ChemComm

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/d0cc02241a

Received 27th March 2020,
Accepted 13th May 2020

DOI: 10.1039/d0cc02241a

rsc.li/chemcomm

ArnD is a deformylase involved in polymyxin resistance†

Taniya Adak,[a] Daniela L. Morales,[b] Alina J. Cook,[a] Jason C. Grigg,[b] Michael E. P. Murphy[b] and Martin E. Tanner *[a]

The modification of lipid A with cationic 4-amino-4-deoxy-L-arabinose residues serves to confer resistance against cationic peptide antibiotics in Gram-negative bacteria. In this work, the enzyme ArnD is shown to act as a metal-dependent deformylase in the biosynthesis of this carbohydrate.

Lipopolysaccharide (LPS) is a major component of the outer leaflet of Gram-negative bacteria.[1–4] LPS consists of three domains; the O-antigen, the core oligosaccharide (OS), and lipid A. Lipid A is a glucosamine disaccharide that is esterified with multiple fatty acid residues and is typically bis-phosphorylated (Fig. 1). The fatty acid residues serve to anchor the LPS to the bacterial outer membrane. Cationic antimicrobial peptides of the innate immune system, and cationic antibiotics, such as polymyxin, exert their activity by initially recognizing the phosphate groups of lipid A, and then by disrupting the outer membrane structure.[5,6] In order to evade the action of these antibiotics, many strains of bacteria, including *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*, decorate the phosphate groups of their lipid A with cationic compounds such as ethanolamine or 4-amino-4-deoxy-L-arabinose (L-Ara4N).[7–11] The presence of positive charges on lipid A repels the antibacterial compounds *via* charge–charge repulsion and confers resistance to the bacteria.

This antibiotic resistance mechanism is particularly problematic in the case of *Burkholderia cenocepacia*, an opportunistic pathogen that causes life-threatening lung infections in cystic fibrosis patients.[12,13] *B. cenocepacia* is intrinsically resistant to many common antibiotics, including the polymyxins.[14,15] This resistance is due partly to the presence of L-Ara4N on both the lipid A and the inner sugars of the OS. Surprisingly, the L-Ara4N modification of the LPS is also required for survival in this organism.[16] In the absence of the modification, the LPS is not transported to the outer membrane where it must serve as an essential component. Therefore, compounds that interfere with L-Ara4N biosynthesis and its attachment to the LPS could be lethal to this organism.

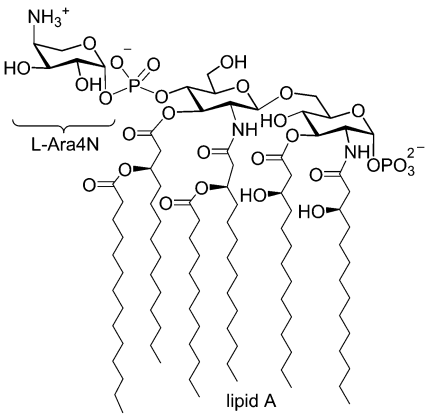


Fig. 1 The structure of lipid A that has been modified by L-Ara4N.

The biosynthesis of L-Ara4N takes place in the cytosol.[9] UDP-glucuronic acid (UDP-GlcUA) is oxidized and decarboxylated by the enzyme ArnA to give a UDP-4-keto-pentose (Fig. 2).[17,18] The transaminase ArnB then utilizes glutamate to produce UDP-L-Ara4N.[19,20] The bifunctional enzyme ArnA then utilizes *N*-10 formyl-THF to formylate the amine and produce UDP-4-formamido-4-deoxy-L-arabinose (UDP-L-Ara4FN).[21–23]

At this point, ArnC transfers the sugar onto an undecaprenyl phosphate with the release of UDP.[21] This anchors the compound to the inner membrane. Removal of the formate group has been postulated to be catalyzed by ArnD, although this activity has never been directly demonstrated.[9,21] Once L-Ara4N undecaprenyl phosphate has been produced it is flipped across the inner membrane where it is attached to lipid A molecules by the transferase ArnT.[24–27] Ultimately these modified lipid A molecules are transported to the outer membrane and incorporated into the LPS. An explanation as to why the sugar is first formylated (by ArnA) and then deformylated by ArnD is that this

[a] Department of Chemistry, University of British Columbia, Vancouver, BC, V6T 1Z1, Canada. E-mail: mtanner@chem.ubc.ca
[b] Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada

† Electronic supplementary information (ESI) available: Synthetic procedures, enzyme production and purification, ^{1}H and ^{13}C NMR spectra. See DOI: 10.1039/d0cc02241a

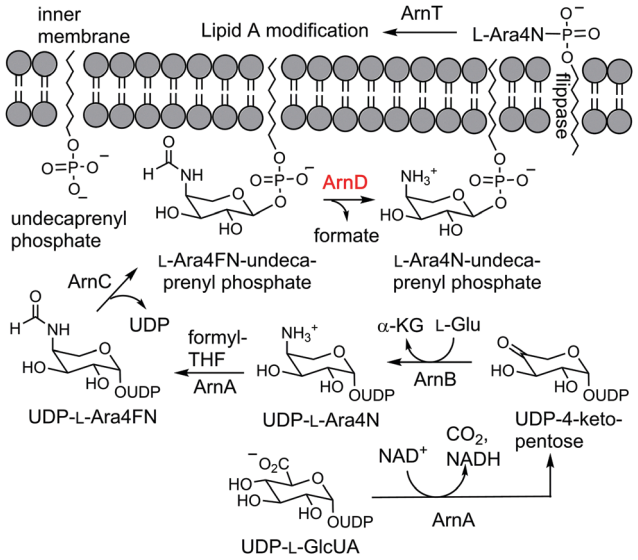
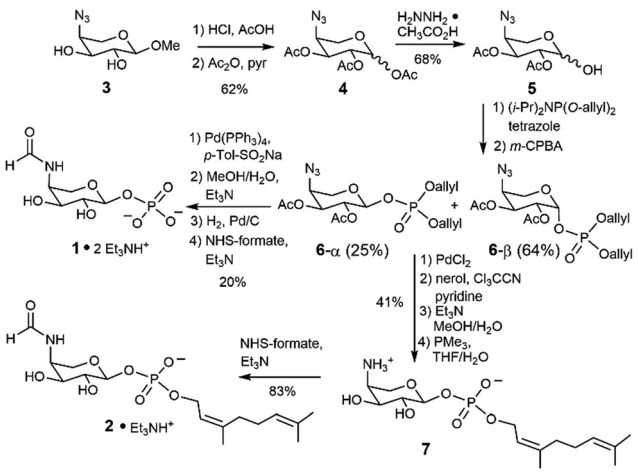


Fig. 2 The biosynthetic pathway for the modification of lipid A by L-Ara4N.

renders the phosphotransferase reaction of ArnC effectively irreversible.[21] While ArnA and ArnB have been relatively well characterized, the reactions on the lipid bearing substrates catalyzed by the putative ArnC and ArnD, have not. The activity of the ArnD gene product has only been inferred from genetic context in the *arn* operon and sequence similarities with related enzymes. In this work, we describe the synthesis of a substrate for the *B. cenocepacia* deformylase and demonstrate ArnD activity for the first time.

The synthetic targets for this work were 4-formamido-4-deoxy-L-arabinose-α-phosphate **1** as well as the prenylated version, 4-formamido-4-deoxy-L-arabinose-α-neryl-phosphate **2** (Scheme 1). The former compound would test for the requirement of a lipid group on the anomeric phosphate, and the latter compound would test whether a neryl group could replace the naturally occurring undecaprenyl group. The synthesis of both compounds began with the literature known azide **3**.[28] Compound **3** was hydrolyzed under acidic conditions and then peracetylated to give compound **4**.[29]



Scheme 1 The synthesis of compounds **1** and **2**.

Selective deprotection of the anomeric acetate using hydrazine acetate gave compound **5**. This was phosphorylated by treatment with diallyl *N*,*N*-diisopropylphosphoramidite followed by oxidation with *m*-CPBA to give compound **6** as a 2:5 mixture of α:β anomers.[30] To prepare compound **1**, the α-anomer of compound **6** was treated with $Pd(PPh_3)_4$, followed by mild basic hydrolysis, to remove the allyl and acetyl protecting groups, respectively.[31] The crude azide was then reduced to an amine by catalytic hydrogenation and the amine was formylated with NHS-formate.[32] Compound **1** was purified by ion-exchange chromatography. For the synthesis of compound **2**, the α-anomer of compound **6** was first deprotected with $PdCl_2$, and then coupled to nerol using trichloroacetonitrile and pyridine, to give the phosphate diester that was partially purified by silica gel chromatography.[33] The acetyl groups were removed using mild basic hydrolysis, and the azide was reduced using trimethylphosphine in $MeOH/H_2O$ to give the amine **7**. A recent report on the ArnT enzyme has described an alternate synthetic approach towards compound **7**.[24] Formylation of the amine was accomplished with NHS-formate which gave compound **2** after reversed-phase chromatography.

The gene encoding for full length *B. cenocepacia* ArnD was overexpressed in *E. coli* to obtain ArnD containing a C-terminal octa-histidine tag. Sequence analysis with XtalPred showed that ArnD is a protein with a surface hydrophobicity score typical of soluble protein. Initial attempts to isolate ArnD in the absence of detergents were unsuccessful. A panel of detergents were tested to solubilize ArnD during cell lyses and inclusion of either 1% *n*-dodecyl β-D-maltopyranoside (DDM) or Triton X-100 greatly enhanced the yield of recombinant protein (30 mg per litre of cell culture). Mass spectrometry of ArnD gave a mass of 35 000 Da (the calculated mass of the construct missing the N-terminal methionine and including the His-tag is 35 000 Da). Protein isolated using DDM was unstable to storage at 4 °C overnight so Triton X-100 was used to solubilize the protein used in the activity assays.

Compounds **1** and **2** were then tested as substrates for ArnD using mass spectrometry to monitor product formation. All attempts to show activity with compound **1** in the presence of various divalent metal ions were unsuccessful. However, when compound **2** was incubated with ArnD in the presence of 0.25 mM Zn^{2+}, the loss of a mass corresponding to that of a formyl group was clearly observed (Fig. 3, panels A and B, $M - H^+$ for **2** = 392, $M - H^+$ for **7** = 364). If the divalent cation was replaced by 0.5 mM EDTA, the activity was essentially eliminated (Fig. 3, panels C and D). Finally, when the incubation was carried out with no added metals or EDTA, a similar level of activity was observed as compared to the reaction run with added metal (data not shown). In order to confirm that the product of the reaction was compound **7**, a larger scale reaction was run, and the product was purified by reversed-phase chromatography.

The ^1H NMR spectrum of the isolated material is shown in Fig. 4A. It compares quite favorably with the spectrum of a synthetic standard of compound **7** (Fig. 4B), although slight chemical shift differences are observed in the signals corresponding to the C-3, C-4 and C-5 protons. This is likely due to

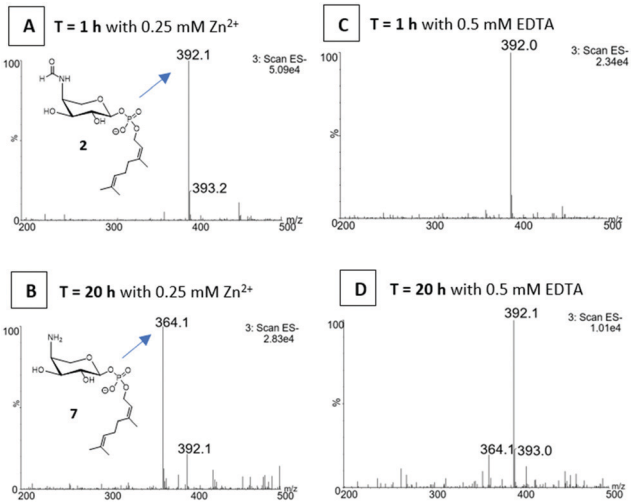


Fig. 3 Identification of the ArnD reaction product by mass spectrometry. (A) Compound **2** incubated with ArnD for 1 h in the presence of 0.25 mM Zn^{2+}. (B) Compound **2** incubated with ArnD for 20 h in the presence of 0.25 mM Zn^{2+}. (C) Compound **2** incubated with ArnD for 1 h in the presence of 0.5 mM EDTA. (D) Compound **2** incubated with ArnD for 20 h in the presence of 0.5 mM EDTA.

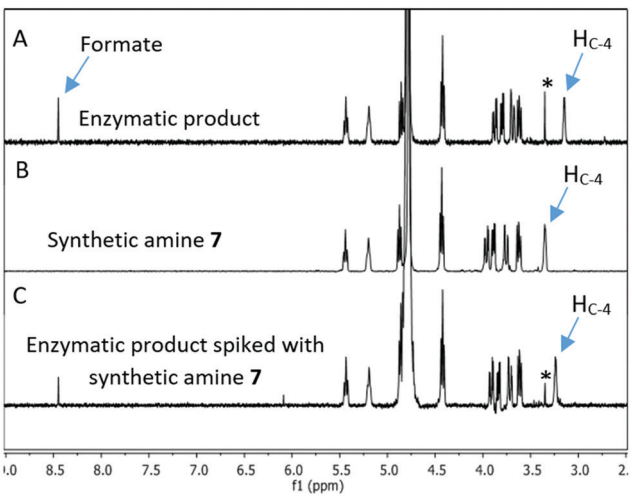


Fig. 4 Identification of the ArnD reaction products by ^{1}H NMR spectroscopy. (A) Shows products purified from the ArnD reaction. (B) Shows a synthetic standard of compound **7**. (C) Shows the enzymatic products spiked with a sample of synthetic compound **7**. * = impurity in enzymatic product.

differences in the pH of the two samples that affects the protonation state of the amine. In order to confirm the compounds are identical, the enzymatically-produced sample was spiked with the synthetic sample and the spectrum was retaken. The resulting spectrum (Fig. 4C) shows all signals are coincident indicating that compound **7** was indeed formed in the ArnD reaction.

The characteristic signals for the formamido proton of compound **2** appear as two singlets at 8.19 and 8.09 ppm in a 5 : 1 ratio due to the presence of two rotamers about the amide bond that slowly interconvert on the NMR time scale (Fig. S17, ESI†). These have been replaced by a single singlet at 8.45 ppm for formic acid in the enzymatic reaction (co-purified in Fig. 4A). Furthermore, the resonance for the C-4 sugar proton that appears at 4.37 ppm in compound **2** (Fig. S17, ESI†) moves upfield in the region of 3.15–3.36 ppm in compound **7** (Fig. 4A–C).

These studies demonstrate that ArnD is the deformylase involved in lipid A modification by Gram-negative bacteria. The observation that EDTA dramatically affects the enzyme activity implies that this is a metalloenzyme. This is somewhat expected as ArnD shows modest sequence homology to known metal-dependent carbohydrate deacetylases such as peptidoglycan deacetylase.[34] The observation that the enzyme retains activity in the absence of added metal suggests that it is purified in a metal-bound state. The absence of any observed activity with compound **1** indicates that the lipid portion is essential for catalysis. Furthermore, the observed activity with compound **2** indicates a neryl group can replace the undecaprenyl group to give a viable substrate. This suggests that the enzyme at least partially extracts the substrate from the inner membrane and has recognition elements for the lipophilic tail. Further studies will focus on identifying the catalytic metal and active site residues, as well as developing ArnD inhibitors.

We thank the Natural Sciences and Engineering Research Council of Canada (NSERC) (MET) and the Cystic Fibrosis Canada Grant 3210 (MEPM) for support.

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 C. R. H. Raetz and C. Whitfield, *Annu. Rev. Biochem.*, 2002, **71**, 635–700.
2 R. P. Sweeney and T. L. Lowary, *Curr. Opin. Chem. Biol.*, 2019, **53**, 37–43.
3 X. Y. Wang and P. J. Quinn, *Prog. Lipid Res.*, 2010, **49**, 97–107.
4 C. Whitfield and M. S. Trent, *Annu. Rev. Biochem.*, 2014, **83**, 99–128.
5 F. Guilhelmelli, N. Vilela, P. Albuquerque, L. D. Derengowski, I. Silva-Pereira and C. M. Kyaw, *Front. Microbiol.*, 2013, **4**, 353.
6 L. T. Nguyen, E. F. Haney and H. J. Vogel, *Trends Biotechnol.*, 2011, **29**, 464–472.
7 R. F. Maldonado, I. Sa-Correia and M. A. Valvano, *FEMS Microbiol. Rev.*, 2016, **40**, 480–493.
8 A. O. Olaitan, S. Morand and J. M. Rolain, *Front. Microbiol.*, 2014, **5**, 643.
9 C. R. H. Raetz, C. M. Reynolds, M. S. Trent and R. E. Bishop, *Annu. Rev. Biochem.*, 2007, **76**, 295–329.
10 P. Srinivas and K. Rivard, *Curr. Infect. Dis. Rep.*, 2017, **19**, 38.
11 M. Vaara, T. Vaara, M. Jensen, I. Helander, M. Nurminen, E. T. Rietschel and P. H. Makela, *FEBS Lett.*, 1981, **129**, 145–149.
12 J. R. W. Govan and V. Deretic, *Microbiol. Rev.*, 1996, **60**, 539–574.
13 V. C. Scoffone, L. R. Chiarelli, G. Trespidi, M. Mentasti, G. Riccardi and S. Buroni, *Front. Microbiol.*, 2017, **8**, 1592.
14 S. A. Loutet, L. E. Mussen, R. S. Flannagan and M. A. Valvano, *Environ. Microbiol. Rep.*, 2011, **3**, 278–285.
15 S. A. Loutet and M. A. Valvano, *Front. Cell. Infect. Microbiol.*, 2011, **1**, 6.
16 M. A. Hamad, F. Di Lorenzo, A. Molinaro and M. A. Valvano, *Mol. Microbiol.*, 2012, **85**, 962–974.
17 S. D. Breazeale, A. A. Ribeiro and C. R. H. Raetz, *J. Biol. Chem.*, 2002, **277**, 2886–2896.
18 P. Z. Gatzeva-Topalova, A. P. May and M. C. Sousa, *Structure*, 2005, **13**, 929–942.
19 S. D. Breazeale, A. A. Ribeiro and C. R. H. Raetz, *J. Biol. Chem.*, 2003, **278**, 24731–24739.

20 M. Lee and M. C. Sousa, *Biochemistry*, 2014, **53**, 796–805.
21 S. D. Breazeale, A. A. Ribeiro, A. L. McClerren and C. R. H. Raetz, *J. Biol. Chem.*, 2005, **280**, 14154–14167.
22 N. A. Genthe, J. B. Thoden and H. M. Holden, *Protein Sci.*, 2016, **25**, 1555–1562.
23 G. J. Williams, S. D. Breazeale, C. R. H. Raetz and J. H. Naismith, *J. Biol. Chem.*, 2005, **280**, 23000–23008.
24 C. Olagnon, J. M. Feria, C. Grunwald-Gruber, M. Blaukopf, M. A. Valvano and P. Kosma, *ChemBioChem*, 2019, **20**, 2936–2948.
25 V. I. Petrou, C. M. Herrera, K. M. Schultz, O. B. Clarke, J. Vendome, D. Tomasek, S. Banerjee, K. R. Rajashankar, M. B. Dufrisne, B. Kloss, E. Kloppmann, B. Rost, C. S. Klug, M. S. Trent, L. Shapiro and F. Mancia, *Science*, 2016, **351**, 608–612.
26 F. Tavares-Carreon, K. B. Patel and M. A. Valvano, *Sci. Rep.*, 2015, **5**, 10773.
27 M. S. Trent, A. A. Ribeiro, S. H. Lin, R. J. Cotter and C. R. H. Raetz, *J. Biol. Chem.*, 2001, **276**, 43122–43131.
28 B. Muller, M. Blaukopf, A. Hofinger, A. Zamyatina, H. Brade and P. Kosma, *Synthesis*, 2010, 3143–3151.
29 M. Blaukopf, B. Muller, A. Hofinger and P. Kosma, *Eur. J. Org. Chem.*, 2012, 119–131.
30 M. Polakova, M. U. Roslund, F. S. Ekholm, T. Saloranta and R. Leino, *Eur. J. Org. Chem.*, 2009, 870–888.
31 S. Marchesan and D. MacMillan, *Chem. Commun.*, 2008, 4321–4323.
32 A. Barre, M. L. Tintas, F. Alix, V. Gembus, C. Papamicael and V. Levacher, *J. Org. Chem.*, 2015, **80**, 6537–6544.
33 T. L. Li, A. Tikad, W. D. Pan and S. P. Vincent, *Org. Lett.*, 2014, **16**, 5628–5631.
34 D. E. Blair, A. W. Schuttelkopf, J. I. MacRae and D. M. F. van Aalten, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 15429–15434.

