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Supporting Information

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Inhibition of *Mycobacterium tuberculosis* pantothenate synthetase by analogues of the reaction intermediate

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Expression of pantothenate synthetase – The plasmid pET30a:*panC* encoding pantothenate synthetase from *M. tuberculosis* was kindly provided by Dr. Wang, University of California Los Angeles. This plasmid adds 44 amino acid residues to the *N*-terminus of the recombinant protein (MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKA–), including a 6×His tag and an enterokinase cleavage site. The plasmid (5 μL) was amplified from XL1(Blue) cells (Novagen) and the gene was sequenced to confirm its identity (Cogenics – Lark Technology). The sequencing revealed two single-point mutations in the gene, corresponding to T2A and E77G mutations on the gene product [see Ref. (*1*)]. C41(DE3) competent cells transformed with the pET30a:*panC* plasmid were grown at 37 °C to an A_{600} of 0.6 in 2xYT medium containing 50 μg/mL kanamycin. After addition of IPTG (0.5 mM) the culture was continued for an additional 12 h at 30 °C.

Purification and Characterization of His_6-PS – Cells (16 g from a 2 L culture) were suspended in 50 mL of 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.007% v/v β-mercaptoethanol and 1 mM PMSF protease inhibitor. Cells were lysed by incubating for 30 min at room T with addition of 0.2 mg/ml of lysozime, 10 μg/ml DNase and 10 mM $MgCl_2$, followed by sonication for a total process time of 3 min. The cell lysate was applied to a Ni-NTA column (GE Healthcare), washed with 50 mM sodium phosphate, pH 8.0, 30 mM imidazole, 300 mM NaCl, and eluted with 250 mM imidazole. Fractions containing protein were buffer-exchanged in 50 mM HEPES-HCl, pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, using a HiPrep 26/10 Desalting column (GE Healthcare), to yield ~55 mg of His_6-PS from 1 L culture. The His-tagged protein was stored at -80 °C and used for biophysical studies and kinetic assays.

Protein concentration was determined by A_{280}, with extinction co-efficient for His_6-PS of 24,000 $M^{-1}cm^{-1}$ obtained by amino acid analysis (PNAC). Amino acid analysis (data not shown) was also consistent with that predicted from the 353 aa protein construct (see Table 1). Electrospray mass spectrometry (ESI-MS) was carried out on a q-Tof mass spectrometer (Sarah Maslen, Department of Chemistry). The mass of the protein, calculated from the predicted amino acid sequence (37 433 Da), compares well with the ESI-MS value (37 431 Da) determined for the recombinant protein. Size exclusion chromatography was carried out using a HiLoad 26/60 SuperDex 200 gel filtration column (GE Healthcare) eluted using 20 mM HEPES-HCl, pH 7.6, 150 mM NaCl at a flow rate of 1.0 ml min^{-1}. The protein run as a dimer, with apparent molecular size of ~86 kDa.

Table 1, SI. Amino acid sequence of *M. tuberculosis* His_6-PS

MHHHHHHSSG LVPRGSGMKE TAAAKFERQH MDSPDLGTDD DDKAMAIPAF HPGELNVYSA
PGDVADVSRA LRLTGRRVML VPTMGALHEG HLALVRAAKR VPGSVVVVSI FVNPMQFGAG
GDLDAYPRTP DDDLAQLRAE GVEIAFTPTT AAMYPDGLRT TVQPGPLAAE LEGGPRPTHF
AGVLTVVLKL LQIVRPDRVF FGEKDYQQLV LIRQLVADFN LDVAVVGVPT VREADGLAMS
SRNRYLDPAQ RAAAVALSAA LTAAAHAATA GAQAALDAAR AVLDAAPGVA VDYLELRDIG
LGPMPLNGSG RLLVAARLGT TRLLDNIAIE IGTFAGTDRP DGYRAILESH WRN

Enterokinase cleavage – For crystallization studies, the N-terminal His_6-tag was cleaved using enterokinase (New England Biolabs). Enterokinase digestion was carried out in a solution containing 20 mM Tris and 15 mM HEPES, pH 8.0, 50 mM NaCl, 2 mM $CaCl_2$, and 1.5 $MgCl_2$ with enterokinase (0.0001% w/w of substrate protein). ESI-MS analysis of the cleaved protein gave a MW of 31 538 Da. This is consistent with the cleavage of the first 43 amino acid N-terminal extension as well as of the last 9 residues from the C-terminus of the protein (–AILESHWRN), as previously reported by Wang and Eisenberg (*1*). The calculated MW of the resulting protein construct is 31 539 Da. The cleaved N-terminal and the C-terminal peptides (MW of 4 804 Da and 1 125 Da, respectively) were both observed in the ESI-MS spectrum.

The reaction solution was filtered, and loaded onto a Trypsin Inhibitor-Agarose column (Sigma, T0637) to remove the enterokinase, after adjusting NaCl concentration to 500 mM. The flow-through was then applied to a Ni-NTA chelating column equilibrated with 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 10 mM imidazole, to separate the digested enzyme from undigested enzyme and cleaved N-terminal tag. Finally, the protein was concentrated down to 1 ml, applied to a HiLoad 26/60 SuperDex 200 gel filtration column, and eluted using 20 mM HEPES-HCl, pH 7.6, 150 mM NaCl at a flow rate of 1.0 ml min^{-1}. The cleaved protein run as a dimer in gel filtration (apparent molecular size of ~70 kDa).

Enzyme kinetic assay – Pantothenate synthetase activity was assayed by coupling the formation of AMP to the oxidation of NADH with myokinase, pyruvate kinase and lactate dehydrogenase as previously described (*2-5*). The decrease in absorbance of NADH at 340 nm (ε = 6,220 M^{-1} cm^{-1}) was measured in a 96-well Bio-Tek Powerwave *XS* plate reader, running the Biotek instrument software KC4 (version 3.2). The temperature of the plate was maintained at 25 ^{o}C throughout the assay. A solution of sodium pantoate (1 M) was prepared by dissolving pantolactone (130 mg, 1.0 mmol) in a solution of NaOH (1 M, 1 ml) as previously described (*6*). The assay contained 100 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1.5 mM potassium phosphoenolpyruvate, 100 nM His_6-PS, 200 μM NADH, 5 units of pyruvate kinase, 5 units of myokinase, and 6 units of lactate dehydrogenase. Pantoate and β-alanine were held at a concentration of 4 mM and the concentrations of ATP and inhibitor were varied as indicated, by using four different inhibitor concentrations and four concentrations of ATP. After incubating the solution for 5 minutes at 25 ^{o}C, the reaction was initiated by addition of pantoate to a final concentration of 4 mM. The final volume per well was 200 μl. The assay was performed in duplicate and mean values are shown. Least-squares fitting of the data to the Michaelis-Menten and LineWeaver-Burk equations were performed using the GraFit software (version 5.0.6, Erithacus Software Limited, http://www.erithacus.com/grafit/).

References

1. Wang, S. and Eisenberg, D. (2003) Crystal structures of a pantothenate synthetase from *M. tuberculosis* and its complexes with substrates and a reaction intermediate, *Protein Sci. 12*, 1097-1108.

2. Miyatake, K., Nakano, Y., and Kitaoka, S. (1973) Isotopic determination and optimum reaction conditions of pantothenic acid synthetase, *Agr. Biol. Chem. 37*, 1205-1207.

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5. Tuck, K. L., Saldanha, S. A., Birch, L. M., Smith, A. G., and Abell, C. (2006) The design and synthesis of inhibitors of pantothenate synthetase, *Org. Biomol. Chem. 4*, 3598-3610.

6. King, H. L., Jr., Dyar, R. E., and Wilken, D. R. (1974) Ketopantoyl lactone and ketopantoic acid reductases. Characterization of the reactions and purification of two forms of ketopantoyl lactone reductase, *J. Biol. Chem. 249*, 4689-4695.

7. The PyMOL Molecular Graphics System, DeLano Scientific, 2002.

Table 2, SI. Crystallographic data collection and refinement statistics.

Table 2	apo	2	3	4
Data collection				
X-ray source	ESRF, ID29	ESRF, ID29	ESRF, ID29	ESRF, ID29
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Cell parameters, Å (α=γ=90°)				
a	48.27	48.42	48.49	48.60
b	70.92	71.24	71.20	71.23
c	81.87	81.81	81.85	82.02
β	99.16	99.46	99.80	99.56
Resolution range, Å (outer shell)	50.0-1.5 (1.53-1.50)	50.0-1.8 (1.84-1.80)	50.0-2.05 (2.10-2.05)	50.0-2.0 (2.05-2.00)
No. of unique reflections	86488	50195	34527	37427
Multiplicity	3.7	3.7	4.6	4.4
R_{merge}, % (outer shell)	5.9 (34.1)	7.0 (35.7)	7.0 (46.6)	9.9 (47.9)
Average $I/\sigma(I)$	13.1	10.9	9.3	8.9
% Reflections with $I/\sigma(I)$>3, (outer shell)	80.4 (43.5)	76.5 (38.2)	75.6 (40.0)	74.4 (38.0)
Completeness, % (outer shell)	99.7 (100)	99.6 (99.1)	98.3 (97.1)	99.9 (100.0)
Mosaicity, °	0.28	0.59	0.50	0.59
Wilson B, $Å^2$	16.3	19.7	31.1	23.0
Refinement				
Resolution range, Å	47.67-1.50	47.78-1.80	44.59-2.03	47.95-2.00
R_{cryst},[a] %	16.9	16.0	16.6	16.8
R_{free},[b] %	19.5	20.9	22.1	21.8
Number of reflections				
Working set	77811	45081	31066	33660
Test set	4327	2550	1730	1872
Number of protein atoms	4194	4218	4146	4187
Number of water molecules	601	447	369	361
Inhibitor	0	2	2	2
Sulfate	4	0	0	2
Glycerol	4	2	1	1
Ethanol	5	2	3	4
Average Isotropic B Value (protein)	17.9	22.8	37.6	31.4
Average Isotropic B Value (waters)	31.2	31.9	44.4	37.6
Average Isotropic B Value (inhibitor)		15.6	28.9	29.9

[a] $R_{cryst} = \Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$, F_{obs} and F_{calc} are observed and calculated structure factor amplitudes
[b] R_{free} as for R_{cryst} using a random subset of the data excluded from the refinement

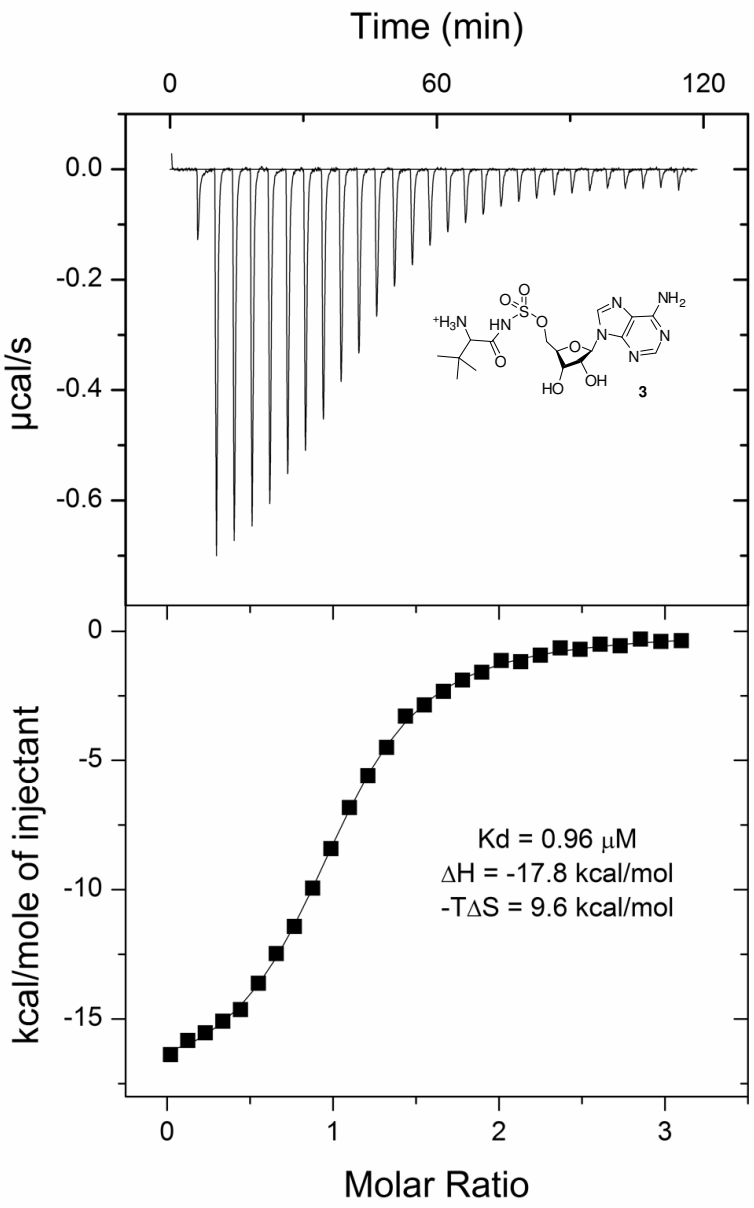

Figure 1, SI. ITC titration of **3** against *M. tuberculosis* His_6-PS.

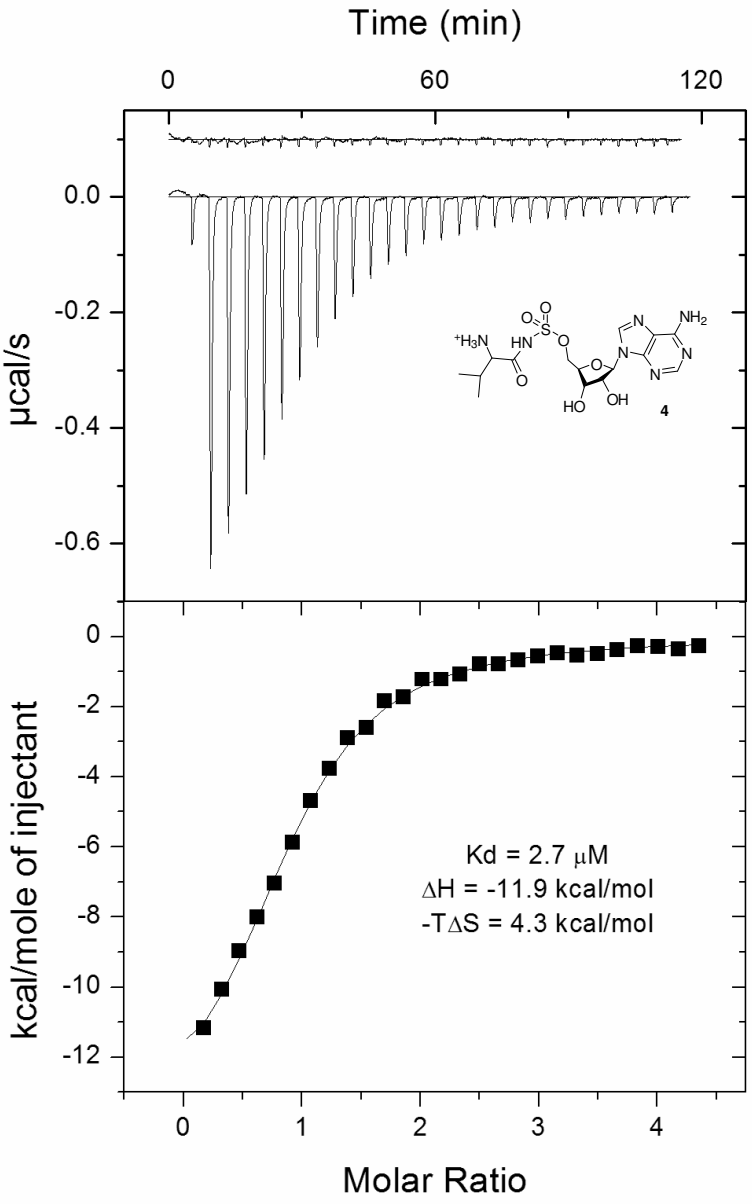

Figure 2, SI. ITC titration of **4** against *M. tuberculosis* His_6-PS.

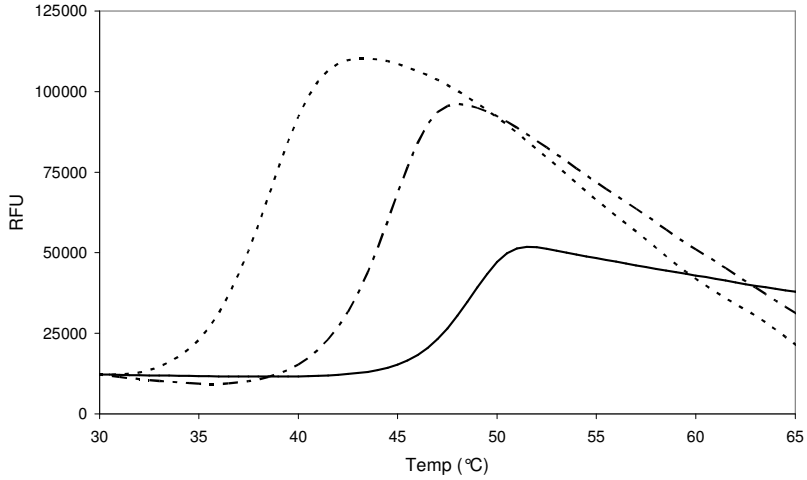

Figure 3, SI. Thermal shift binding assays.

Dotted curve: apo enzyme, T_m = 38 °C; dotted-dashed curve: enzyme in the presence of 60 μM of **3**, T_m = 45.5 °C; solid curve: in the presence of 28 μM of **2**, T_m = 48.5 °C.

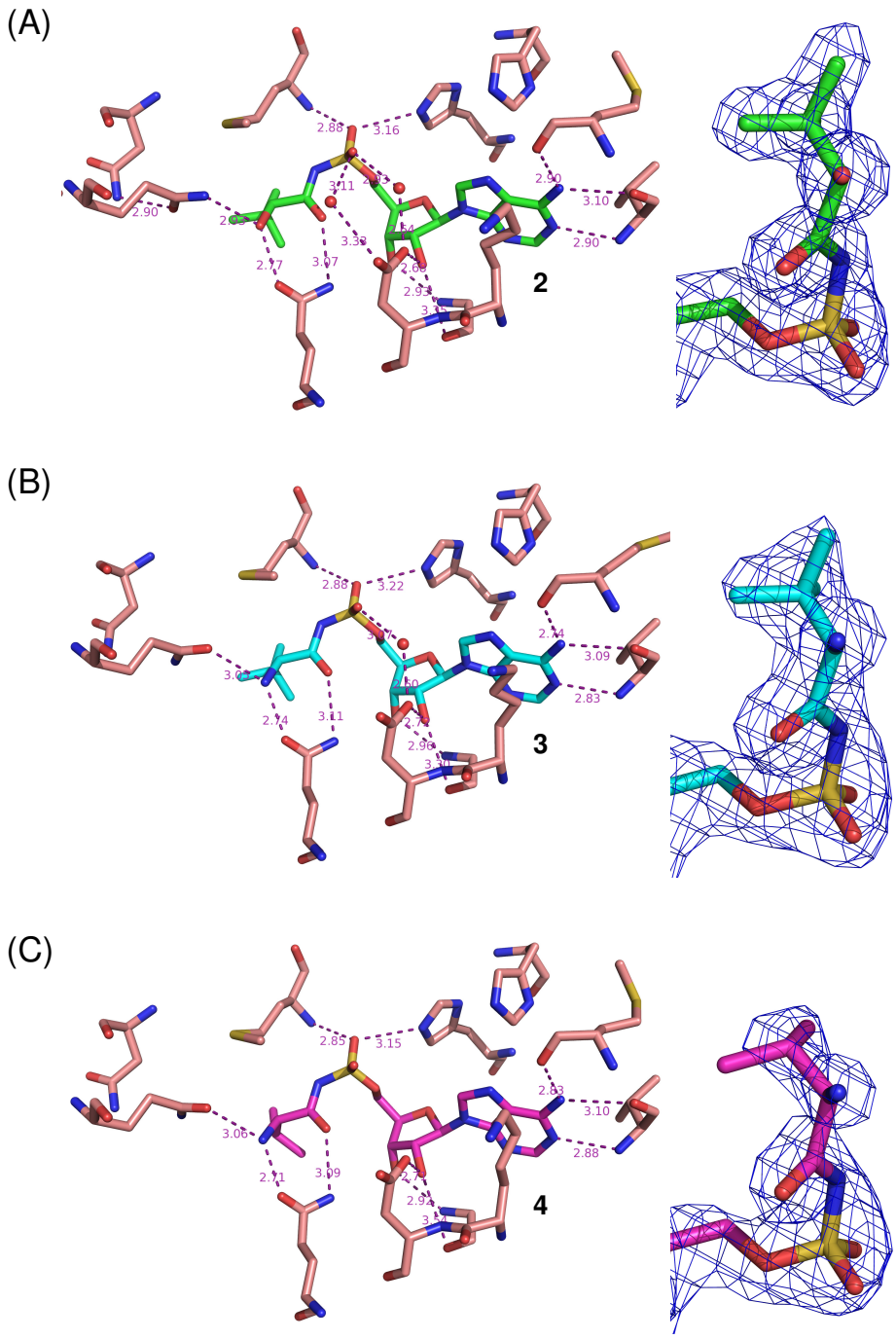

Figure 4, SI. Detailed binding interactions of the inhibitors **2** (A), **3** (B) and **4** (C) in the active site of one subunit of pantothenate synthetase. Inhibitors are shown as stick with green (**2**), cyan (**3**) or magenta (**4**) carbon atoms, nitrogen in blue, oxygen in red and sulphur in orange. Key protein residues are shown with pink carbons and key water molecules are shown as red spheres. Hydrogen bonds are indicated with dark purple dashed lines and H-bond distances are indicated. Omit difference electron density ($F_o - F_c$) superimposed around **2** (A), **3** (B) and **4** (C) are shown in blue and are contoured at 3σ (insert figures on the right). The densities unambiguously identify the absolute stereochemistry at the hydroxyl (**2**) and amine (**3** and **4**) group. The figures were generated and rendered using Pymol v. 0.99 (*7*).

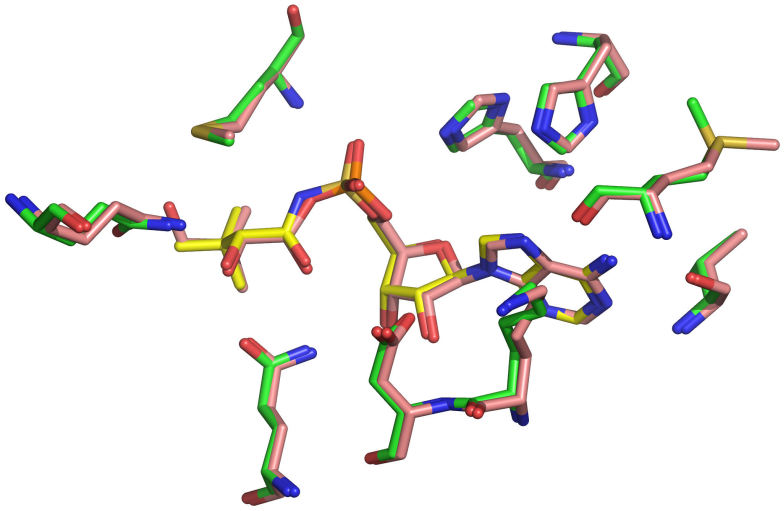

Figure 5, SI. Superposition of the crystal structures of *M. tuberculosis* pantothenate synthetase with bound pantoyl adenylate **1** [PDB code 1n2h (*1*)] and sulfamoyl analogue **2** (this work). Pantoyl adenylate and inhibitor **2** are shown as sticks with pink and yellow carbon atoms, respectively, nitrogen in blue, oxygen in red, phosphorus in dark orange and sulphur in orange. Key protein residues in the structures with pantoyl adenylate and **2** bound are shown with pink and green carbon atoms, respectively.