Supporting Information

An improved synthesis of biotinol-5'-AMP: Implications for antibacterial discovery

1. Biological analysis 1

1.1 Recombinant protein production 1

1.1 *In vitro* biotinylation assay 2

1.2 *In vitro* antimicrobial assays 2

1.3 Drug susceptibility testing of mycobacterial species 2

1.4 Assay of cell culture cytotoxicity 3

2. Chemical Synthesis 3

2.1 General methods 3

2.2 Synthesis of Biotinol-5′-AMP *via* method 1 (see Scheme 1) 3

2.3 Synthesis of Biotinol-5′-AMP *via* method 2 (see scheme 2) 5

3. References 8

1. Biological analysis

1.1 Recombinant protein production

The cloning and purification of recombinant BPL from *Escherichia coli*, *Staphylococcus aureus* and *Homo sapiens* have been described. [1-3]. Cloning and purification of BPL from *Klebsiella pneumonia* and *Acinetobacter calcoaceticus* is described in [4]. The gene encoding BPL from *Mycobacterium tuberculosis* H37Rv was purchased from GENEART® Gene Synthesis (LifeTechnologies) and subcloned in pET16b protein expression vector for recombinant expression in *E. coli* BL21 (DE3). A hexahistidine-tag and TEV cleavage site was engineered into the construct to facilitate purification by immobilized metal ion affinity chromatography, as described in [5]. Material eluting from the IMAC column was detected by UV absorbance was pooled and subsequently exchanged into Buffer A (25mM Tris pH 8.5, 0.1mM EDTA, 5% glycerol, 1mM DTT) using a 50 ml desalting cartridge. The sample was further purified with a 20 ml Q-Sepharose column pre-equilibrated with Buffer A. The protein was fractionated with a gradient of 0 to 400 mM NaCl using Buffer B (25 mM Tris pH 8.5, 0.1 mM EDTA pH 8.0, 1 mM DTT, 5% glycerol, 400 mM NaCl).

1.1 *In vitro* biotinylation assay

Quantitation of BPL catalysed ^{3}H-biotin incorporation into a biotin domain substrate was performed as previously described.[5-6] Briefly, the reaction mixture contained 50 mM Tris-HCl pH 8.0, 3 mM ATP, 4.5 µM biotin, 0.5 µM, ^{3}H-biotin, 5.5 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA and 10 µM of the 103 amino acid biotin domain of *S. aureus* pyruvate carboxylase. The reaction was the initiated with the addition of enzyme. The final concentration of BPL employed in the individual reactions were as follows: *Escherichia coli* at 2.85 nM, *Staphylococcus aureus* at 6 nM*; Klebsiella pneumonia* at 100 nM, *Acinetobacter calcoaceticus* at 8.75 nM; *Mycobacterium tuberculosis* at 20 nM and *Homo sapiens* at 9.2 nM. The reaction was performed for 10 minutes at 37 °C, during which time the reaction was linear at saturating substrate concentrations. The reaction was then terminated by alliquoting 4 µL of the reaction mixture onto Whatman filter paper pre-treated with biotin and trichloroacetic acid. The filters were washed twice with 10 % (v/v) ice-cold tricholoracetic acid and once with 100 % ethanol before air-drying. Quantitation of ^{3}H-biotin labeled BCCP was performed using a liquid scintillation counter.

The K_i value of each compound was determined from a dose-response curve by varying the concentration of the inhibitor under the same enzyme concentration. Data were analysed with GraphPad Prism software using the Morrison equation for a tight binding inhibitor [7], where K_M is the affinity of the competitive substrate for the enzyme (ie biotin, see Table 1) and [S] is the concentration of biotin in the assay (ie 5 µM). The mode of inhibition was investigated by varying the concentrations of inhibitor alongside varying the concentrations of ^{3H}-biotin. The data was plotted as double reciprocal plots and assessed using Lineweaver-Burk analysis. Data reported here are the means of three independent assays (n = 3).

1.2 *In vitro* antimicrobial assays

Antimicrobial activity of the compounds was determined by a microdilution broth method as recommended by the CLSI (Clinical and Laboratory Standards Institute, Document M07-A8, 2009, Wayne, Pa.) with cation-adjusted Mueller-Hinton broth (Trek Diagnostics Systems, U.K.). Biotinol-5′-AMP **2** was prepared in water before two-fold serial dilutions in the growth media. Trays were inoculated with 5 x 10^4 CFU of each strain in a volume of 100 µL and incubated at 35 °C for 16- 20 hours. Growth of the bacterium was quantitated by measuring the absorbance at 620 nm. The minimal inhibitory concentration (MIC) was obtained from experiments performed in triplicate.

1.3 Drug susceptibility testing of mycobacterial species

A selection of *Mtb* strains was tested for anti-TB activity using the Mycobacteria Growth Indicator Tube (MGIT) and BACTEC™ 960 TB automated incubation and reading technology (BD, Sparks, Md, USA). Growth of *Mtb* is monitored using an oxygen-quenched fluorochrome embedded in silicone at the bottom of the MGIT. During growth of microorganisms, free oxygen is utilized; upon depletion of the free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light. For the present study, Biotinol-5′-AMP **2** was diluted in water and added to the MGIT at final concentrations varying from 5.0 to 0.0625 ug/ml. Assays were preformed in triplicate to determine the MIC. The Mycobacterium Reference Laboratory is a member of the WHO Supranational Reference Laboratory network, and participates in

external quality control programmes for drug susceptibility testing through WHO, and the Australian Mycobacterium Reference Laboratory Network.

1.4 Assay of cell culture cytotoxicity

HepG2 cells were suspended in Dulbecco-modified Eagle's medium containing 10% fetal bovine serum and then seeded in 96-well tissue culture plates at either 5,000, 10,000, or 20,000 cells per well. After 24 h, cells were treated with varying concentrations of Biotinol-5′-AMP **2**. After treatment for 24 or 48 h, WST-1 cell proliferation reagent (Roche Applied Science) was added to each well and incubated for 0.5 h at 37 °C. The WST-1 assay quantitatively monitors the metabolic activity of cells by measuring the hydrolysis of the WST-1 reagent, the products of which are detectable at absorbance 450 nm.

2. Chemical Synthesis

2.1 General methods

All reagents and solvents were obtained from standard commercial sources. Reactions were monitored by TLC using precoated plates (silica gel 60 F_{254}, 250 µm, Merck, Darmstadt, Germany), spots were visualised under ultraviolet light at 254 nm and with basic potassium permanganate. Column chromatography was performed with silica gel (40-63 µm 60 Å), Davisil, Grace, Germany.

^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 (300 MHz), Varian Inova 600 MHz or Varian 500 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to the residue signals ($CDCl_3$ was 7.26 ppm for ^{1}H and 77.23 ppm for ^{13}C and D_2O was 4.79 for ^{1}H) and 85% phosphoric acid external standard with 0 ppm for ^{31}P. Low resolution mass spectra (LRMS) were recorded on Finnigan MAT LCQ. High resolution mass spectra (HRMS) were recorded on Agilent 6500 QTOF.

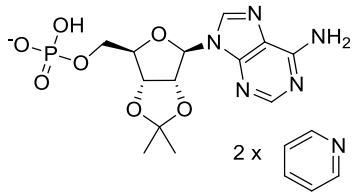
2.2 Synthesis of Biotinol-5′-AMP *via* method 1 (see Scheme 1)

Triethylamine bicarbonate buffer solution (TEAB)

Triethylamine bicarbonate buffer was prepared as follows: triethylamine (278 ml, 202 g, 2 mol) was added portion-wise to de-ionised water (500 mL). To the stirring solution was added portion-wise dry ice to give a pH>8. The buffer was diluted with water (500 mL), sealed and stored at 4 ^{o}C in a fridge. The triethylamine bicarbonate buffer was diluted with de-ionised water to the specified concentrations, dry ice was added to adjust to

approximately pH 7 and the buffer was degassed *in vacuo* and used as eluting solvent for Sephadex DEAE A25 ion exchange purification.

[(3a*R*,4*R*,6a*R*)-4-(6-aminopurin-9-yl)-2,2-dimethyl-3*a*,4,6,6a-tetrahydrofuro[3,4-*d*][1,3]dioxol-6-yl]methyl hydrogen phosphate (6)



To an ice cooled solution of adenosine (600 mg, 1.96 mmol) and water (20 µL) in triethyl phosphate (10 mL) was added drop-wise a solution of phosphorous oxychloride (324 mg, 2.14 mmol) in triethyl phosphate (10 mL). The reaction mixture was stirred for 2 h under nitrogen atmosphere and then sealed and placed in a 4 ^{o}C fridge overnight. The reaction mixture was poured into 2 M triethylamine bicarbonate buffer solution (50 mL) and stirred for 20 min. Additional triethylamine bicarbonate buffer solution was added to give a pH>7 and the solution was diluted with de-ionised water to give a TEAB concentration of 0.05 M (total volume 3 L). The solution was purified by Sephadex A25 DEAE column using a step-wise gradient of 0.05 to 0.5 M TEAB solution. Fractions containing the target compound, as determined by TLC, were pooled and lipolyzed to give the triethylamine salt of **5** as a clear oil. The triethylamine salt of **5** was dissolved in water, filtered through a Dowex 50 x 8 pyridium column and washed with de-ionised water (100 ml). Fractions containing UV active spots, as judge by TLC, were pooled and concentrated *in vacuo* to give a light yellow oil (1.27 g, 57%).

^{1}H NMR (300 MHz, D_2O): δ 8.47 (4H, d, *J* = 5.1 H), 8.12-8.16 (3H, m), 7.90 (1H, s), 7.62-7.67 (4H, m), 5.97 (1H, d, *J* = 3 Hz), 5.16 (1H, dd, *J* = 3.3, 6 Hz), 5.00 (1H, dd, *J* = 2.1, 6 Hz), 4.44-4.49 (1H, m), 3.90-3.93 (2H, m), 1.50 (3H, s), 1.28 (3H, s); ^{13}C NMR (75 MHz, D_2O): 150.6, 148.2, 147.3, 145.6, 142.3, 141.3, 127.6, 118.7, 114.9, 91.7, 85.9, 84.6, 81.8, 74.9, 65.4, 26.3, 24.5; ^{31}P NMR (100 MHz, D_2O): δ -22.86. HRESI-MS for $C_{13}H_{18}N_5O_7P$+ H: calculated 388.1022, measured 388.1049.

Biotinol-5′-AMP (2)

To a suspension of biotinol (168 mg, 0.73 mmol) and adenosine 5'-monophosphate **6** (375 mg, 0.80 mmol) in dry pyridine (10 mL) was added DCC (226 mg, 1.10 mmol) and the mixture stirred under nitrogen atmosphere for 24 h. Acetic acid (15 mL) was added, the mixture stirred at 80 ^{o}C for 2 h, and then concentrated *in vacuo. Purification of the residue* by Sephadex A25 DEAE eluting with a gradient of aqueous triethylamine bicarbonate (0.05 – 0.35 M, pH 7-8) gave the triethylamine salt of biotinol-5′-AMP **2** as a clear gum (73 mg). The triethylamine salt of **2** was desalted by semi-preparative HPLC (see table 1 below for HPLC conditions and Method 2 for characterisation data).

[(3a*R*,4*R*,6a*R*)-4-(6-Aminopurin-9-yl)-2,2-dimethyl-3a,4,6,6a-tetrahydrofuro[3,4-*d*][1,3]dioxol-6-yl]methyl 5-[(3a*S*,6a*R*)-2-oxo-1,3,3a,4,6,6a-hexahydrothieno[3,4-*d*]imidazol-4-yl]pentyl hydrogen phosphate (9)

To a suspension of biotinol (168 mg, 0.73 mmol) and adenosine 5′-monophosphate **6** (375 mg, 0.80 mmol) in dry pyridine (10 mL) was added DCC (226 mg, 1.10 mmol) and mixture was stirred under nitrogen atmosphere for 24 h. The reaction mixture was concentrated *in vacuo* and the residue purified by Sephadex A25 DEAE eluting with a gradient of aqueous triethylamine bicarbonate (0.05 – 0.35 M, pH 7-8). As judged by TLC, fractions containing the biotinol-5′-AMP **2** were pooled and concentrated *in vacuo* to give **9** as a clear gum (110 mg). This material was suspended in a mixture of acetic acid and water (2:3), stirred at 60 ^{o}C for 4 h, cooled and finally concentrated *in vacuo.* The residue was purified following the method as described for biotinol-5'-AMP **2**.

^{1}H NMR (300 MHz, D_2O): δ 8.37 (1H, s), 8.20 (1H, s), 6.22 (1H, d, *J* = 3.0 Hz), 5.41 (1H, dd, *J* = 3.0, 6.0 Hz), 5.16-5.19 (1H, m), 4.59-4.65 (1H, m), 4.49-4.53 (1H, m), 4.24-4.28 (1H, m), 3.98-4.01 (2H, m), 3.49-3.54 (2H, m), 3.16 (12H, quart, *J* = 8 Hz) 3.04-3.09 (1H, m), 2.88 (1H, dd, *J* = 5.1, 13 Hz), 2.67 (1H, d, *J* = 13 Hz), 1.63 (3H, s), 1.42 (3H, s) 1.20-1.31 (26H, m); HRESI-MS for: $C_{23}H_{34}N_7O_8PS$: 600.2000, calculated. 600.2005.

2.3 Synthesis of Biotinol-5′-AMP *via* method 2 (see scheme 2)

((3a*R*,4*R*,6*R*,6a*R*)-6-(6-benzamido-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl (5-((3a*S*,4*S*,6a*R*)-1-(bis(4-methoxyphenyl)(phenyl)methyl)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl) methyl phosphate (13a)

A solution of biotinol **12** (100 mg, 0.19 mmol) and adenosine **11**[8] (118 mg, 0.21 mmol) in dry acetonitrile (2 ml) was added to 5-(ethylthiol)-1*H*-tetrazole (49 mg, 0.38 mmol) and the solution was stirred for 1 h under nitrogen atmosphere. Reaction was monitored by TLC and upon consumption of **11**, 5 M *tert*-butyl hydrogen peroxide (0.38 mL, 1.88 mmol) was added and the solution stirred for 15 min. Saturated aqueous sodium metabisulfite (5 mL) was added with stirring for an additional 30 min. The mixture was diluted with water (15 mL) and extracted with dichloromethane (5 x 15 ml). The organic layers were pooled, washed with brine (1 x 75 mL), dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was purified by silica gel chromatography eluting with 5% methanol in dichloromethane to give **13a** as light yellow solid (112 mg, 59%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.98 (0.5H, bs),10.52 (0.5H, bs), 8.70 (0.5H, s), 8.67 (0.5H, s), 8.11 (0.5H, s), 7.9-7.94 (2H, m), 7.09-7.25 (12H, m), 6.99 (0.5H, bs), 6.70-6.76 (4H, m), 6.39 (0.5H, bs), 6.23 (0.5H, d, *J* = 2.1 Hz), 6.20 (0.5H, d, *J* = 2.1 Hz), 5.61 (0.5H, dd, *J* = 2.1, 6.3 Hz), 5.57 (0.5H, dd, *J* = 2.1, 6.3 Hz), 5.33 (0.5H, dd, *J* = 2.7, 6.3 Hz), 5.30 (0.5H, dd, *J* = 2.7, 6.3 Hz), 3.98-4.51 (5H, m), 3.74-2.78 (9H, m), 3.10-3.18 (1H, m), 2.41 (1H, dd, *J* = 4.5, 13.2 Hz), 2.23-2.29 (1H, m), 1.62-1.75 (5H, m,), 1.38-1.45 (5H, m), 1.26-1.33 (4H, m); ^{31}P NMR (200 MHz, $CDCl_3$): δ -0.64; LRMS (M+Na): 1042.2

((3a*R*,4*R*,6*R*,6a*R*)-6-(6-Benzamido-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methyl (5-((3a*S*,4*S*,6a*R*)-1-(bis(4-methoxyphenyl)(phenyl)methyl)-5-oxido-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl) methyl phosphate 13b

To a solution of biotinol **12** (100 mg, 0.19 mmol) and adenosine **11** (118 mg, 0.21 mmol) in dry acetonitrile (2 mL) was added 5-(ethylthiol)-1*H*-tetrazole (49 mg, 0.38 mmol) and the solution was stirred for 1 h, under nitrogen atmosphere. Reaction was monitored by TLC and upon consumption of **12**, 5 M aqueous *tert*-butyl hydrogen peroxide (0.38 mL, 1.88 mmol) was added with stirring for 15 min. The mixture was diluted with water (15 mL) and then extracted with dichloromethane (5 x 15 mL). The organic layers were pooled, washed with brine (1 x 75 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by silica gel chromatography eluting with 5% methanol in dichloromethane to give **13b** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.38 (0.5H, bs), 10.05 (0.5H, bs), 8.71 (0.5H, s), 8.69 (0.5H, s), 8.16 (0.5H, s), 8.15 (0.5H, s), 7.95(2H, t, *J* = 9 Hz), 7.45-7.51 (1H, m), 7.09-7.35 (13H, m), 7.00 (0.5H, bs), 6.75-6.79 (4H, m), 6.52 (0.5H, bs), 6.16 (0.5, d, *J* = 2 Hz), 6.14 (0.5, d, *J* = 2 Hz), 5.47 – 5.49 (1H, m), 5.15 – 5.19 (1H, m), 4.67-4.75 (1H, m), 4.48 – 4.51 (1H, m), 4.33 – 4.38 (1H, m), 4.26 – 4.30 (1H, m), 4.14 – 4.21 (1H, m), 3.90 – 4.04 (2H, m), 3.77 – 3.78 (7H, m), 3.74 (1.5 H, d, *J* = 11 Hz), 3.69 ((1.5 H, d, *J* = 11 Hz), 2.92 – 3.02 (2H, m), 2.41 (1H, dd, *J* = 7, 15 Hz), 1.72 – 1.81 (2H, m), 1.34 – 1.63 (14H, m); ^{31}P NMR (200 MHz, $CDCl_3$): δ -0.72 LRMS (M+H): 1058.2

Biotinol-5′-AMP (2)

To a solution of compound **13a** (50 mg, 0.05 mmol) in dichloromethane (1 mL) was added TFA (0.1 mL) and the solution was stirred for 3 h. The reaction mixture was concentrated *in vacuo* and the residue dissolved in a mixture of methanol and tetrahydrofuran (1:1, 0.8 mL). Aqueous ammonium solution (32%, 0.4 mL) was added, the solution stirried overnight, and then concentrated *in vacuo*. The residue was filtered through 1 cm silica gel column eluting with methanol. The resulting solution was concentrated and resuspended in dry acetone (1 mL). Sodium iodide (6 mg, 0.04 mmol) was added and the mixture stirred under reflux for 6 h.

This mixture was cooled and concentrated *in vacuo* and the residue purified by HPLC (using gradient as describe in table 1 below) to give **2** as a white solid (11mg, 41%).

^{1}H NMR (300 MHz, D_2O): δ 8.63 (1H, s), 8.43 (1H, s), 6.19 (1H, d, *J* = 5.4 Hz), 4.52 – 4.57 (m, 2H), 4.36 – 4.40 (m, 1H), 4.32 (dd, *J* = 4.2, 8.1 Hz, 1H), 4.01 – 4.13 (m, 2H), 3.74 – 3.82 (m, 2H), 3.13 – 3.21 (m, 1H), 2.91 (dd, *J* = 4.8, 13.2 Hz, 1H), 2.70 (d, *J* = 13.2 Hz, 1H), 1.45 – 1.59 (m, 3H), 1.14 – 1.40 (m, 5H).

^{13}C NMR (126 MHz, dmso) δ 163.26, 156.31, 152.85, 149.87, 139.88, 119.41, 87.70, 83.41, 73.69, 70.75, 66.14, 61.50, 59.65, 55.90, 49.04, 30.09, 28.67, 28.64, 25.56. ^{31}P NMR (32 MHz, DMSO-d_6): δ -22.73; HRESI-ME for $C_{20}H_{31}N_7O_8P_S$: 560.1691, calc. 560.1692.

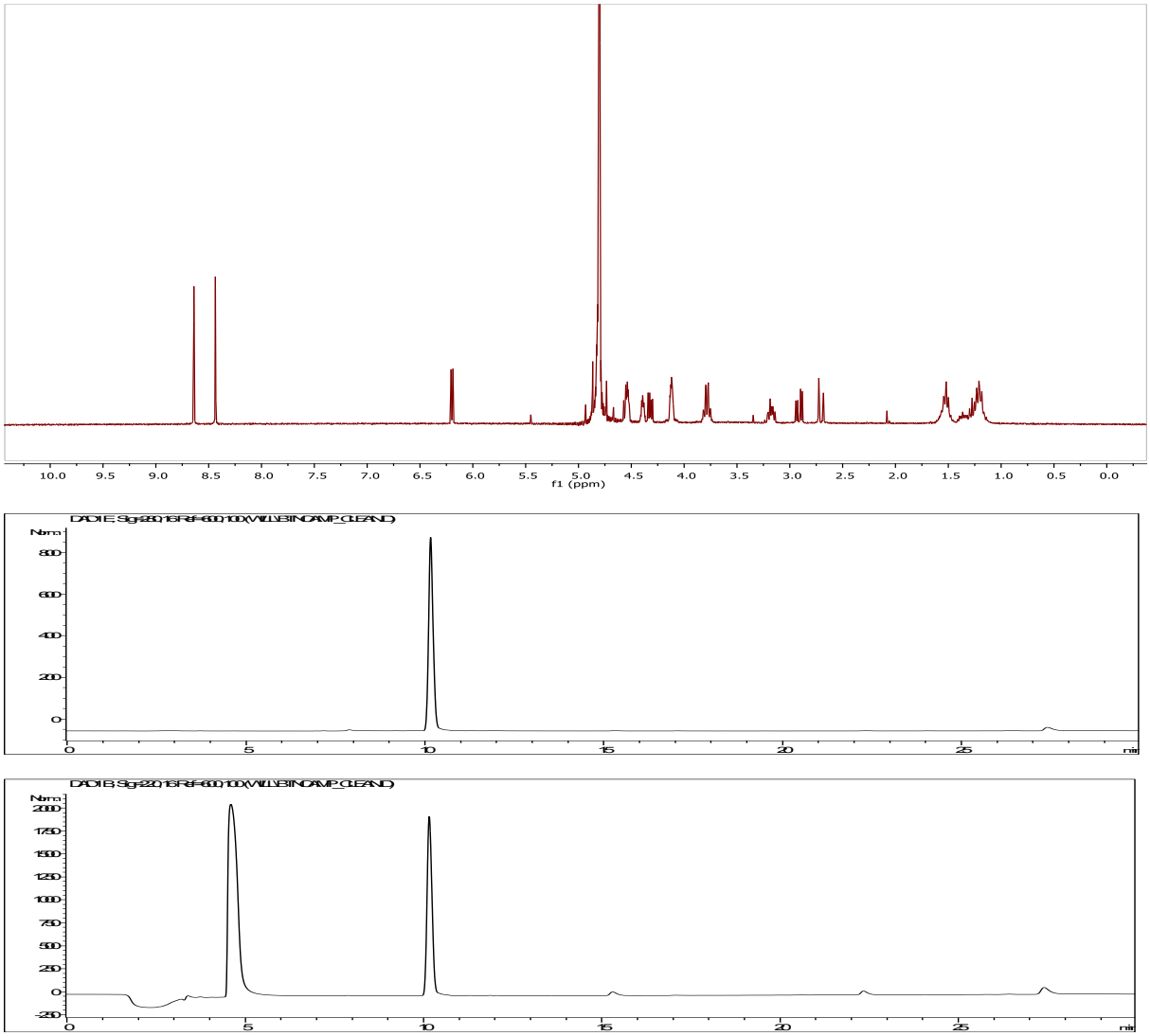


Figure legend: ^{1}H NMR (200 MHz, D_2O) of biotinol-5'-AMP **2**(Top); HPLC chromatogram of biotinol-5'-AMP **2** detected at 280nm (middle) and 220nm (bottom), biotinol-5'-AMP **2** eluted at 10.2 min and DMSO loading solvent at 4.8 min (see table 1 below for HPLC method)

Table 1: HPLC[a] gradient timetable for the purification of Biotinol-5'-AMP **2**

Time	Solvent A[b]	Solvent B[c]
0 min	100	0
5 min	100	0
30 min	35	65
40 min	10	90

[a] The sample was dissolved in DMSO (1 mg/ ml) and eluted on a Discovery BIO Wide Pore RP-C5, 15 cm x 4.6 mm, 5µm using a flow rate = 1 ml/min. [b] Solvent A: 0.1% TFA in Milliq water, [c] Solvent B: 0.08% TFA in MeCN.

3. References

1. Chapman-Smith, A.; Mulhern, T. D.; Whelan, F.; Cronan, J. E., Jr.; Wallace, J. C., The C-terminal domain of biotin protein ligase from E. coli is required for catalytic activity. *Prot. Sci.* **2001,** *10* (12), 2608-17.
2. Pendini, N. R.; Polyak, S. W.; Booker, G. W.; Wallace, J. C.; Wilce, M. C., Purification, crystallization and preliminary crystallographic analysis of biotin protein ligase from Staphylococcus aureus. *Acta crystallographica. Section F, Structural biology and crystallization communications* **2008,** *64* (Pt 6), 520-3.
3. Mayende, L.; Swift, R. D.; Bailey, L. M.; Soares da Costa, T. P.; Wallace, J. C.; Booker, G. W.; Polyak, S. W., A novel molecular mechanism to explain biotin-unresponsive holocarboxylase synthetase deficiency. *J. Mol. Med. (Berl)* **2012,** *90* (1), 81-8.
4. Yap, M. Y. Structural characterisation of biotin protein ligase and its interacting partners: a novel antibiotic. PhD thesis, Monash University, 2014.
5. Soares da Costa, T. P.; Tieu, W.; Yap, M. Y.; Pendini, N. R.; Polyak, S. W.; Sejer Pedersen, D.; Morona, R.; Turnidge, J. D.; Wallace, J. C.; Wilce, M. C.; Booker, G. W.; Abell, A. D., Selective inhibition of biotin protein ligase from Staphylococcus aureus. *J. Biol. Chem.* **2012,** *287* (21), 17823-32.
6. Polyak, S. W.; Chapman-Smith, A.; Brautigan, P. J.; Wallace, J. C., Biotin protein ligase from Saccharomyces cerevisiae. The N-terminal domain is required for complete activity. *J. Biol. Chem.* **1999,** *274* (46), 32847-54.
7. Copeland, R., Tight binding inhibition. In *Evaluation of Enzyme Inhibitors in Drug Discovery, 2nd ed.*, Wiley: 2013; pp 245-282.
8. Desjardins, M.; Garneau, S.; Desgagnés, J.; Lacoste, L.; Yang, F.; Lapointe, J.; Chênevert, R., Glutamyl Adenylate Analogues Are Inhibitors of Glutamyl-tRNA Synthetase. *Bioorg. Chem.* **1998,** *26* (1), 1-13.