

## Supporting information

Enzymatic logic of anthrax stealth siderophore biosynthesis: AsbA catalyzes ATP-dependent condensation of citric acid and spermidine

Daniel Oves-Costales, Nadia Kadi, Mark J. Fogg, Lijiang Song, Keith S. Wilson, and Gregory L. Challis\*

**Complete reference 2:** Inglesby, T.V.; O'Toole, T.; Henderson, D. A.; Bartlett, J. G.; Ascher, M. S.; Eitzen, E.; Friedlander, A. M.; Gerberding, J.; Hauer, J.; Hughes, J.; McDade, J.; Osterholm, M. T.; Parker, G.; Perl, T. M.; Russell, P. K.; Tonat, K. *J. Am. Med. Assoc.*, **2002**, 287, 2236-2252.

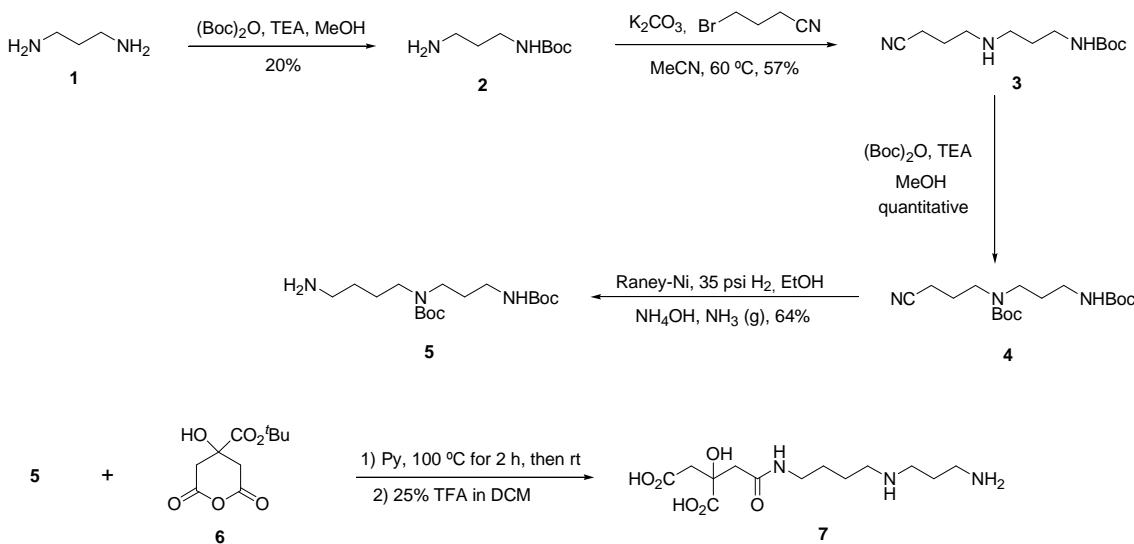
### 1 Materials, methods and procedures

#### 1.1 Synthesis of *N*<sup>1</sup>-(3,4-dihydroxybenzoyl)-spermidine (N-[3-(4-amino-butylamino)-propyl]-3,4-dihydroxy-benzamide)

*N*-[3-(4-Amino-butylamino)-propyl]-3,4-bis-benzyloxy-benzamide trifluoroacetate salt, prepared according to the procedure described by O. Phanstiel IV *et al.*,<sup>1</sup> (60 mg, mmol) was dissolved in a water/EtOH mixture (0.3:1) containing 30 mg of 10% Pd/C. The mixture was subjected to H<sub>2</sub> at atmospheric pressure and stirred at room temperature for 5 hours. The catalyst was removed by filtration of the mixture through celite and the solvent was removed under reduced pressure to give a solid that was purified by HPLC (Agilent eclipse XDB-C18 100 x 21 mm 5  $\mu$ , 70% water / MeCN isocratic). The spectroscopic data agreed well with the published literature data.<sup>2</sup>

#### 1.2 Synthesis of *N*<sup>8</sup>-citryl-spermidine (2-{[4-(3-Amino-propylamino)-butylcarbamoyl]-methyl}-2-hydroxy-succinic acid) 7

The synthesis of *N*<sup>8</sup>-citryl-spermidine **7** was accomplished via the route outlined in Scheme 1. Procedures for the synthesis of each compound and spectroscopic data are described below.



**Scheme 1.** Route employed for the synthesis of *N*<sup>8</sup>-citryl-spermidine **7**

**1.2.1 (3-Aminopropyl)carbamic Acid *tert*-butyl ester 2.** 1,3-Diaminopropane **1** (3.7 g, 0.05 mol) was dissolved in a solution of 10% triethylamine in methanol (110 mL). A solution of di-*tert*-butyl dicarbonate (3.70 g, 0.017 mol) in MeOH (10 mL) was added dropwise to this mixture, which was subsequently stirred overnight at room temperature. The methanol and the triethylamine were removed under reduced pressure to afford an oily residue that was dissolved in dichloromethane (100 mL) and washed with 10% Na<sub>2</sub>CO<sub>3</sub> (2 x 100 mL). The organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered and the solvent removed under reduced pressure to yield an oil that was purified by flash column chromatography (5:10:85 NH<sub>4</sub>OH:MeOH:CHCl<sub>3</sub>). White solid, 20% yield. Mp (uncorrected): 106–108 °C;  $\nu_{\text{max}}$  (neat) / cm<sup>-1</sup> 3359, 2931, 1680, 1522, 1328 and 1246;  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 400 MHz) 1.45 (s, 9H, -<sup>t</sup>Bu), 1.62 (tt, 2H, -CH<sub>2</sub>-, <sup>3</sup>J 6.6, <sup>3</sup>J 6.6), 2.77 (t, 2H, -CH<sub>2</sub>NH<sub>2</sub> <sup>3</sup>J 6.6), 3.22 (m, 2H, -CH<sub>2</sub>NH(BOC)), 4.97 (bs, 1H, -NH(BOC));  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 100 MHz) 28.3, 33.4, 38.4, 39.7, 78.9 and 156.0; HRMS: Calculated for C<sub>8</sub>H<sub>19</sub>N<sub>2</sub>O<sub>2</sub><sup>+</sup> (M+H)<sup>+</sup>: 175.1441; found: 175.1438. <sup>1</sup>H NMR data for **2** agreed well with those reported in the literature.<sup>3</sup>

**1.2.2 [3-(3-Cyano-propylamino)-propyl]-carbamic acid *tert*-butyl ester 3.** To a solution of the amine **2** (2 g, 11.5 mmol) in anhydrous MeCN (40 mL) was added potassium carbonate (5 g, 0.036 mol) and the suspension was stirred at room temperature for 10 min. Then, a solution of 4-bromo butyronitrile was added and the mixture was stirred at 60 °C for 24 h. The mixture was filtered to remove the insoluble salts and the filtrate was evaporated under reduced pressure to afford a semi-solid that was purified by flash column chromatography (5:10:85 NH<sub>4</sub>OH:MeOH:CHCl<sub>3</sub>). Colourless oil, 57% yield.  $\nu_{\text{max}}$  (neat) / cm<sup>-1</sup> 3325, 2930, 1689, 1515, 1364, 1249 and 1167;  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 400 MHz) 1.46 (s, 9H, -<sup>t</sup>Bu), 1.66 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>NH(BOC)), 1.83 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>CN), 2.48 (t, 2H, -CH<sub>2</sub>CN, <sup>3</sup>J 7.1), 2.69 (t, 2H, -CH<sub>2</sub>NH-, <sup>3</sup>J 6.5), 2.75 (t, 2H, -CH<sub>2</sub>NH-, <sup>3</sup>J 6.7 Hz), 3.22 (m, 2H, -CH<sub>2</sub>NH(BOC)), 5.12 (bs, 1H, -NH(BOC));  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 100 MHz) 14.8, 25.7, 28.3, 29.8, 38.9, 47.3, 47.9, 79.0, 119.6 and 156.0; HRMS: Calculated for C<sub>12</sub>H<sub>24</sub>N<sub>3</sub>O<sub>2</sub><sup>+</sup> (M+H)<sup>+</sup>: 242.1863; found: 242.1863.

**1.2.3 (3-*tert*-Butoxycarbonylaminopropyl)-(3-cyanopropyl)-carbamic acid *tert*-butyl ester 4.** To a solution of **3** (1.4 g, 5.8 mmol) in 10% triethylamine / methanol (v/v, 40 mL) was added dropwise a solution of di-*tert*-butyl dicarbonate (2 g, 9.17 mmol) in methanol (15 mL). The mixture was stirred at room temperature overnight. Then, the solvents were removed under reduced pressure to afford an oily residue that was dissolved in DCM (100 mL) and washed twice with 10% Na<sub>2</sub>CO<sub>3</sub> (2 x 100 mL). The organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered and the solvent removed under reduced pressure to yield an oil that was purified by flash column chromatography (50% EtOAc in hexanes). Colourless oil, 100% yield.  $\nu_{\text{max}}$  (neat) / cm<sup>-1</sup> 3361, 2974, 1681, 1511, 1417, 1365, 1248 and 1161;  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 400 MHz) 1.37 (s, 9H, -<sup>t</sup>Bu), 1.40 (s, 9H, -<sup>t</sup>Bu), 1.60 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>NH(BOC)), 1.82 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>CN), 2.29 (t, 2H, -CH<sub>2</sub>CN, <sup>3</sup>J 7.1), 3.04 (m, 2H, -CH<sub>2</sub>NH(BOC)), 3.19 (m, 2H, -CH<sub>2</sub>N(BOC)-), 3.23 (t, 2H, -CH<sub>2</sub>N(BOC)-, <sup>3</sup>J 7.0);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 125 MHz) 14.6, 24.5, 28.3, 37.4, 43.9, 44.6, 45.5, 79.0, 80.2, 119.1, 155.6 and 155.9; HRMS: Calculated for C<sub>17</sub>H<sub>31</sub>N<sub>3</sub>NaO<sub>4</sub><sup>+</sup> (M+Na)<sup>+</sup>: 364.2207; found: 364.2221. <sup>1</sup>H NMR data for **2** agreed well with those reported in the literature.<sup>3</sup>

**1.2.4 {3-[(4-Amino-butyl)-*tert*-butoxycarbonyl-amino]-propyl}-carbamic acid *tert*-butyl ester 5.** Nitrile **4** (2 g, 5.86 mmol) was dissolved in ethanol (80 mL). NH<sub>4</sub>OH

(8 mL) and Raney nickel (6.4 g, wet) were added and ammonia gas was bubbled through the solution for 30 min at 0°C. The resulting suspension was hydrogenated at 35 psi for 24 h using a Par apparatus. Then, air was bubbled through the solution and the Raney nickel was removed by filtration through a sintered glass funnel keeping the catalyst wet at all times. The solvents were removed under reduced pressure. The oily residue was dissolved in DCM and washed with 10% Na<sub>2</sub>CO<sub>3</sub> (2 x 50 mL). The organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered and evaporated under reduced pressure to afford the product. Colourless oil, 64% yield.  $\nu_{\text{max}}$  (neat) / cm<sup>-1</sup> 3360, 2972, 2929, 1685, 1414, 1364 and 1247;  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 400 MHz) 1.35 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 1.37 (s, 9H, -'Bu), 1.39 (s, 9H, -'Bu), 1.48 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>N(BOC)-) 1.59 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>NH(BOC)), 2.63 (t, 2H, -CH<sub>2</sub>NH<sub>2</sub>, <sup>3</sup>J 7.0), 3.02 (m, 2H, -CH<sub>2</sub>NH(BOC)), 3.08 (m, 2H, -CH<sub>2</sub>N(BOC)-), 3.18 (m, 2H, -CH<sub>2</sub>N(BOC)-), 5.29 (bs, 1H, -NH-);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 100 MHz) 25.2, 25.8, 28.2, 30.8, 37.3, 41.7, 43.5, 46.7, 79.3 and 155.1; HRMS: Calculated for C<sub>17</sub>H<sub>36</sub>N<sub>3</sub>O<sub>4</sub><sup>+</sup>: 346.2700; found: 346.2713. <sup>1</sup>H NMR data for **2** agreed well with those reported in the literature.<sup>3</sup>

**1.2.5 2-{[4-(3-Amino-propylamino)-butylcarbamoyl]-methyl}-2-hydroxy-succinic acid trifluoroacetate salt **7**.** A solution of compound **5** (100 mg, 0.289 mmol) and 4-Hydroxy-2,6-dioxo-tetrahydro-pyran-4-carboxylic acid *tert*-butyl ester **6**<sup>4</sup> (80 mg, 0.346 mmol) in anhydrous pyridine (10 mL) was stirred at 100°C for 2 h and then at room temperature overnight. The solvent was removed under vacuum and the crude material was used in the next step without purification. It was dissolved in a solution of 25% TFA in DCM (2 mL total volume) and stirred at room temperature for 3 hours. Then, the solvent was removed under vacuum and the residue was purified by HPLC (Agilent Eclipse XDB-C18 column (150 X 4.6 mm, 5 $\mu$ ) detecting absorption at 210 nm using the elution profile in table 1.

**Table 1.**

Time (min)	Water (0.1% TFA)	MeCN (0.1% TFA)	Flow (mL / min)
0	85	15	4.5
10	85	15	4.5
20	0	100	4.5
25	0	100	4.5

The collected fractions were analyzed by ESI-MS and those containing the compound with *m/z* 320 were freeze-dried (retention time 5.10 min).  $\delta_{\text{H}}$  (D<sub>2</sub>O, 400 MHz) 1.58 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>NHCO-), 1.71 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>NH-), 2.09 (m, 2H, -CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2.69 (d, 1H, -CHHCONH-, <sup>2</sup>J 14.5), 2.80 (d, 1H, -CHHCONH-, <sup>2</sup>J 14.5), 2.81 (d, 1H, -CHHCO<sub>2</sub>H, <sup>2</sup>J 15.8), 3.0-3.20 (m, 7H, -CH<sub>2</sub>NH<sub>2</sub>, -CH<sub>2</sub>NH-, -CH<sub>2</sub>NH-, -CH<sub>2</sub>NHCO-), 3.23 (t, 2H, -CH<sub>2</sub>NHCO-, <sup>3</sup>J 6.7);  $\delta_{\text{C}}$  (D<sub>2</sub>O containing 2 drops of CD<sub>3</sub>CN, 400 MHz) 23.3, 24.2, 26.0, 37.1, 38.9, 43.8, 44.9, 45.2, 47.8, 74.8, 116.9 (<sup>1</sup>J<sub>CF</sub> 292 Hz), 163.3 (<sup>2</sup>J<sub>CF</sub> 35 Hz), 171.6, 174.0 and 177.1; HRMS: Calculated for C<sub>13</sub>H<sub>26</sub>N<sub>3</sub>O<sub>6</sub><sup>+</sup>: 320.1816; found: 320.1821

### 1.3 Cloning and overexpression of AsbA in *E. Coli*.

**1.3.1** The coding sequence of *asbA* was amplified by polymerase chain reaction (PCR) from *B. anthracis* (Ames) genomic DNA using KOD Hot Start DNA polymerase (Novagen) and complementary gene-specific primers to which were appended

sequences to facilitate ligation independent cloning (LIC)<sup>5</sup> (underlined) 5'-CCAGGGACCAGCAATGAAGCATGCGAAACAAATCGCG-3', 5'-GAGGAGAAGGCGCGTTATGAACGAACAGCCACTTCTCTAAC-3'. For LIC, the PCR amplification product was treated with LIC qualified T4 DNA polymerase (Novagen) in the presence of dATP to generate 5' single stranded overhangs at both ends of the fragment, through the enzyme's combined 3'-5' exonuclease and DNA polymerase activities. Complementary 5' single stranded overhangs were generated similarly in the cloning vector, a LIC-adapted pET-28a (Novagen) (pET-YSBLIC3C<sup>6</sup>) where the encoded thrombin cleavage site had previously been mutated to encode a site for HRV 3C protease cleavage. The vector pET-YSBLIC3C was digested with the restriction endonuclease *Bse*RI, purified and incubated with LIC qualified T4 DNA polymerase in the presence of dTTP to generate the required single stranded overhangs. The vector and PCR products were annealed and used to transform *E. coli* NovaBlue cells (Novagen) to kanamycin resistance. Single colonies were selected, grown overnight and plasmids isolated using a QIAprep plasmid miniprep kit (Qiagen). Incubation of the plasmids with *Nco*I and *Bse*RI restriction endonucleases verified the presence of the gene insert. Correct sequence of the cloned gene was confirmed by DNA sequencing (Molecular Biology Service, Department of Biological Sciences, University of Warwick). One correct clone named pDOC0001 was used to transform *E. coli* BL21Star (DE3) (Invitrogen).

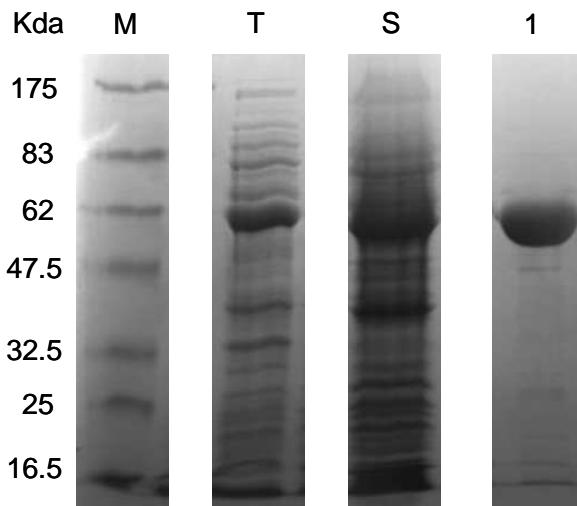
For overexpression of *asbA*, 300 mL of LB medium supplemented with kanamycin (50 µg/mL) was inoculated with a 0.5 mL overnight culture of *E. coli* BL21Star(DE3) / pDOC0001 and incubated in an orbital shaker at 190 rpm and 37°C. Incubation continued until the optical density at 600 nm of the culture reached 0.6, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce expression of *asbA*. The culture was then incubated overnight at 190 rpm and 15° C.

**1.3.2 Purification of His<sub>6</sub>-AsbA.** Cells were harvested by centrifugation, the pellet was resuspended in 15 mL of 20 mM Tris buffer-HCl, pH 8.0, 250 mM NaCl, 20 mM imidazole and 10 % glycerol, and lysed using a French Press (17,000 psi internal cell pressure). After removal of cellular debris by centrifugation (18,000 x g for 20 min, at 4° C), the supernatant was applied to a 1mL HiTrap™ HP affinity column (Nickel Sepharose High Performance, GE Healthcare) equilibrated with a solution containing 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 20 mM imidazole, and 10% glycerol. Unbound proteins were removed by washing with 10 mL of equilibration buffer and the His<sub>6</sub>-AsbA fusion protein was eluted with 3 mL of elution buffer (20 mM Tris-HCl, pH8.0, 250 mM NaCl, 300 mM imidazole, 10% glycerol).

Fractions were analysed by electrophoresis (10% SDS-PAGE, Figure 1), and those containing His<sub>6</sub>-AsbA were pooled, washed and concentrated to 1 mL using Amicon® Ultra filtration with a 30,000 MWCO membrane (Millipore) in buffer containing 20 mM Tris-HCl, pH 8.0, 250 mM NaCl and 10 % glycerol. His<sub>6</sub>-AsbA was then aliquoted and frozen at -80° C. The protein was used for all subsequent experiments without further purification, unless otherwise noted.

**1.3.3 Confirmation of His<sub>6</sub>-AsbA identity.** Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. The identity of purified His<sub>6</sub>-AsbA, was confirmed by peptide mass fingerprinting of tryptic digests of the protein (The Biological Mass Spectrometry and Proteomics Facility in the

Department of Biological Sciences, University of Warwick). 15 Predicted tryptic fragments of AsbA were identified by this analysis.



**Figure 1:** 10% SDS-PAGE analysis of His<sub>6</sub>-AsbA overproduction and purification. Lane M = molecular weight standards, Lane T = total protein after lysis, lane S = soluble protein fraction after lysis, lane 1 = protein eluted from Ni-NTA column with 300mM imidazole.

**1.4 Incubation of His<sub>6</sub>-AsbA with citric acid and spermidine.** 1.5 mM citric acid, 3 mM ATP, 7.5 mM MgCl<sub>2</sub>, 5 mM spermidine, 100 mM Tris-HCl, 3 µM His<sub>6</sub>-AsbA (after Ni-NTA purification and concentration) in a final volume of 300 µL were incubated for 90 minutes at 37 °C. The reaction was initiated by addition of the enzyme and was stopped by addition of 150 µL of a 5% trichlororoacetic acid solution. The corresponding control was carried out in the same conditions, using denatured His<sub>6</sub>-AsbA (heated at 100 °C for 20 minutes prior to addition to the incubation mixture).

LC-MS analysis of the reaction mixture was carried out using a reverse phase column (Synergi 4u fusion-RB 80, 250 x 4.60 mm, 4 micron, Phenomenex) connected to an Agilent 1100 HPLC instrument. The outflow was routed to a Bruker HCT+ ion trap spectrometer fitted with an electrospray source operating in positive ion mode. The compounds were eluted using the profile in table 2. The retention time for compound 7 is 26.7 minutes.

**Table 2.**

Time (min)	Water (0.1% TFA)	MeOH (0.1% TFA)	Flow (mL / min)
0	100	0	0.25
5	100	0	0.25
25	0	100	0.25
30	0	100	0.25

**1.5 Incubation of His<sub>6</sub>-AsbA with citric acid and 3,4-dihydroxybenzoylspermidine.** 1.5 mM citric acid, 3 mM ATP, 7.5 mM MgCl<sub>2</sub>, 100 mM Tris-HCl, 3 µM His<sub>6</sub>-AsbA (after Ni-NTA purification and concentration) and different concentrations of 3,4-dihydroxybenzoylspermidine (from 3 to 45 mM) in a final volume of 300 µL were incubated for 90 minutes at 37°C. The reaction was initiated by addition of the enzyme and was stopped by addition of 150 µL of a 5%

trichloroacetic acid solution. LC-MS analysis of the reaction mixture was carried out using the same conditions described in **1.4**. No 3,4-DHB-CT-SP formation was observed in any of the incubations carried out.

In order to rule out a hypothetical inactivation of AsbA due to the presence of any impurities in the synthetic standard of 3,4-DHB-SP, a competitive incubation, using 1.5 mM citric acid, 3 mM ATP, 7.5 mM MgCl<sub>2</sub>, 13.3 mM spermidine, 13.3 mM 3,4-DHB-SP, 100 mM Tris-HCl, 4.2 µM His<sub>6</sub>-AsbA (after Ni-NTA purification and concentration) in a final volume of 150 µL was carried out for 105 minutes at 37 °C. LC-MS analysis of the reaction mixture showed the presence of CT-SP, but not 3,4-DHB-CT-SP.

**1.6 Scaled-up incubation of His<sub>6</sub>-AsbA with citric acid and spermidine.** **Purification and characterization of CT-SP.** 1.5 mM citric acid, 3 mM ATP, 7.5 mM MgCl<sub>2</sub>, 5 mM spermidine, 100 mM Tris-HCl, 3.5 µM His<sub>6</sub>-AsbA (after Ni-NTA purification and concentration) in a final volume of 9 mL were incubated for 15 hours at 37 °C. The reaction was stopped with 4.5 mL of 5% trichloroacetic acid solution. The crude mixture was purified on a reverse phase column (Synergi fusion-RB 80, 250 x 10 mm, 4 micron, Phenomenex) attached to an Agilent 1100 HPLC instrument fitted with a quaternary pump and VWD detector, detecting absorption at 210 nm. The elution profile in table 3 was used.

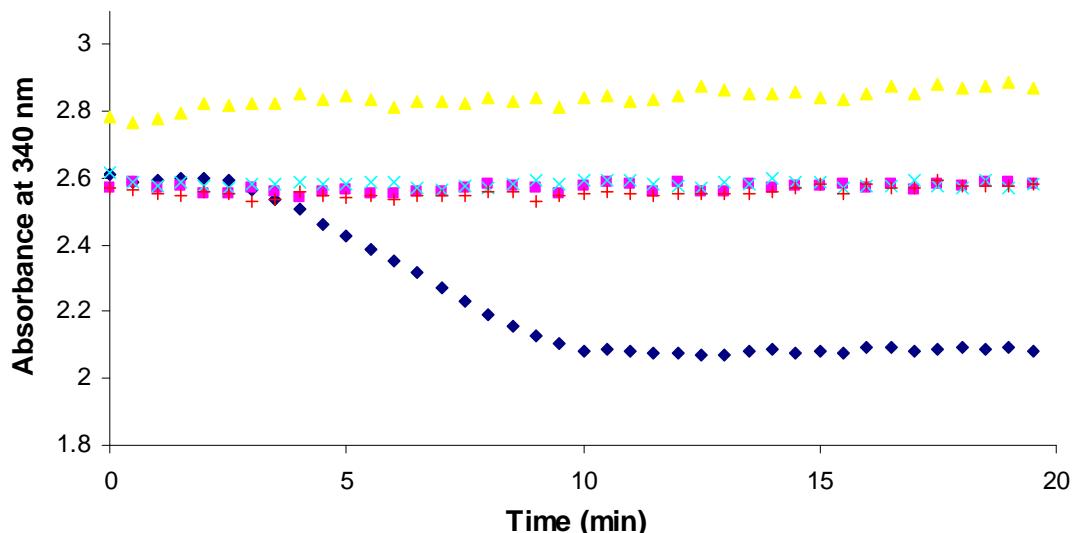
**Table 3.**

Time (min)	Water (0.1% TFA)	MeOH (0.1% TFA)	Flow (mL / min)
0	90	10	1.3
25	0	100	1.3
30	0	100	1.3

The collected fractions were analysed by ESI-MS and those containing the compound with *m/z* 320 were freeze dried. A further purification was carried out with this fraction using the same column with an isocratic mixture of 70% MeOH (0.1% TFA) in water (0.1% TFA) at a flow rate of 1.3 mL / min. The fractions containing the compound with *m/z* 320 were freeze dried (retention time 11 minutes).

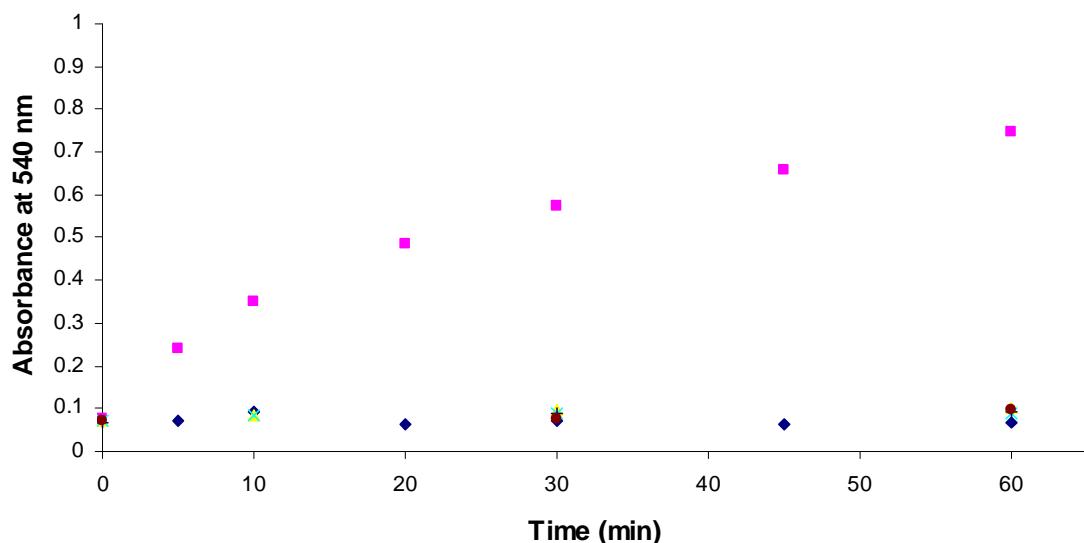
The CT-SP thus obtained was analysed by ESI-MS/MS (Bruker HCT+ spectrometer equipped with an electrospray source in positive and negative ion mode), ESI-TOF-MS (Bruker MicroTof) and NMR spectroscopy (<sup>1</sup>H, COSY and HMBC; Bruker Avance 700 spectrometer equipped with a TCI cryoprobe). The data obtained agreed well with the data obtained for the synthetic standard of CT-SP.

**1.7 Analysis of AMP and ADP formation in incubation mixtures.** For analysis of AMP formation, reactions containing 25 mM Tris-HCl buffer (pH 8.0), 3 mM ATP, 15 mM MgCl<sub>2</sub>, 1.5 mM phosphoenolpyruvate, 1.0 mM NADH, 3.5 µM His<sub>6</sub>-AsbA (after Ni-NTA purification and concentration), 12.6 units of lactate dehydrogenase, 8.4 units of pyruvate kinase, 4 units of myokinase, 2 mM citric acid and 7.1 mM amine (spermidine or 3,4-DHB-SP) in a total volume of 140 µL were incubated at 37 °C for 20 min in a quartz cuvette in a Varian Cary 1 UV-Vis spectrometer. The decrease in absorbance at 340 nm with time due to oxidation of NADH was monitored. Two control reactions were carried out using either boiled His<sub>6</sub>-AsbA, or α-ketoglutarate in place of citric acid. For the ADP assay, the same procedure was used except that myokinase was omitted from the reaction. The results of these experiments are shown in Figure 2.



**Figure 2.** Change in absorbance at 340 nm with time for different incubation mixtures; ▲ AMP assay with 3,4-DHB-SP; ♦ AMP assay with spermidine; ■ ADP assay with spermidine; ✕ AMP assay using boiled His<sub>6</sub>-AsbA and spermidine; + AMP assay using  $\alpha$ -ketoglutarate in place of citric acid.

**1.8 Incubation of His<sub>6</sub>-AsbA with citric acid and hydroxylamine.** 3.0 mM citric acid, 3 mM NTP (ATP, TTP, CTP or GTP), 15 mM MgCl<sub>2</sub>, 133 mM hydroxylamine, 100 mM Tris-HCl, 2.3  $\mu$ M His<sub>6</sub>-AsbA (after Ni-NTA purification and concentration) in a final volume of 300  $\mu$ L were incubated for different time periods at 37°C. The reactions were initiated by addition of the enzyme and were stopped by addition of 300  $\mu$ L of an aqueous solution of 3.3% trichloracetic acid, 10% FeCl<sub>3</sub>•6H<sub>2</sub>O and 0.7 M HCl. The reaction mixtures were centrifuged and absorbance at 540 nm of each supernatant was recorded on a Beckman Coulter DU 7400 UV-Vis spectrometer. The results of these experiments are shown in Figure 3.



**Figure 3.** Change in absorbance at 540 nm with time for different incubation mixtures; ■ with ATP and MgCl<sub>2</sub>; ♦ with ATP, heat-inactivated His<sub>6</sub>-AsbA and MgCl<sub>2</sub>; ▲ with CTP and MgCl<sub>2</sub>; ✕ with GTP and MgCl<sub>2</sub>; + with TTP and MgCl<sub>2</sub>; ● with ATP and without MgCl<sub>2</sub>.

## References

1. Gardner, R. A.; Kinkade, R.; Wang, C.; Phanstiel IV, O. *J. Org. Chem.* **2004**, *69*, 3530-3537.
2. Lee, J. Y.; Janes, B. K.; Passalacqua, K. D.; Pfleger, B. F.; Bergman, N. H.; Liu, H.; Håkansson, K.; Ravindranadh, V. S.; Courtney, C. A.; Cendrowski, S.; Hanna, P. C.; Sherman, D. H. *J. Bacteriol.* **2007**, *189*, 1698-1710.
3. Roblot, G.; Wylde, R.; Martin, A.; Parello, J. *Tetrahedron* **1993**, *49*, 6381-6398.
4. Fadeev, E. A.; Luo, M.; Groves, J. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3771-3774.
5. Aslandis, C.; de Jong, P. J. *Nucleic Acids Res.* **1990**, *18*, 6069.
6. Alzari, P. M.; Berglund, H.; Berrow, N. S.; Blagova, E.; Busso, D.; Cambillau, C.; Campanacci, V.; Christodoulou, E.; Eiler, S.; Fogg, M. J.; Folkers, G.; Geerlof, A.; Hart, D.; Haouz, A.; Herman, M. D.; Macieira, S.; Nordlund, P.; Perrakis, A.; Quevillon-Cheruel, S.; Tarandieu, F.; van Tilburgh, H.; Unger, T.; Luna-Vargas, M. P. A.; Velarde, M.; Willmanns M.; Owens, R. J. *Acta Crystallogr. D* **2006**, *62*, 1103-1113.

## Abbreviations

CT-SP:  $N^8$ -citryl-spermidine (2-{{4-(3-Amino-propylamino)-butylcarbamoyl}-methyl}-2-hydroxy-succinic acid)

3,4-DHB-SP:  $N^1$ -(3,4-dihydroxybenzoyl)-spermidine (N-[3-(4-amino-butylamino)-propyl]-3,4-dihydroxy-benzamide)

3,4-DHB-CT-SP:  $N^1$ -(3,4-dihydroxybenzoyl)- $N^8$ -citryl spermidine (2-({4-[3-(3,4-dihydroxy-benzoylamino)-propylamino]-butylcarbamoyl}-methyl)-2-hydroxy-succinic acid)

## 2 Spectroscopic data

- ESI-TOF-MS spectrum of  $N^8$ -citryl-spermidine (from incubation with His<sub>6</sub>-AsbA) (p. 9)
- MS/MS spectrum of  $N^8$ -citryl-spermidine (synthetic standard) in positive ion mode (p. 10)
- MS/MS spectrum of  $N^8$ -citryl-spermidine (from incubation with His<sub>6</sub>-AsbA) in positive ion mode (p. 11)
- MS/MS spectrum of  $N^8$ -citryl-spermidine (synthetic standard) in positive ion mode (p. 12)
- MS/MS spectrum of  $N^8$ -citryl-spermidine (from incubation with His<sub>6</sub>-AsbA) in positive ion mode (p. 13)
- <sup>1</sup>H, <sup>13</sup>C, COSY, HMBC, HMQC spectra for  $N^8$ -citryl-spermidine (synthetic standard)

