**Supplementary information for:**

# Mechanism of regulation of pantothenate biosynthesis by the PanD-PanZ.AcCoA complex reveals an additional mode of action for the antimetabolite N-pentyl pantothenamide (N5-Pan)

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**Contents**

**Figure S1** Overlay of structure of ADC(T57V)-PanZ complex (green and cyan) and ADC(WT)-PanZ complex (yellow and white).

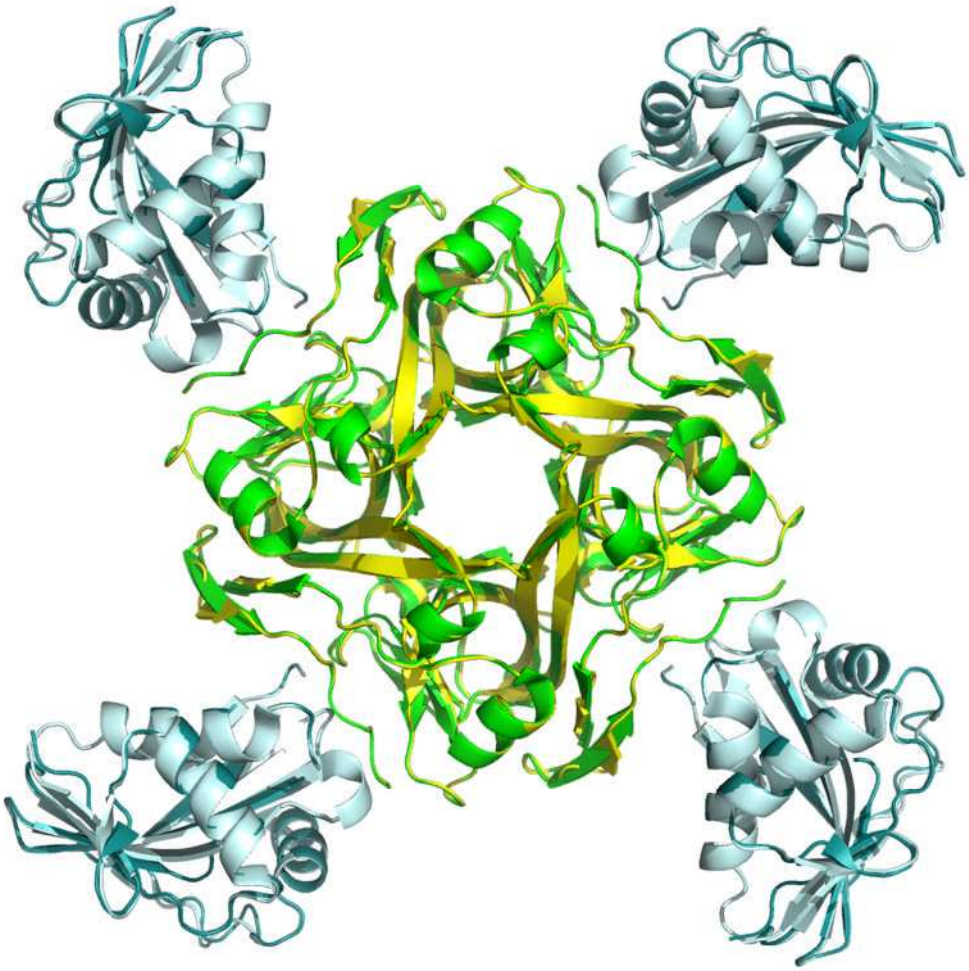


**Figure S2 a** *F*o-*F*c difference electron density (green mesh) contoured at 3 rmsd and 2*F*o-*F*c electron density (grey mesh) contoured at 1 rmsd showing the presence of additional density in the region of the pyruvoyl group (green mesh). **b** final electron density, contoured as in (a) with a ketone hydrate modeled.

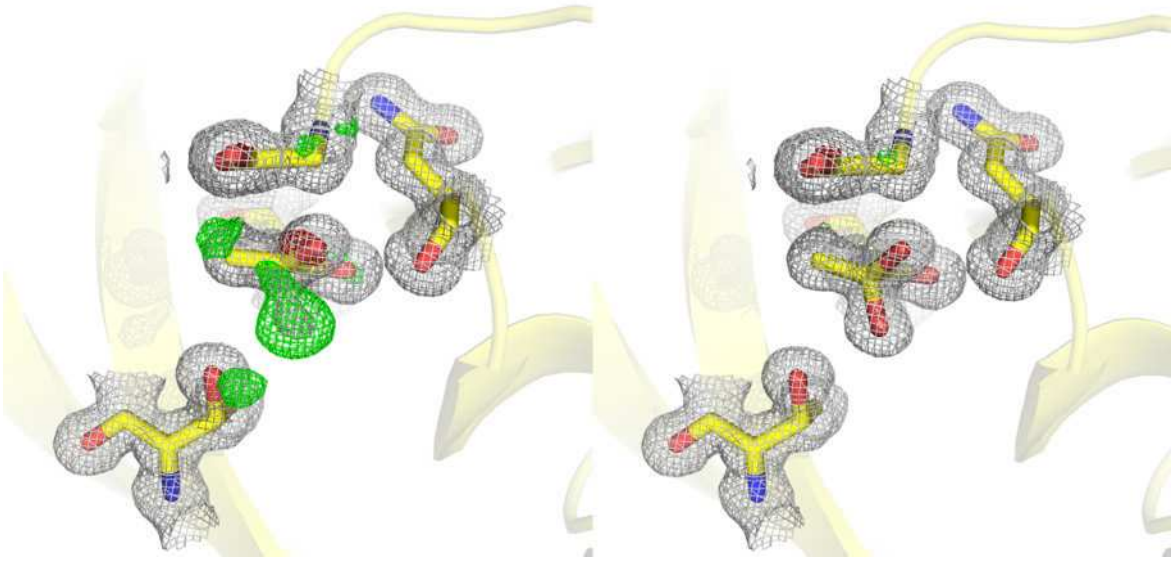


**Figure S3 Size exclusion chromatography analysis of complex formation by PanD and PanZ. a&b** SEC analysis of PanD (a black), PanD(K119A) (a red), PanZ (b black) and PanZ(R73A) (b red). All proteins elute as single species; analysis of peak fractions by SDS-page reveals that PanD electrophoreses as a tetramer if samples are not preboiled. **c** SEC analysis of WT PanD-PanZ complex. The complex elutes as a single species, Western blotting analysis confirms the presence of PanZ in the complex, trace activated PanD is visible in the boiled fractions. **d** SEC analysis of PanD(K119A) interaction with PanZ. PanZ chiefly elutes as separate species but a small proportion elutes in a complex with PanD(K119A). **e** SEC analysis of PanZ(R73A) interaction with PanD. The proteins elute individually.

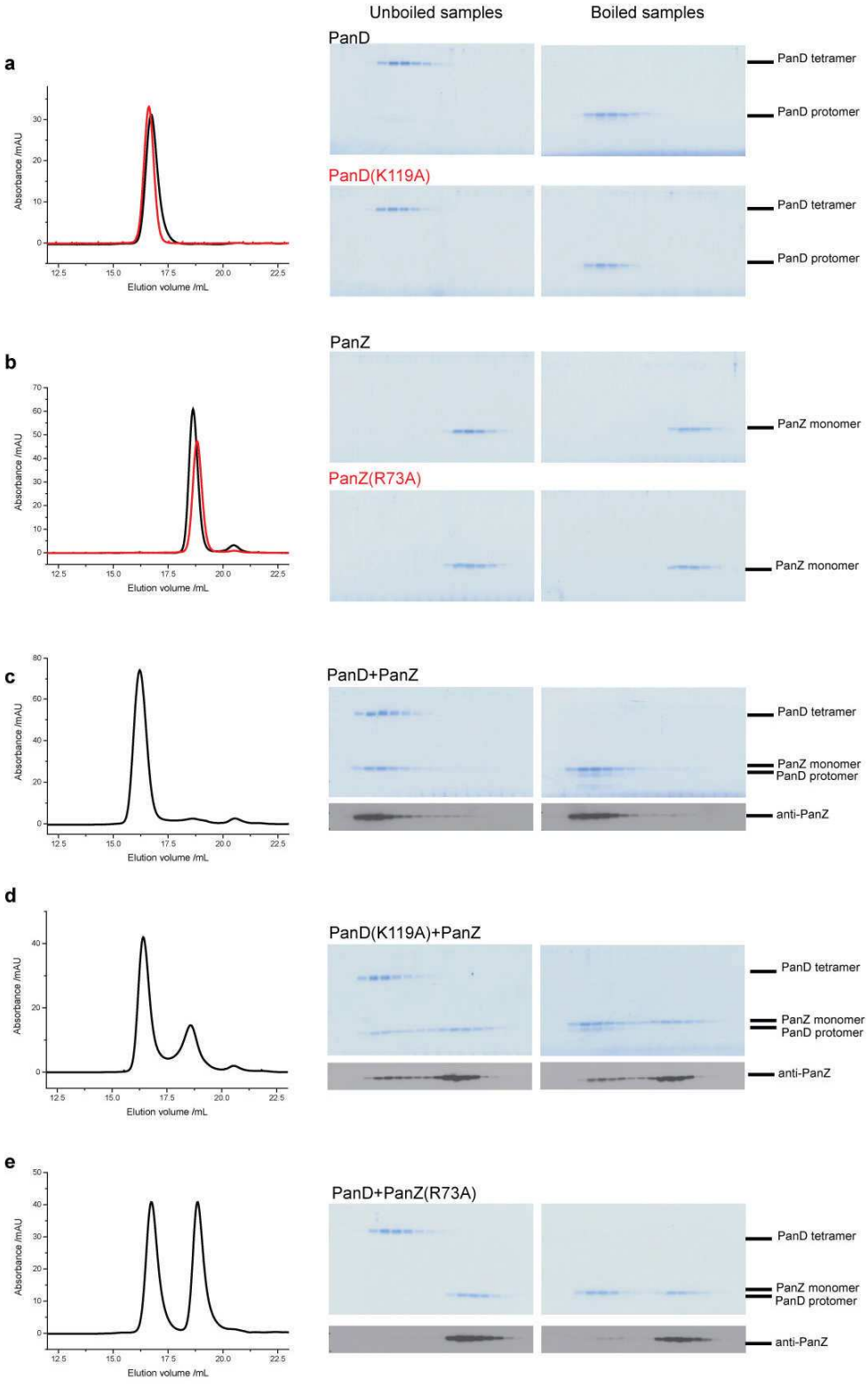


**Figure S4 Relationship of Arg73 to AcCoA binding site in PanZ.** Binding site for AcCoA in the PanD(WT)-PanZ.AcCoA crystal structure. Residue Arg73 forms a salt-bridge with residue Glu103 adjacent to the pantothenate moiety of AcCoA, potentially forming both van der Waals interactions and stabilizing the helix which forms the adenine binding pocket. 2*F*o-*F*c electron density contoured at 1 rmsd for AcCoA, Arg73 and Glu103.

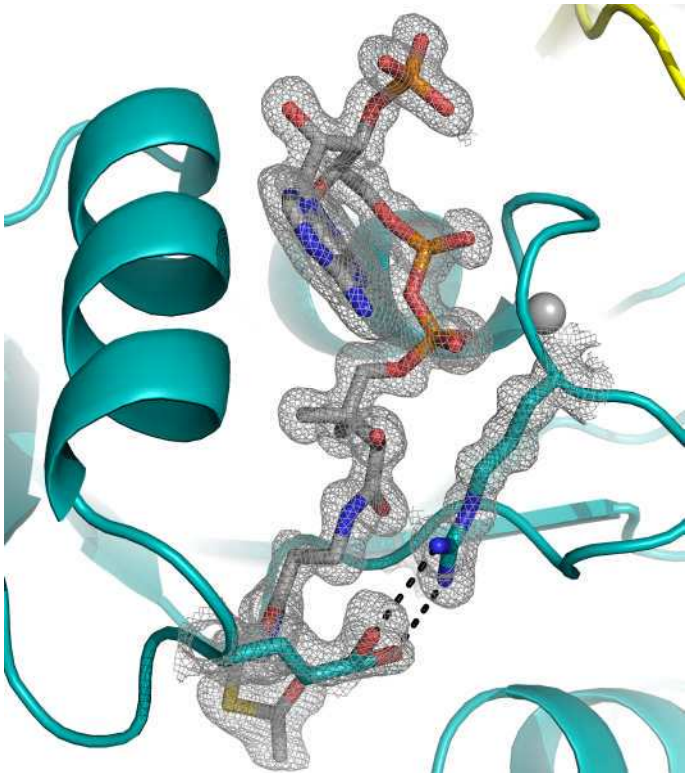


**Figure S5** Global fitting of EtdtCoA binding to PanZ by isothermal titration calorimetry. The sample of EtdtCoA contained trace impurities leading to a significant sloping heat of dilution (see red lines S5a). Four independent titrations were globally fitted in SEDPHAT to a single set of thermodynamic parameters but with independent baseline height and slope correction. Substoichiometric binding is due to copurification of the AcCoA with PanZ. **a** 85 µM EtdtCoA titrated into 10 µM PanZ, **b & c** 85 µM EtdtCoA titrated into 18 µM PanZ, **d** 85 µM EtdtCoA titrated into 20 µM PanZ

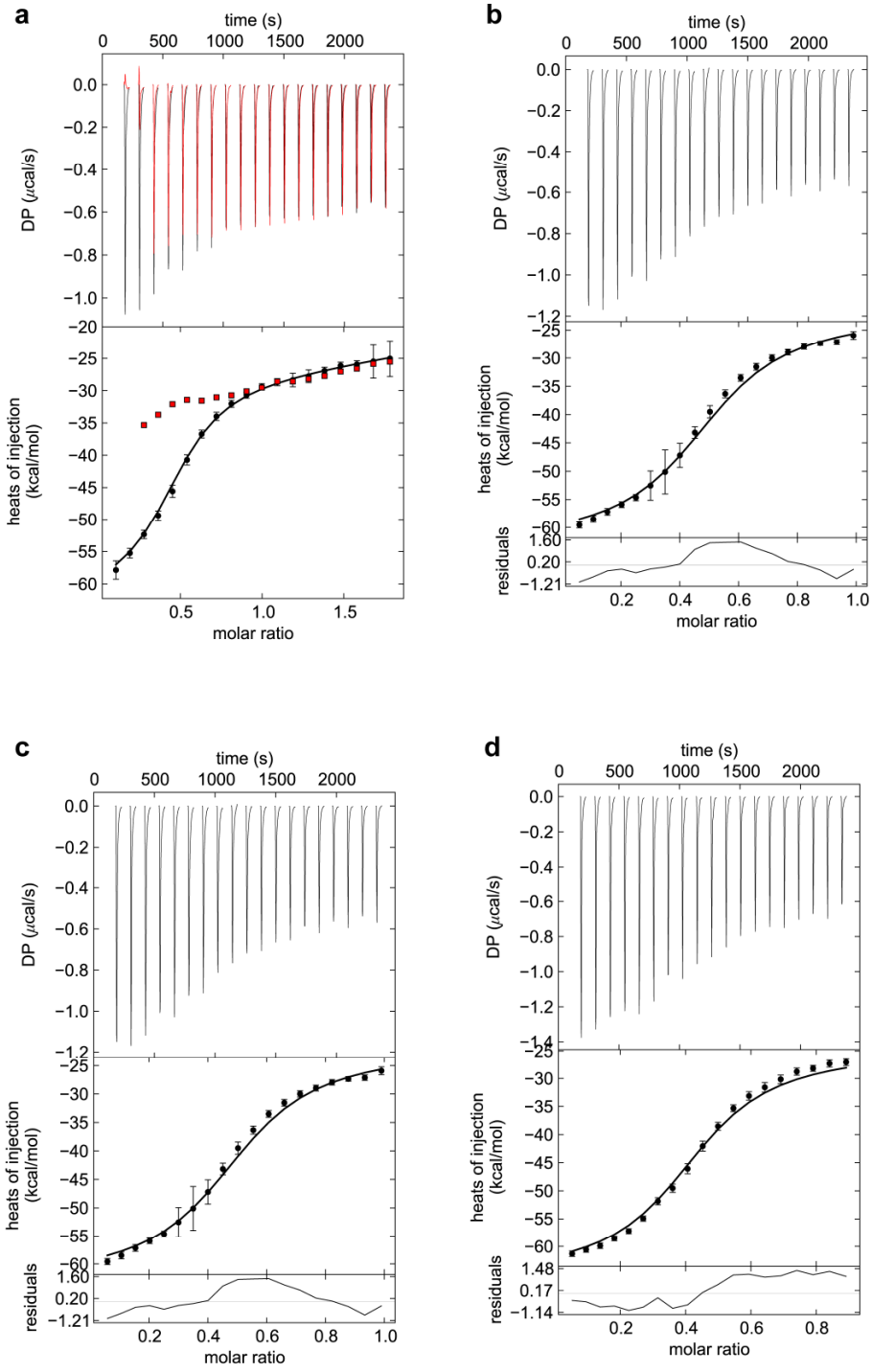


**Figure S6** Estimation of minimum inhibitory concentration for growth of *E. coli* MG1655 and strains in which the *panD* locus has been mutated determined by growth in M9 media for 20 h at 37 ºC. Pentyl pantothenamide inhibits growth of wild-type *E. coli* at 4 mg/mL (Black squares). Mutation of the *panD* locus to *panD*(K119A) which reduces the PanD-PanZ interaction affinity is insufficient to generate resistance (red circles) whereas incorporation of the *Bacillus subtilis* gene at the *panD* locus (SN218) is sufficient to generate resistance (blue triangles). Data are fitted to a Logistic model for growth inhibition with shared upper and lower limits.

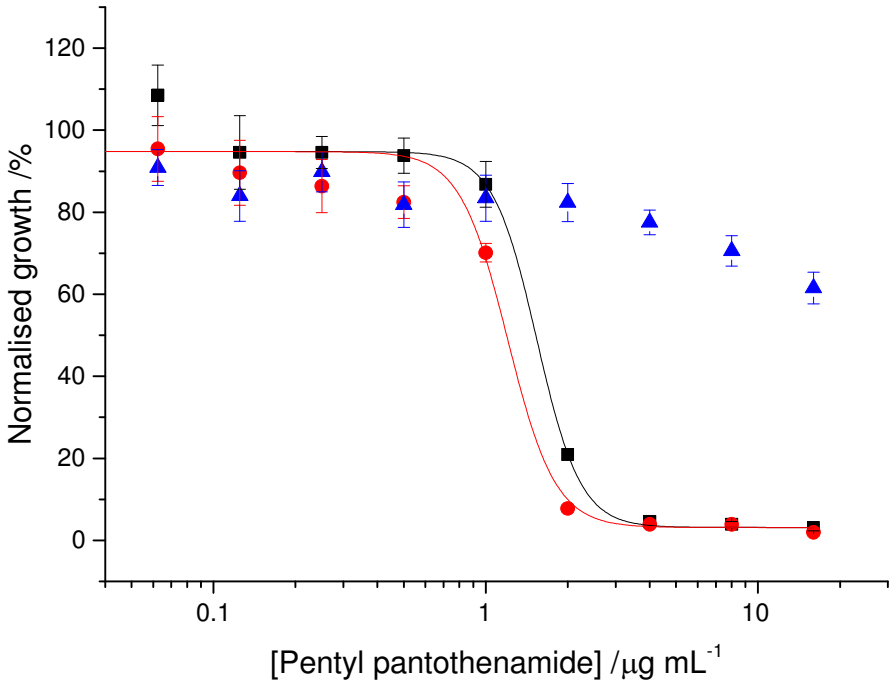
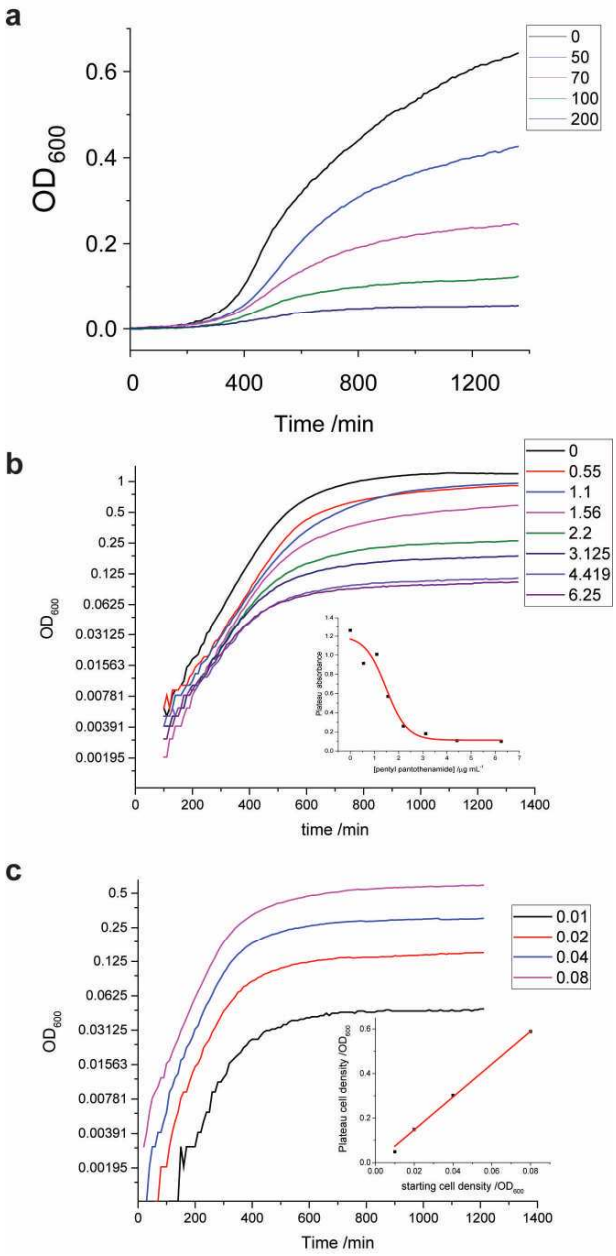


**Figure S7 Analysis of growth of *E. coli* MG1655 inhibited by pentyl pantothenamide. a β-Alanine supplementation inhibits growth of E. coli** Growth of MG1655 in the presence of increasing concentrations of pentyl pantothenamide and 0.5 mM β-alanine indicates that cells grow to a lower final density in the presence of β-alanine, suggesting that overproduction of pantothenate under these conditions is detrimental to optimal growth. **b Pentyl pantothenamide does not effect growth rate**. Growth of MG1655 in increasing concentrations of pentyl pantothenamide (data also shown in figure 3b).The exponential growth rate is broadly unaffected by addition of pentyl pantothenamide, only the plateau position is affected (inset). **c Final plateau growth density is a function of seeding density**. Growth of *E. coli* MG1655 in the presence of 10 μg $mL^{-1}$ pentyl pantothenamide at varied seeding densities indicates a linear relationship between seeding density and the final growth plateau.



***Additional experimental methods***

Table A: Primers used for construction of *panD* mutants.

| | |
|---|---|
| panDmutU | aaagtcgcgagccgtctccgcATGCATtaa (NsiI: ATGCAT) |
| panDmutL | atatCTTAAGaacggattcgctggagacga (AflII: CTTAAG) |
| panD(K14A)U | CGCGTGgccGTGACTCATGCGGACCTGCAC |
| panD(K14A)L | AGTCACggcCACGCGGTGGAGTTTGCCCTG |
| panD(K53A)U | AACGGCgccCGTTTCTCCACTTATGCCATC |
| panD(K53A)L | GAAACGggcGCCGTTGGTGACATTCCAGAT |
| panD(K115A)U | GAAATGgccCGTACCGCGAAAGCGATTCCG |
| panD(K115A)L | GGTACGggcCATTTCATTGTCGCCTTCAAA |
| panD(K119A)U | ACCGCGgccGCGATTCCGGTACAGGTTGCT |
| panD(K119A)L | AATCGCggcCGCGGTACGTTTCATTTCATT |

**Construction of pBAD24*panZ*(R73A)**

Table B: Primers used for construction of mutant PanZ expressing plasmids.

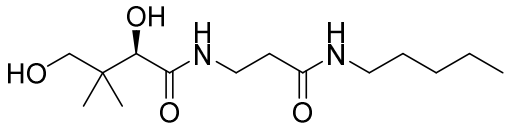
| | |
|---|---|
| panZBADU40 (EcoRI: GAATTC) | acacGAATTCcccATGAAGCTGACCATCATTCGATTAGAA |
| panZL3 | atatGCATGCcggcctacaagatccaaaga (SphI: GCATGC) |
| panZ(R43A)U | GCCGCGgcTTTTAACGAGCGCCTGCTCGCT |
| panZ(R43A)L | GTTAAAAgcCGCGGCGTAGATACGGTGGTT |
| panZ(N45A)U | CGTTTTgcCGAGCGCCTGCTCGCTGCCGTG |
| panZ(N45A)L | GCGCTCGgcAAAACGCGCGGCGTAGATACG |
| panZ(T72A)U | GAAGTCgCCCGCCGTCGCGGTGTGGGGCAA |
| panZ(T72A)L | ACGGCGGGcGACTTCCCGCACGCGCAGGGA |
| panZ(R73A)U | GTCACCgcCCGTCGCGGTGTGGGGCAATAT |
| panZ(R73A)L | GCGACGGgcGGTGACTTCCCGCACGCGCAG |

**Size-exclusion chromatography analysis**

Size-exclusion chromatography analysis was carried out as described previously. Following purification, proteins were analysed using an Akta Prime Plus liquid chromatography system at a flow rate of 0.2 mL/min of 50 mM Hepes/KOH, 150 mM KCl, pH 7.6. For protein-interaction analysis 60 μM PanD and 15 μM PanZ were mixed and incubated at 4 ºC for 60 min prior to analysis. Western blotting against PanZ was carried out using a rabbit polyclonal antibody to PanZ produced under contract by Biogate Co., Japan.

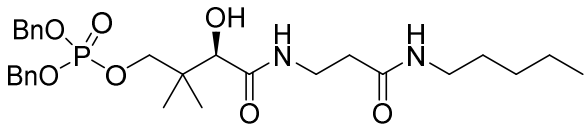
**Synthesis of small molecules**

**N-pentyl pantothenamide**



Calcium pantothenate (2.0 g, 8.3 mmol) was dissolved in $H_2O$ (~20 mL) and passed through an Amberlite IR-120 ($H^+$) column. This was washed with 2 × 20 mL $H_2O$ and eluants were combined and lyophilised overnight to leave pantothenic acid as a colourless oil. The oil was dissolved in DMF (10 mL) and amylamine (1.16 mL, 10 mmol) and diphenylphosphine azide (2.24 mL, 10 mmol) were added. The mixture was cooled to 0 °C and triethylamine (1.39 mL, 1.2 mmol) was added followed by stirring for 5 hours. The DMF was removed under reduced pressure to leave a white oil which was purified by flash chromatography three times (silica; 5% *v/v* methanol-$CH_2Cl_2$ and gradient elution 1-10% *v/v* methanol-$CH_2Cl_2$) due to co-elution in the presence of traces of DMF. Fractions were combined and evaporated under reduced pressure to yield a colourless oil which was lyophilised overnight to leave a colourless solid on standing (0.84 g, 35%). TLC ($CH_2Cl_2$/MeOH/AcOH 94:5:1 (*v/v*)): $R_f$: 0.19; $^1$H NMR (500 MHz, $CDCl_3$): δ 7.45 (1H, t (br), *J*= 6.3 Hz, COHCON*H*), 6.09 (1H, t (br), *J*= 5.4 Hz, N*H*), 4.26 (1H, d, *J*= 5.5 Hz, HC-O*H*), 3.99 (1H, d, *J*= 5.3 Hz, $CH_2O$*H*), 3.73 (1H, t, *J*= 5.6 Hz, *H*C-OH ), 3.56 (2H, m, C*H*$_2$OH), 3.49 (2H, d, *J*= 5.5 Hz, C*H*$_2$CH$_2$CO), 3.21 (2H, q, *J*= 7.2, CH$_2$C*H*$_2$CO), 2.43 (2H, t, *J*= 6.0 Hz, NHC*H*$_2$-C$_4$H$_9$), 1.49 (2H, m, C*H*$_2$-C$_3$H$_7$), 1.30 (4H, m, C$_2$*H*$_4$-CH$_3$), 1.00 (3H, s, CH$_2$C*H*$_3$CH$_3$), 0.93 (3H, s, CH$_2$CH$_3$C*H*$_3$), 0.90 (3H, t, *J*= 6.7, CH$_2$C*H*$_3$); $^{13}$C NMR (125 MHz, $CDCl_3$): δ 197.8, 173.4, 171.2, 77.7, 70.9, 39.7, 35.8, 35.4, 29.1, 29.0, 22.2, 21.4, 20.4, 13.8; IR $\nu_{max}$/cm$^{-1}$: 3280 (N-H), 3000-2800 (OH, broad), 1642 (C=O); HRMS (*m/z*): $[M+Na]^+$ calcd for $C_{25}H_{30}NO_7Na$, 311.1941; found, 311.1945.

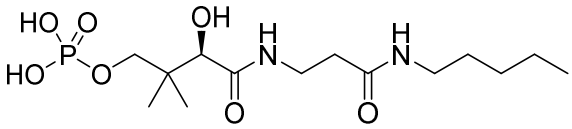
**N-pentylpantothenamide-4'-O-dibenzylphosphate**



N-pentylpantothenamide (300 mg, 1.04 mmol) was dissolved in dry acetonitrile (12 mL) under $N_2$. A tetrazole solution in acetonitrile (0.45 M, 2.31 mL, 1.04 mmol) and dibenzyl-N,N-diisopropyl-phosphoramidite (0.35 mL, 1.04 mmol) were added and the reaction mixture stirred for 5 min at room temperature. mCPBA (269.2 mg, 1.56 mmol) was added and the

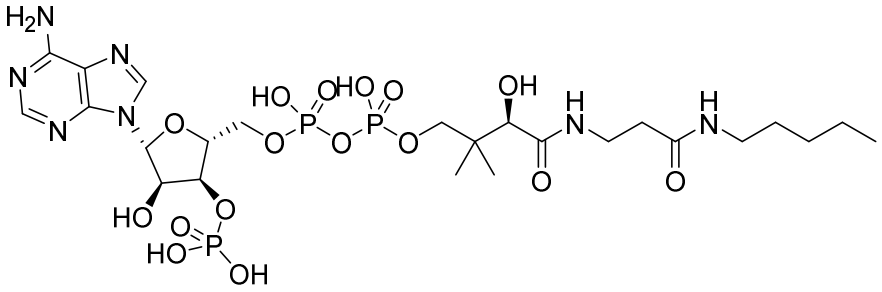
solution stirred for 2 h at room temperature. The product was concentrated *in vacuo*, extracted with ethyl acetate (3 × 20 mL), and the combined organic extracts washed with 1M $H_2SO_4$ (2 × 5 mL) and 1M $NaHCO_3$ (2 × 5 mL) before drying ($MgSO_4$) and concentration *in vacuo*. The product was purified by flash column chromatography ($MeOH/CH_2Cl_2$ 7:93) to yield N-pentylpantothenamide-4'-O-dibenzylphosphate as a colourless oil (415.3 mg, 72.7%) $^1H$ NMR (500 MHz, $CDCl_3$): δ 7.57 (1H, t(br), J = 6.2 Hz, NH), 7.51 (1H, s(br), OH), 7.49 (1H, s(br), OH), 7.42-7.24 (10H, m, $H_{15}$, $H_{16}$, $H_{17}$), 6.87 (1H, t(br), J = 5.7 Hz, NH), 5.16-4.98 (4H, m, $H_{14}$), 4.04 (1H, dd, J = 9.9, 6.1 Hz, $H_1$), 3.97 (1H, s, $H_4$), 3.72 (1H, dd, J = 9.9, 6.1 Hz, $H_1$) 3.57 (2H, q, J = 6.2 Hz, $H_6$), 3.2 (2H, q, J = 6.5 Hz, $H_7$), 2.56-2.53 (2H, m, $H_9$), 1.48 (2H, p, J = 7.3 Hz, $H_{10}$), 1.33-1.22 (4H, m, $H_{11}$, $H_{12}$), 1.05 (3H, s, $H_3$), 0.91-0.83 (6H, m, $H_3$, $H_{13}$); IR ($V_{max}/cm^{-1}$): 3300 (N-H), 3095-2873 (OH, broad), 1638 (C=O);

**N-pentylpantothenamide-4'-O-phosphate**




N-pentylpantothenamide-4'-O-dibenzylphosphate (415.3 mg, 0.75 mmol) was dissolved in 9:1 $CH_3OH/H_2O$ (20 mL) and stirred under $N_2$. Pd/C (10% (*wt/wt*), 41.5 mg) was added and the suspension was maintained under a hydrogen atmosphere (1 atm, balloon) at room temperature with stirring for 6 h. A second portion of Pd/C (10% (*wt/wt*), 41.5 mg) was added and the before stirring under $H_2$ for a further 2h. The solution was filtered through celite and concentrated *in vacuo*. The product was extracted with water (3 × 20 mL) and lyophilised to yield the title compound as an orange powder (47.69 mg, 17.1 %) $^1H$ NMR (500 MHz, $D_2O$): δ 3.95 (1H, s, $H_4$), 3.78 (1H, dd, J = 9.7, 4.8 Hz, $H_1$), 3.59 (1H, dd, J = 9.7, 4.8 Hz, $H_1$) 3.43 (2H, m, $H_6$), 3.07 (2H, m, $H_7$), 2.4 (2H, t, J = 6.4 Hz, $H_9$), 1.41 (2H, p, J = 7.0 Hz, $H_{10}$), 1.21 (4H, m, $H_{11}$, $H_{12}$), 0.9 (3H, s, $H_3$), 0.83 (3H, s, $H_3$), 0.79 (3H, t, J = 6.8 Hz, $H_{13}$); $^{13}C$ NMR (100 MHz, $D_2O$): δ 174.79, 173.60, 133.80, 129.61, 128.70, 74.40, 71.59, 39.51, 38.33, 28.33, 27.90, 21.68, 20.58, 18.60, 13.24; $^{31}P$ NMR (400 MHz, $D_2O$): δ 0.12; IR ($V_{max}/cm^{-1}$): 3310 (N-H), 3097-2871 (OH, broad), 1637 (C=O);

**Ethyldethiacoenzyme A**



N-pentyl 4-dibenzylphosphopantothenamide (10.5 mM) was added to a mixture of both CoaD (9 μM) and CoaE (11 μM) in 50 mM Tris-HCl buffer (pH 7.6) in the presence of $MgCl_2$ (5 mM) and ATP (50 mM) (Final volume 900 μL) before incubation at 37 °C for 2-4 hours Production of EtdtCoA was analysed by LCMS. CoaD and CoaE were precipitated by heating at 95 °C for 15 min and removed by centrifugation. The supernatant was purified by reverse phase HPLC ($C_{18}$-silica) using a 5-95% 50 mM $KH_2PO_4$ (pH 5.3)/MeOH gradient to remove separation of the ATP/AMP from the EtdtCoA. The MeOH was removed *in vacuo* and the samples adjusted to pH 7.6 using 5 M NaOH before being lyophilized. The product was resuspended in a minimum volume of $H_2O$ and desalted using a Sephadex G-10 column to yield the title compound (1.54 mg, 21%). EtdtCoA was quantified by UV absorption at 257 nm. ($\varepsilon_{257}$ = 16840 $M^{-1}$ $cm^{-1}$). $^1H$ NMR (750 MHz, $D_2O$) 8.45 (1H, s, purine-2H), 8.17 (1H, s, purine-8H), 6.07 (1H,d 6.9, ribose-1'H), 4.72 (1H, br s, ribose-4'H), 4.47 (1H br s, ribose-2'H), 4.13 (2H, br s, ribose-5'H), 3.90 (1H, s, ribose-4'H), 3.71 (1H, dd, J = 9.5,4.9 Hz, $C(CH_3)_2.CH_2OP$), *3.68 (0.6H, ddd, glycerol)*, 3.63 (1H, s, $C(CH_3)_2CH(OH)CO$), *3.55 (1H, dd, glycerol)*, *3.46 (1H, dd, glycerol)*, 3.43 (1H, dd 9.5,4.9, $-C(CH_3)_2.CH_2OP$), 3.37 (1H, dt, J= 14, 6.7 Hz, $NHCH_2CH_2CO$), 3.33 (1H, dt, J= 14, 6.7 Hz, $NHCH_2CH_2CO$), 2.98 (2H, dt J= 6.9, 3.0 Hz, $NHCH_2CH_2CH_2$), 2.33 (2H, t, J= 6.6 Hz, $NHCH_2CH_2CO$), 1.31 (2H, p, J = 7.0 Hz, $NHCH_2CH_2CH_2$) 1.08 (4H, m, $CH_2CH_2CH_3$), 0.76 (3H, s, $C(CH_3)_2$), 0.72 (3H, t, J = 6.9 Hz, $CH_2CH_2CH_3$), 0.62 (3H, s, $C(CH_3)_2$); HRMS (*m/z*): $[MH]^+$ 778.1961 ($C_{24}H_{43}N_7O_{16}P_3$ requires 778.1979), $[MNa]^+$ 800.1782 ($C_{24}H_{42}NaN_7O_{16}P_3$ requires 800.1799), $[M-H+2Na]^+$ 822.1601 ($C_{24}H_{41}Na_2N_7O_{16}P_3$ requires 822.1618), $[M-2H+3Na]^+$ 844.1425 ($C_{24}H_{40}Na_3N_7O_{16}P_3$ requires 844.1437).

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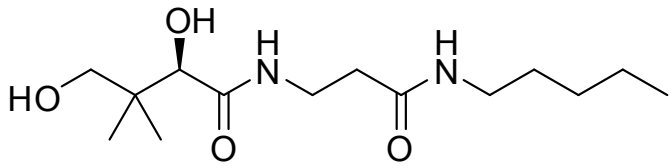

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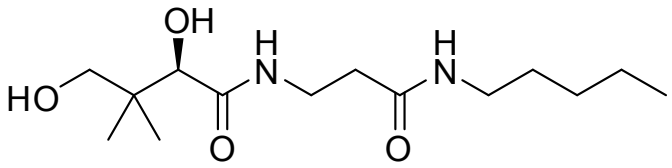

# School of Chemistry Mass Spectrometry Service

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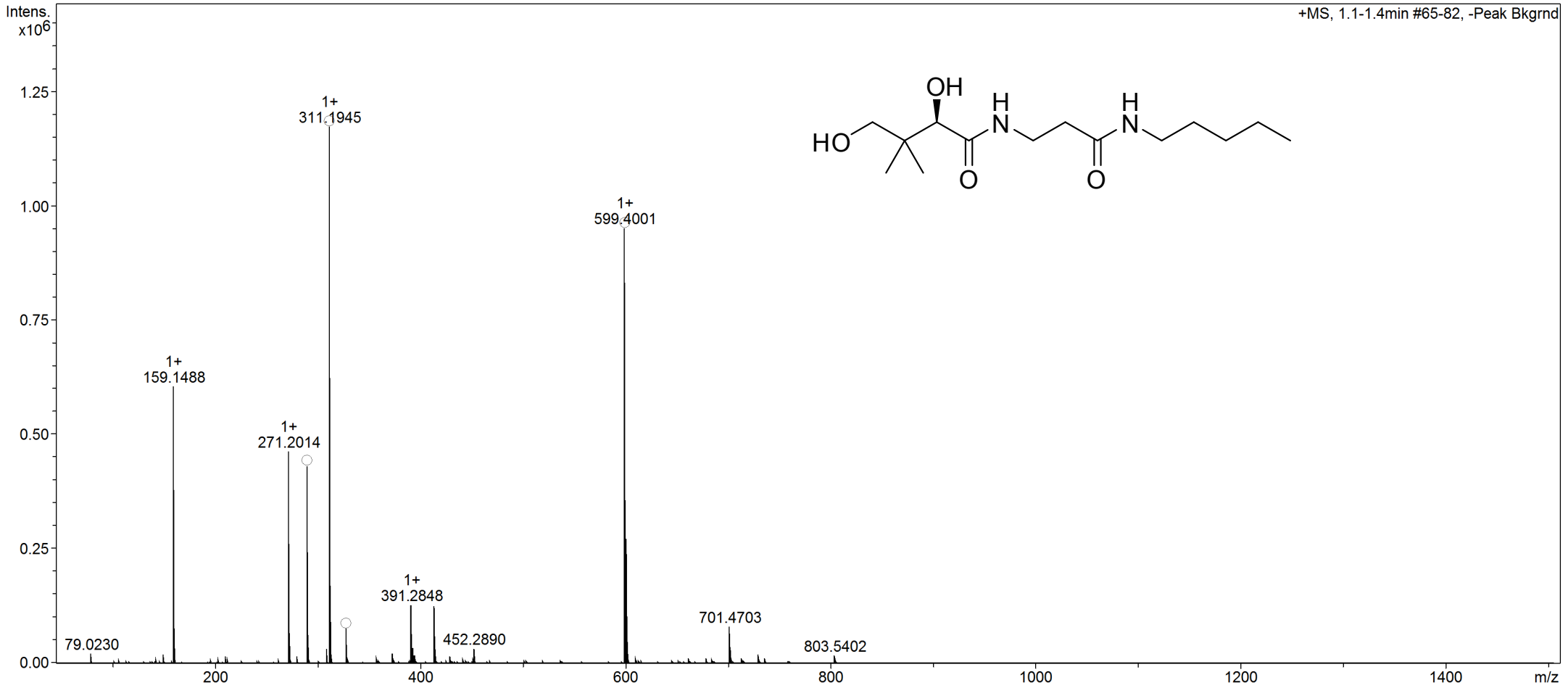

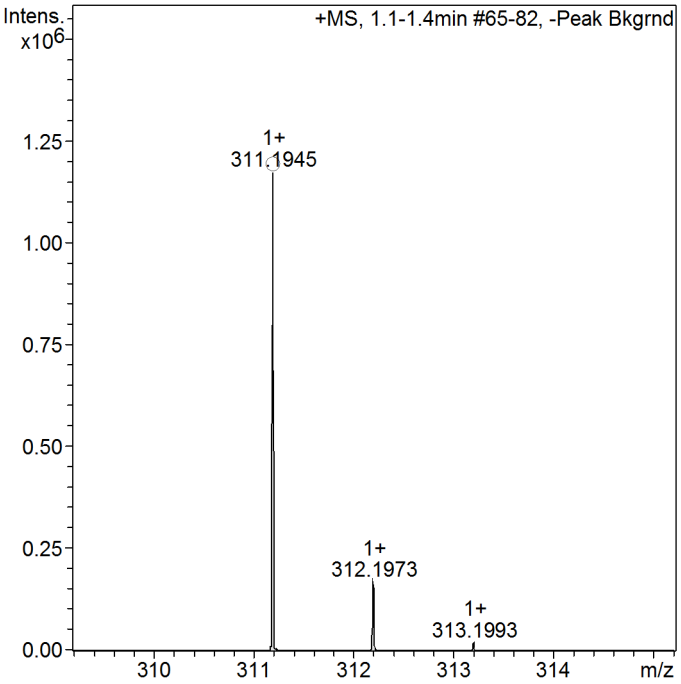


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| CHNO and adducts considered implicitly | |
| Formula Search Minimum | |
| Formula Search Maximum | |
| Algorithm Parameters | |
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| Electron Configuration | even |
| Estimate No of Carbons | yes |
| Filter by H/C Ratio | 0< H/C < 3 |
| Number of Double Bonds & Rings | 0< rings&DB < 80 |

## Confirm/Find Formula Results

The section below shows the results of formula calculation. If an expected formula was provided and found these are the results that are listed. If no formula was provided or no matches were found the system has attempted to determine the formula constrained by the parameters listed to the left

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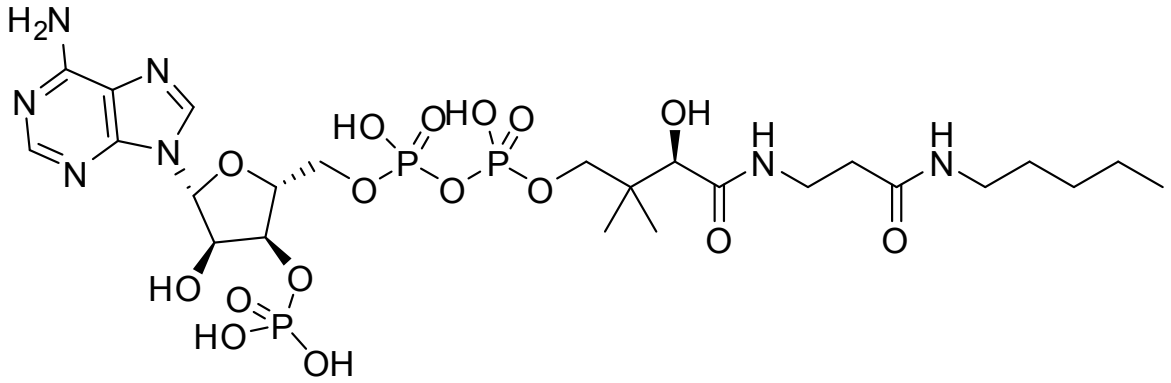
| 311.194541 | C14H28N2NaO4 | 1+ | 311.194128 | 0.4 | 1.3 | 11.9 | 100.00 | | M+Na |
| 327.167961 | C14H28KN2O4 | 1+ | 327.168065 | 0.1 | 0.3 | 5.9 | 100.00 | | M+K |
| 599.400086 | C28H56N4NaO8 | 1+ | 599.399035 | -1.1 | -1.8 | 21.2 | 100.00 | | 2M+Na |

qgxD7ig6TtiK$p5kFJZIRQ.3.fid
1D_Test



# School of Chemistry Mass Spectrometry Service

| | | | | | |
|---|---|---|---|---|---|
| **SampleID** | 20170731 sample1 | | | **Submitter** | Zoe Arnott |
| **Sample Description** | | | | | |
| **Analysis Name** | D:\Data\srisridharan\cm10zlpa\20170731sample1_211147_RE3_01_35420.d | | | **Supervisor** | Sri Sridharan |
| **Method** | 3a_AccMass_Loop_Positive.m | | | **Acquisition Date** | 31/07/2017 13:32:30 |
| **Instrument** | maXis impact | **Source Type** | ESI | **Ion Polarity** | Positive |
| **Scan Begin** | 50 m/z | **Scan End** | 1500 m/z | | |