcare). For UV-visible assays the concentration of enzyme was 40 µM and the spectrophotometer was blanked with 20 mM Tris (pH 7.5) containing 150 mM NaCl at 25 °C. Quartz cuvettes from NSG Precision Cells, Type 18-BM with a lightpath of 10 mm and a sample volume of 500 µl were used and the spectra were collected from 800 nm to 200 nm.

SPT activity by measuring CoASH release using DTNB assay:

SPT activity was measured using the DTNB assay as previously described [3]. Assays contained enzyme, substrates and DTNB with final concentrations as followed: 0.25 µM *Sp*SPT, 25 mM L-serine, 250 µM palmitoyl-CoA, 0.2 mM DTNB in 20 mM Hepes buffer, pH 8.0 at 25 °C. Control SPT:PLP samples before removal of PLP, regeneration of PLP or addition of PL rates

were normalised to 100% relative activity. Activity assays were carried out in duplicate and the error bars are the standard error of the mean (SEM) values for each separate experiment. A typical experiment to determine the K_m value for L-serine contained 0.42 µM SPT, 0.1 – 50 mM L-serine, 250 µM palmitoyl CoA and 0.2 mM DTNB in 50 mM potassium phosphate buffer pH 7.5. K_m values were calculated directly from Michaelis-Menten plots using Sigma Plot.

SPT activity by measuring KDS formation:

SPT activity was monitored by incorporation of U- ^{14}C L-serine (PerkinElmer) into ^{14}C-KDS. [3] A final enzyme concentration of 25µM SPT (in 20 mM Tris buffer, pH 7.5, 150 mM NaCl) was incubated with 20 mM ^{14}C L-serine (7400 Bq, 0.2 µCi,) and 250 µM palmitoyl-CoA in a final volume of 1 mL. The reaction was incubated at 37 °C for 20 minutes and then the reaction was quenched by the addition of NH_4OH (final concentration 0.4 M). This was then extracted with an equal volume of $CHCl_3$:CH_3OH (2:1, v:v). The sample was centrifuged at 13, 000 rpm for 5 minutes and the aqueous phase was discarded. The organic phase was left to evaporate at 50 °C. The resulting lipid residue was re-suspended in 15 µl of $CHCl_3$:CH_3OH (2:1, v:v) and spotted onto a Silica Gel 60 F_{254} TLC plate. Separation was carried out with a mobile phase of $CHCl_3$:CH_3OH (2:1, v:v). The TLC was visualised using an AR-2000^{TM} imaging scanner.

Analysis of SPT:PL by mass spectrometry:

The PL-bound enzyme was reduced with 1 mM sodium borohydride for 30 minutes at 25 °C. The shift in absorbance that accompanied the reduction of the imine to an amine was monitored by UV-vis spectroscopy. The sample was then treated with trypsin at a final trypsin:SPT sample ratio of 1:50.

Online LC-MS of SPT Typtic Digest:

Online Mass Spectrometry - On-line liquid chromatography was performed using an Ultimate 3000 HPLC system (Dionex) equipped with a monolithic PS-DVB (500 µm X 50 mm) reverse-phase analytical column (Dionex) coupled to electrospray ionization (nESI). Protein digests (5 pmoles) were loaded onto the column (maintained at 60°C) followed by a 30 min linear gradient from 2 to 70% acetonitrile (flow rate 20 µl/min). All mass spectra were recorded on SolariX FT-ICR mass spectrometer equipped with a 12 T superconducting magnet (Bruker Daltonics). Transient data size was set to 1Mword for each acquisition.

Online Tandem Mass Spectrometry - Online tandem MS was performed on the SolariX FT-ICR Instrument. After the initial LC-MS run specific peptides of interest were selected for

fragmentation. The protein digest was separated using an identical gradient to the above. However, the mass resolving quadrupole was set to select for a specific *m/z* throughout the LC-MS run and MS/MS was performed using collision-induced dissociation (CID).

Data Analysis - All spectra were externally calibrated using ES tuning mix (Agilent Technologies) and analyzed using DataAnalysis software (Bruker Daltonics). For analysis of the tryptic digest, the SNAP 2.0 algorithm was used for automated peak picking in order to create a list of peptide masses. For CID experiments, fragment lists were created (using SNAP 2.0) and the resulting mass lists were searched against the relevant primary sequences using Prosight PTM software. Error tolerances were set to 10ppm. Isotope distributions of specific charge states were predicted from theoretical empirical formulas. These were overlaid upon the recorded experimental data as scatter plots, with the theoretical apex of each isotope peak designated by a circle.

Determination of dissociation constants, substrate and product quinonoid detection and SPT activity assays:

Binding assays typically contained 40 µM enzyme in 20 mM Tris (pH 7.5). Varying amounts of L-serine (0-60 mM) was added and after addition of the substrate, the reaction mixture was allowed to equilibrate for 15 min at 25 °C. The K_d values were calculated from plots of Δ425 versus L-serine concentrations by fitting to a hyperbolic saturation curve using Sigma Plot software.

$$\Delta A_{obs} = \frac{\Delta A_{max}\,[\text{serine}]}{K_d + [\text{serine}]}$$

where ΔA_{obs} represents the observed change in absorbance at 422 nm, and ΔA_{max} is the maximal absorbance change, [serine] represents L-serine concentration, and the K_d is the dissociation constant[1]. To form the substrate quinonoid, 50 mM L-serine and 1.5 mM S-(2-oxoheptadecyl)-CoA were added to the enzyme. After addition, the reactants were mixed and allowed to equilibrate for 15 min at 25 °C. To form the product quinonoid, 25 µM KDS was added to the enzyme and allowed to equilibrate for 15 min at 25 °C.

Synthesis of S-(2-oxoheptadecyl)-CoA:

Palmitoyl Chloride was purchased from Alfa Aesar, coenzyme A (free hydrate) was purchased from Bosche Scientific all other chemicals were purchased form Aldrich and used without further purification. 1H NMR spectra were recorded on a Bruker OPEN400 (400 MHz) spectrometer. Chemical shifts (δ) are quoted in parts per million (ppm) downfield of tetramethylsilane, using residual protonated solvent as internal standard (D_2O at 4.79 ppm).

Abbreviations used in the description of resonances are: s (singlet), d (doublet), t (triplet), q (quartet), app (apparent), br (broad).

Synthesis of 1-chloro-heptadecan-2-one: Trimethylsilyldiazomethane (6 mL, 2M in Hexane) was diluted into acetonitrile (30 mL) and cooled to 0 ^{o}C. Palmitoyl chloride (1.7g, 6 mmol) in acetonitrile (6 mL) was added dropwise. The reaction mixture was stirred at room room temperature for 2 hours. The solvent was removed under reduced pressure. The resulting powder (1.96 g) was suspended in diethyl ether (20 mL) before the dropwise addition of hydrogen chloride solution (7 mL, 14 mmol, 2M in diethyl ether). The reaction mixture was stirred for 30 minutes before all volatile components were removed under vacuum. The crude product was purified by column chromatography on silica gel (5% Ethyl Acetate/Hexane) to yield 1-chloro-heptadecan-2-one (1.3 g, 75%) as a white solid that displayed spectroscopic data consistent with those reported previously.[2]

Synthesis of S-(2-oxoheptadecyl)-CoA: Li_2CO_3 (3 g, 0.41 mmol) was dissolved in water (10 mL). The solution was purged with Argon for 30 minutes before coenzyme A (100 mg, 0.12 mmol) was added. The reaction mixture was purged with argon for a further 30 minutes. 1-chloro-heptadecan-2-one (173mg, 0.6 mmol) was dissolved in toluene (1 mL). The ketone solution was then diluted with ethanol (19 mL) and added dropwise to the coenzyme A solution. The reaction mixture was left stirring under an atmosphere of argon for 18 hours. The reaction mixture was poured into acetone (500 mL) and the resulting white solid collected. The solid was then washed with ethanol (100 mL) and diethyl ether (2x100 mL) and dried under vacuum to yield a white solid (49 mg, 40%) that displayed spectroscopic data consistent with those reported previously. [1] The 1H nmr spectrum is shown in Supplementary figure S6 below.

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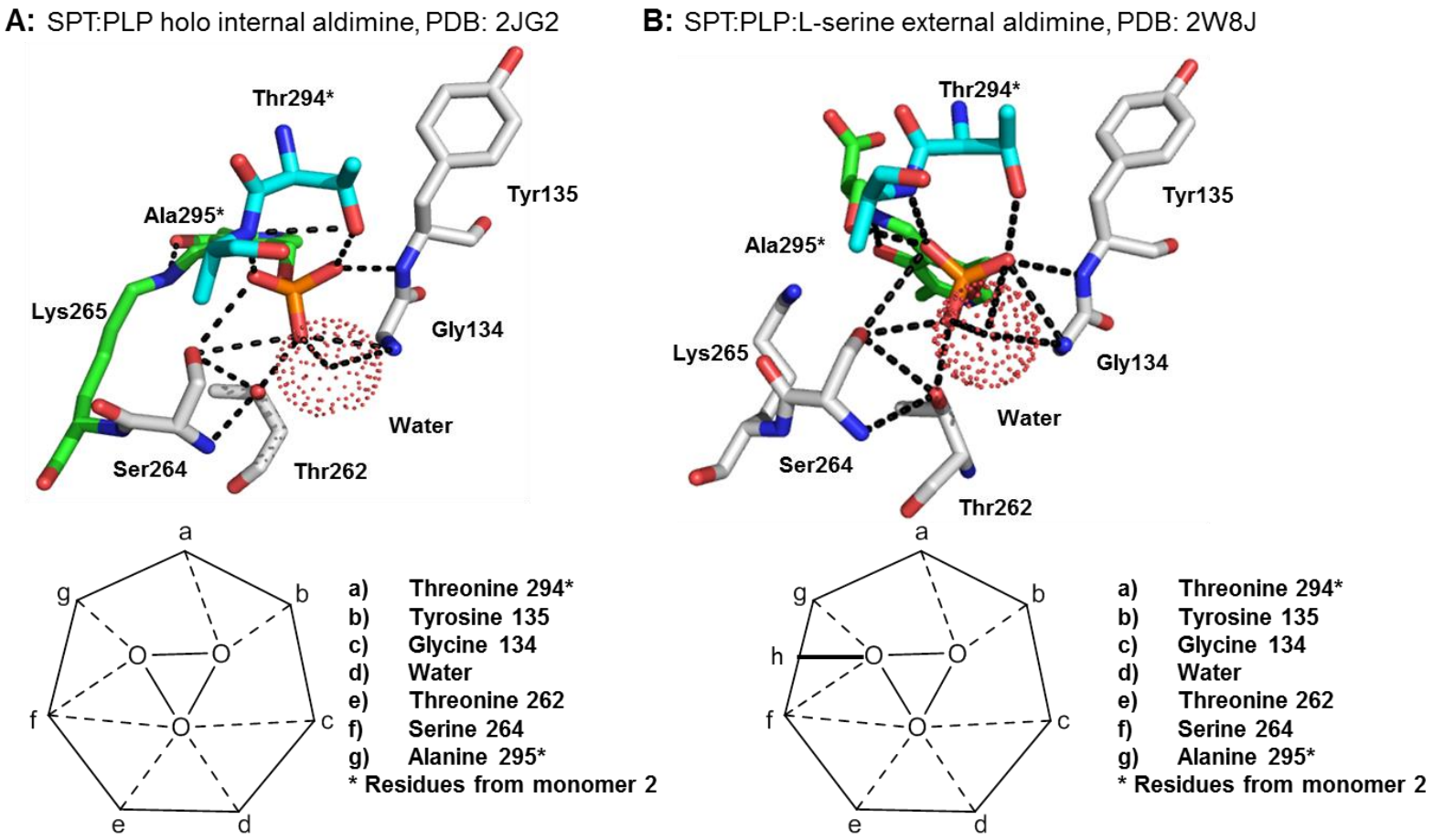
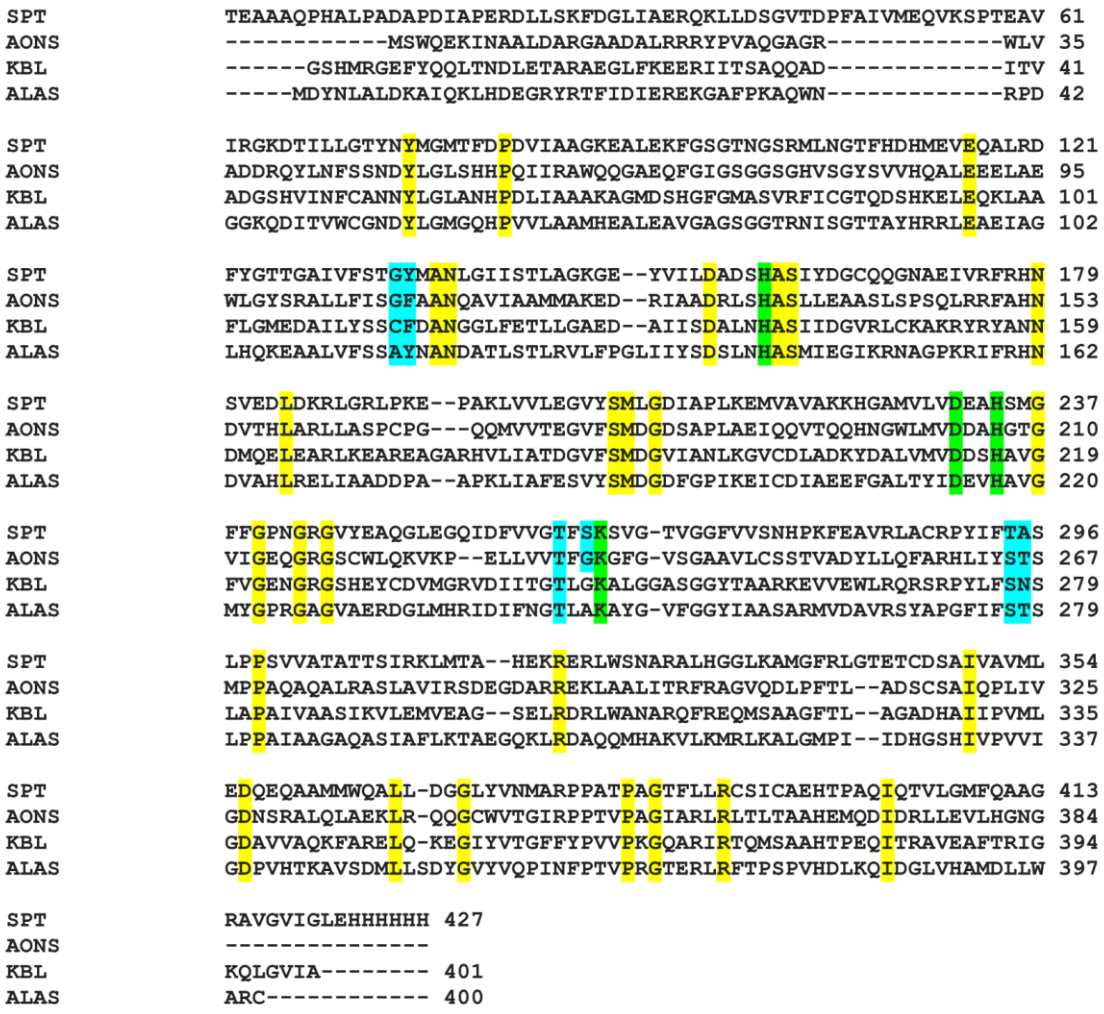
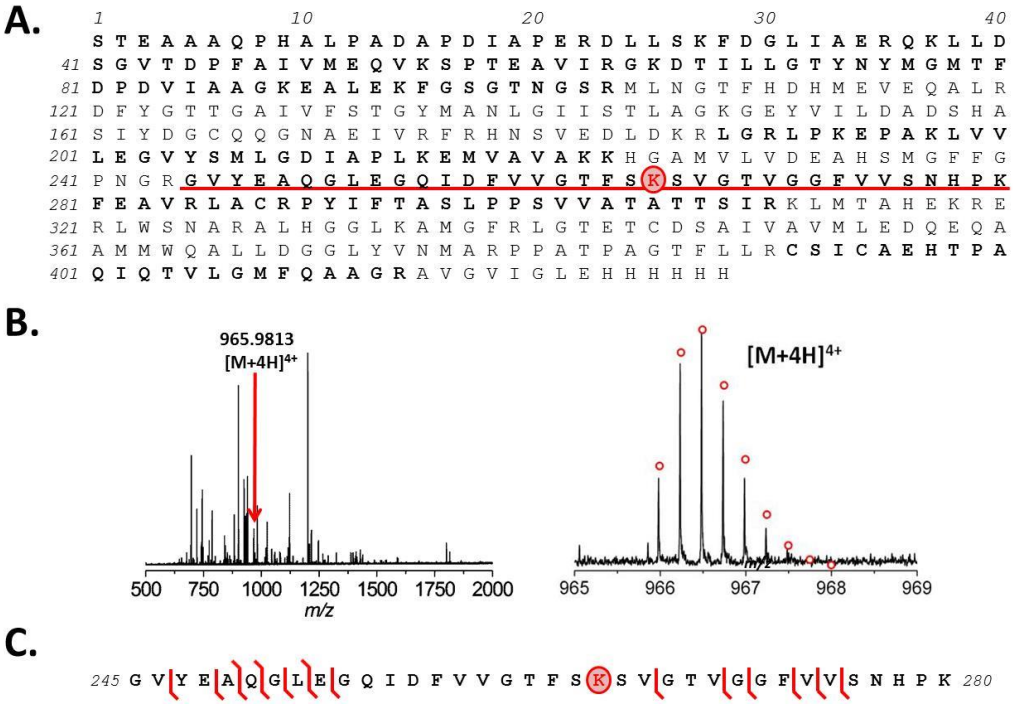


Supplementary figure S1:(A) SPT:PLP internal aldimine, holo-form (PDB: 2JG2). **(B)** SPT:PLP:L-serine external aldimine (PDB: 2W8J). This is a rotated view of that shown in Fig 1(A).



Supplementary figure S2: Sequence analysis of AOS family of PLP dependent enzymes; SPT, *S. paucimobilis* Serine PalmitoylTransferase (Uniprot code: Q93UV0); AONS, *E. coli* AminoOxoNonanoate Synthase (P12998); KBL, *E. coli* KetoButyrate Ligase (P0AB77), ALAS, *R. capsulatus* AminoLevulinic Acid Synthase (P18079). The sequence alignment was generated using CLUSTALW2. [6, 7] Conserved residues are shown in yellow, residues involved in PLP binding are in green and residues involved in the phosphate binding cup are shown in cyan.



Supplementary figure S3. Peptide mass fingerprinting of SPT:PL confirms that PL binds to Lys265.

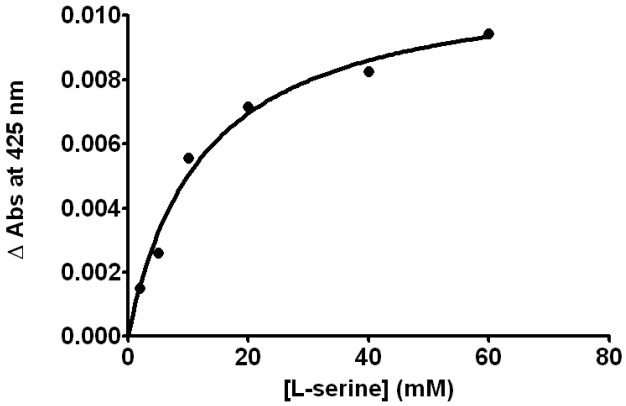
(A) Sequence coverage achieved when analysing the trypsin digest of SPT:PL. Amino acids highlighted in bold were observed in the peptide mass fingerprint (total coverage - 55%). Lys265, the PLP-bound SPT active site residue is highlighted in red. A singles species, with monoisotopic neutral mass 3859.94 Da, displayed a mass consistent with the tryptic peptide G245-K280 with the covalent addition of PL (underlined).

(B) The observed mass spectrum of the trypsin digest of PL-modified SPT (*left*) and the observed isotope distribution of the species consistent with PL-modified G245-K280 peptide (*right*). The species was observed in the $[M+4H]^{4+}$ charge state at monoisotopic *m/z* 965.9813. The theoretical isotopic distribution is overlaid on the experimental data as red circles ($[C_{168}H_{257}N_{43}O_{52}+ C_8H_9NO_2 + 4H]^{4+}$; calculated monoisotopic *m/z* 965.9928 (4+); error 11 ppm).

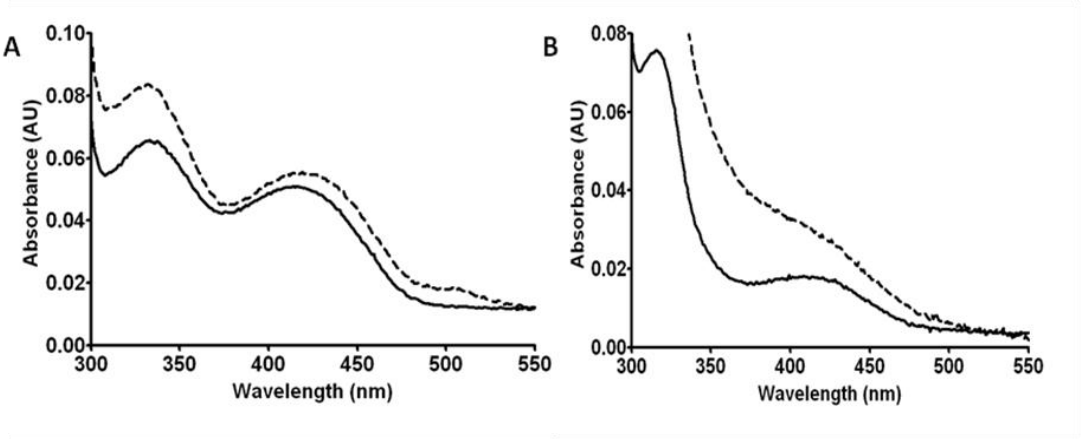
(C) Collision induced dissociation fragmentation map of the PL-modified G245-K280 peptide. The resulting *b* and *y* ion fragments (data not shown) represented in red ticks, were mapped onto the peptide sequence of SPT using ProSight-PTM. Fragmentation allowed the assignment of the PL modification to a 14 amino acid section of the protein containing Lys265.

Enzyme	$k_{cat} \times 10^2$ s^{-1}	K_m^{Ser} mM	K_m^{PCoA} µM	k_{cat}/K_m^{Ser} $M^{-1}s^{-1}$	k_{cat}/K_m^{PCoA} $M^{-1}s^{-1}$
SPT:PLP	154 ±4.1	1.6 ±0.2	35.6 ±6.4	962.5	43258
SPT:PLP regenerated	147 ±4.0	2.7 ±0.3	42.2 ±7.4	544.4	34834
SPT:PL regenerated	13.1 ±0.67	5.0 ±1.0	33.0 ±8.6	26.3	3970
SPT:L-pen (apo)	-	-	-	-	-

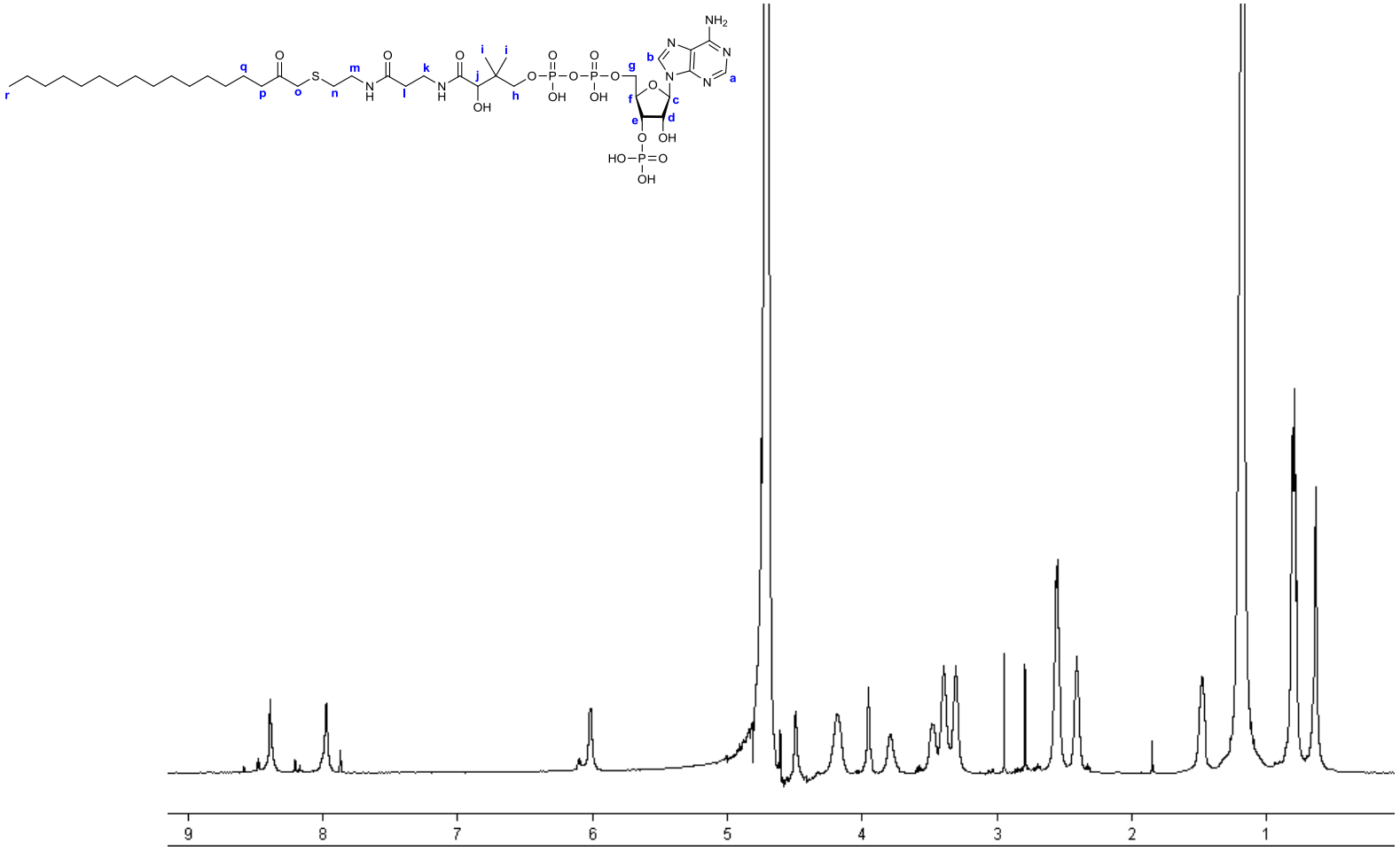
Supplementary Table: Kinetic parameters for the SPT:PLP wild type, SPT:PLP reloaded and SPT:PL reloaded enzyme.



Supplementary figure S4: Dissociation constant for SPT:PL measured at 425 nm with increasing L-serine concentrations.



Supplementary figure S5: (A) UV-vis spectra of SPT:PLP (solid line), after addition of 25 µM KDS (dashed line) (B) UV- vis spectra of SPT:PL (solid line), after addition of 25 µM KDS (dashed line). Conditions: 20 mM Tris, pH 7.5 and 40 µM enzyme.



Supplementary figure S6: NMR analysis of S-(2-oxoheptadecyl)-CoA**.** 1H NMR (400 MHz, D2O) δ 8.37 (1H, **b**), 7.95 (1H, **a**), 6.00 (1H, **c**), 4.49 (1H, **f**), 4.16 (2H, **g**), 3.94 (1H, **j**), 3.79 (1H, **h**), 3.48 (1H, **k**), 3.37 (2H, **o**), 3.29 (2H, **m**), 2.57 (2H, **n**), 2.55 (2H, **p**), 2.40 (2H, **l**), 1.48 (2H, **q**), 0.8 (3H, **i**), (0.78 (3H, **i**), 0.63 (3H, **r**)

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