Enantioselective desymmetrisation of citric acid catalysed by the substrate-tolerant petrobactin biosynthetic enzyme AsbA†

Daniel Oves-Costales, Lijiang Song and Gregory L. Challis*

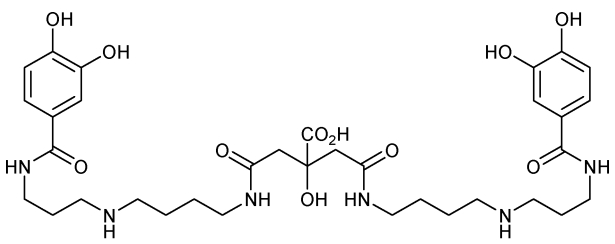
Received (in Cambridge, UK) 24th December 2008, Accepted 28th January 2009
First published as an Advance Article on the web 16th February 2009
DOI: 10.1039/b823147h

AsbA catalyses the highly enantioselective desymmetrisation of citric acid *via* ATP-dependent condensation with spermidine, as well as the condensation of citric acid with several spermidine analogues and the condensation of the citric acid analogue tricarballylic acid with spermidine, suggesting that it may be a useful biocatalyst for asymmetric synthesis.

Catalytic enantioselective desymmetrisation of *meso* compounds is a powerful strategy in asymmetric synthesis for the generation of homochiral organic compounds. Most of these processes are catalysed by homochiral metal-based catalysts.[1] There are relatively few examples of such transformations catalysed by enzymes.[2] Probably the most widely-exploited strategy for enantioselective desymmetrisation of *meso* compounds using enzymes involves the use of hydrolases to catalyse stereoselective hydrolytic and transesterification reactions.[2] Another strategy is the use of redox processes, such as the asymmetric Baeyer–Villiger oxidation of prochiral cyclic ketones by $FADH_2$-dependent monooxygenases.[2,3]

Petrobactin **1** is a mixed catechol–citrate siderophore found in numerous *Bacillus* species[4] that has been shown to be required for growth and virulence of *B. anthracis* in iron-depleted media in a mouse model.[5] Importantly, recent investigations have shown that petrobactin is able to evade siderocalin, a protein from the innate immune system that can sequester the apo- and ferric forms of different siderophores and thus disrupt the bacterial iron-uptake process.[6] These data suggest that inhibitors targeting the biosynthesis and uptake of **1** may be useful anti-anthrax agents. The production of petrobactin in *B. anthracis* is directed by the *asbABCDEF* gene cluster (Fig. 1), which encodes a unique combination of nonribosomal peptide synthetases (NRPSs) and NRPS-independent siderophore (NIS) synthetases and has been extensively studied over the last two years.[7] The biosynthetic roles of each of the enzymes encoded by this gene cluster are now relatively well understood (Fig. 1). We recently showed that the NIS synthetase AsbA catalyses the ATP-dependent condensation of citric acid with N^8 of spermidine to afford N^8-citryl-spermidine **2**.[7c] This raised an interesting question about the stereoselectivity of this desymmetrisation reaction. Here we report that it is highly enantioselective and that AsbA can catalyse similar reactions with an analogue of citric acid and several analogues of spermidine.

As citric acid is a prochiral molecule, its desymmetrisation by the AsbA-catalysed condensation with spermidine would yield either the enantiomer **2a**, the enantiomer **2b**, or a mixture of both. To elucidate the stereochemical outcome of this reaction we envisioned exploiting *si*-citrate synthase, which catalyses the enantiospecific addition of acetyl-CoA enolate to the *si* face of the keto group of oxaloacetic acid followed by thioester hydrolysis, to prepare homochiral (3*R*)-[1,2-$^{13}C_2$]citric acid **3** from [1,2-$^{13}C_2$]acetyl-CoA **4** (Fig. 2).[8] We envisaged that the products deriving from the AsbA-catalysed condensation of spermidine with the labelled and unlabelled carboxyl groups of (3*R*)-[1,2-$^{13}C_2$]citric acid **3** (*i.e.* those corresponding to the prochiral carboxyl groups in unlabelled citric acid) could be discriminated by ^{13}C NMR spectroscopy. Thus, in a one-pot experiment, we incubated *si*-citrate synthase, acetyl-CoA synthetase, purified recombinant AsbA, [1,2-$^{13}C_2$]acetic acid, coenzyme A, ATP, oxaloacetic acid and spermidine for a total of 6 hours at 22 °C (see ESI† for details). The resulting ^{13}C-labelled N^8-citryl-spermidine **2** was purified from the reaction mixture by reverse-phase HPLC, and its identity was confirmed by HRMS (found 322.1899, calculated 322.1883), and 1- and 2-D NMR experiments. The ^{13}C NMR spectrum showed two doublets at 44.5 and 175.6 ppm (see ESI†). A coupling constant of 55 Hz was observed for each doublet. These data showed that the major product of the reaction (>28 : 1 based on the signal to noise ratio of the NMR spectrum) is either **2a** or **2b** and that the AsbA-catalysed desymmetrisation of citric acid is highly enantioselective. To determine which of the enantiomers **2a** or **2b** is the product of the labelling reaction we measured the ^{13}C NMR spectrum of a mixture of the labelled product and synthetic racemic unlabelled N^8-citryl-spermidine (Fig. 2). Together with HMBC NMR data obtained for the pure ^{13}C-labelled N^8-citryl-spermidine **2**, this allowed us to unequivocally assign the identity of this compound as (3*R*)-N^8-citryl-spermidine **2a** (Fig. 2).



1

We have previously reported that the AsbA-catalysed condensation reaction occurs *via* adenylation of the prochiral carboxyl groups in citric acid and subsequent nucleophilic attack of N^8 of spermidine on the activated carbonyl carbon

Department of Chemistry, University of Warwick, Coventry, UK CV4 7AL. E-mail: g.l.challis@warwick.ac.uk; Fax: +44 (0)247 652 4112; Tel: +44 (0)247 657 4024

† Electronic supplementary information (ESI) available: Experimental procedures and spectroscopic data. See DOI: 10.1039/b823147h

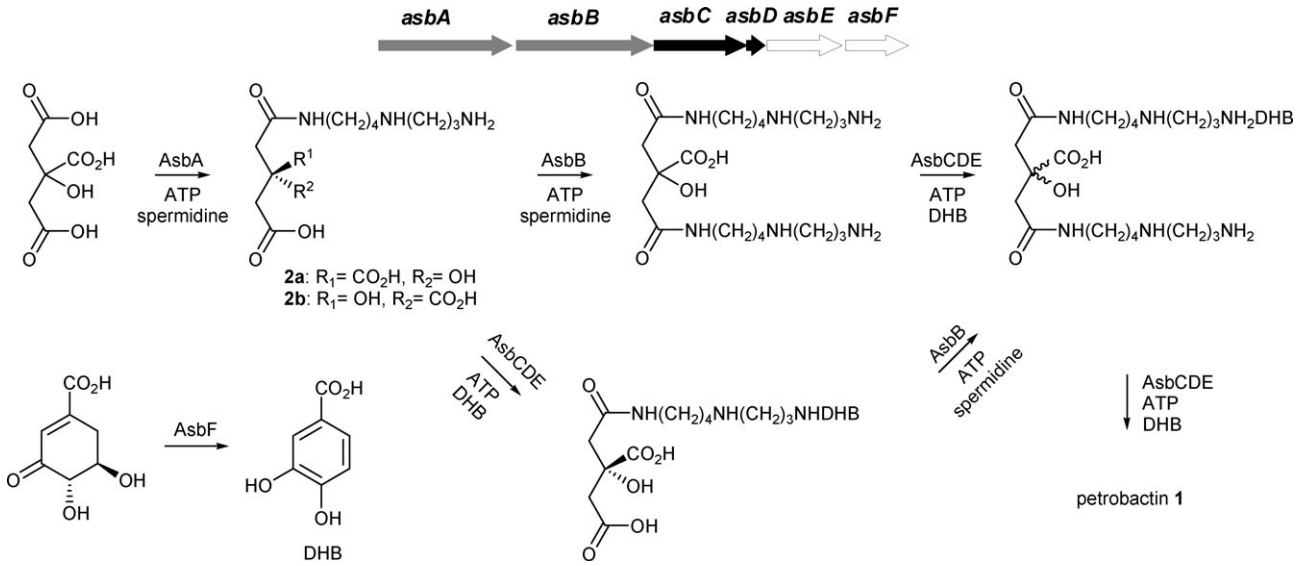


Fig. 1 Organisation of the *asbABCDEF* gene cluster that directs petrobactin production in *B. anthracis* (genes encoding NIS synthetases are in grey and genes encoding NRPS-like proteins are in black) and the reactions catalysed by the encoded petrobactin biosynthetic enzymes.

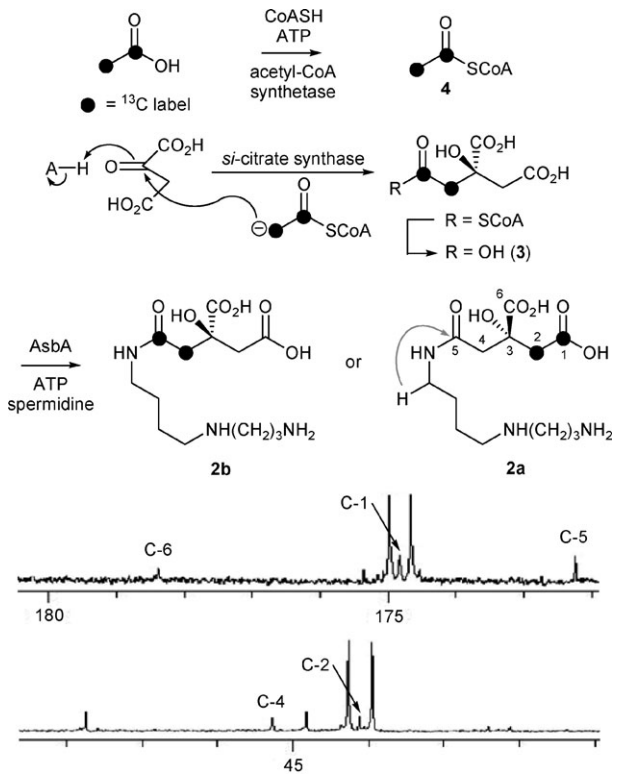


Fig. 2 Elucidation of stereocontrol in the AsbA-catalyzed condensation of citric acide and spermidine. The grey arrow in **2a** shows a key correlation observed in the HMBC spectrum of purified ^{13}C-labelled N^8-citryl-spermidine. Portions of the ^{13}C NMR spectrum of a mixture of labelled **2a** and racemic synthesised **2** revealing doublets flanking the signals for C-1 and C-2 are shown below the reaction scheme.

atom.[7c] The data reported here indicate that the adenylation of citric acid must be highly enantioselective. Only the *pro-R* carboxyl group reacts with ATP and subsequent reaction of the resulting adenylate with spermidine affords (3*S*)-N^8-citryl-spermidine **2a**.

Based on the above data it seems reasonable to assume that the stereochemical outcome of the reaction would be unaffected by substitution of spermidine with other di- and triamines. Thus, we incubated purified recombinant AsbA, citric acid, Mg^{2+} and ATP with several spermidine analogues at 37 °C for 90 minutes. We also carried out control reactions using heat-inactivated AsbA. LC-MS analyses indicated that in all cases compounds with *m*/*z* corresponding to those expected for the N^8-citryl-spermidine analogues (absent from the control reactions) were produced. The reactions where significant quantities of analogues were produced were scaled-up and the products were partially purified using reverse-phase HPLC. The identities of the resulting analogues were confirmed as **5–8** by HRMS and MS/MS analyses (Fig. 3 and ESI†). Although LC-MS analysis of incubations using 1,4-butanediamine, 1,3-propanediamine and N^1-(3,4-dihydroxybenzoyl)-spermidine in place of spermidine showed traces of compounds with *m*/*z* expected for analogues **9**, **10** and **11** (Fig. 3), no attempt to purify these was undertaken due to the small amounts of material produced.[9] Taken together the data on the stereochemical outcome of the AsbA-catalysed condensation of citric acid and spermidine, and the ability of AsbA to catalyse condensation of citric acid with several di- and triamine analogues of spermidine suggest that this enzyme may be a versatile biocatalyst for the preparation of highly enantiomerically-enriched citrate derivatives *via* a novel type of desymmetrisation process.

We also examined the capacity of AsbA to utilise citric acid analogues. Thus, we incubated purified recombinant AsbA at 37 °C for 90 minutes with spermidine, Mg^{2+}, ATP and each of the following carboxylic acids: 2-ketoglutaric acid, 3-ketoglutaric acid, D-glutamic acid, L-glutamic acid, DL-isocitric acid, glutaric acid and tricarballylic acid. LC-MS analyses of the reaction mixtures indicated that among the citrate analogues tested only tricarballylic acid (an analogue of citric acid lacking the C-3 hydroxyl group) is a productive substrate for AsbA. The resulting compound was partially

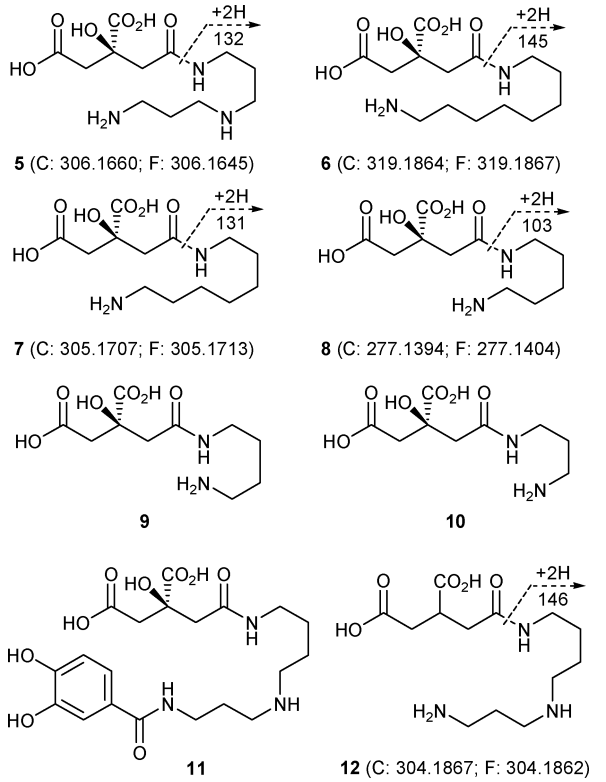


Fig. 3 Structures of citric acid and tricarballylic acid derivatives produced using analogues of spermidine and citric acid in AsbA-catalysed reactions. Diagnostic fragment ions observed in MS/MS spectra of compounds **5–8** and **12** and calculated (C) and found (F) parent ion m/z values are indicated.

purified by reverse-phase HPLC from a scaled-up incubation and HRMS and MS/MS analyses were consistent with structure **12** (Fig. 3 and ESI†). From these experiments we conclude that AsbA is significantly more selective towards citric acid than spermidine as a substrate. It would be interesting to investigate whether AsbA shows the same high degree of enantioselectivity in the desymmetrisation of tricarballylic acid as in the desymmetrisation of citric acid. The lack of a convenient method for the preparation of homochiral labelled tricarballylic acid prevented us from pursuing this question in the present study.

In conclusion, we have shown that the petrobactin biosynthetic enzyme AsbA catalyses the highly enantioselective desymmetrisation of citric acid *via* the condensation of one of its prochiral carboxyl groups with spermidine. The enzyme can also catalyse the condensation of several di- and triamine analogues of spermidine with citric acid and the condensation of the citric acid analogue tricarballylic acid with spermidine. These data indicate that AsbA could be developed into a useful and novel biocatalyst for the preparation of homochiral citric acid derivatives. To the best of our knowledge there are only two other methods for the preparation of homochiral citric acid derivatives. One involves the regio- and enantioselective esterase-catalysed hydrolysis of citrate triesters.[10] The other involves resolution by repeated fractional crystallisation of the brucine salts of 1,2-diesters of citric acid.[11] The biotransformation reported here is highly complimentary to these procedures and provides direct access to homochiral products from commercially available starting materials. It will be interesting to investigate further spermidine analogues in the AsbA-mediated catalytic enantioselective desymmetrisation of citric acid to see whether an expanded range of compounds can be produced. It will also be interesting to examine whether other NIS synthetases possess similar stereoselectivity.

This work was supported by a grant from the BBSRC (grant ref. BB/FO13760/1).

Notes and references

1 (*a*) M. Willis, *J. Chem. Soc., Perkin Trans. 1*, 1999, 1765–1784; (*b*) T. Rovis, in *New Frontiers in Asymmetric Catalysis*, ed. M. Koichi and M. Lautens, John Wiley & Sons, Hoboken, USA, 2007, ch. 9, pp. 275–311.
2 E. Garcia-Urdiales, I. Alfonso and V. Gotor, *Chem. Rev.*, 2005, **105**, 313–354.
3 M. D. Mihovilovic, *Curr. Org. Chem.*, 2006, **10**, 1265–1287.
4 A. T. Koppisch, S. Dhungana, K. K. Hill, H. Boukhalfa, H. S. Heine, L. A. Colip, R. B. Romero, Y. Shou, L. O. Ticknor, B. L. Marrone, L. E. Hersman, S. Iyer and C. E. Ruggiero, *BioMetals*, 2008, **21**, 581–589.
5 S. Cendrowski, W. MacArthur and P. Hanna, *Mol. Microbiol.*, 2004, **51**, 407–417.
6 R. J. Abergel, M. K. Wilson, J. E. L. Arceneaux, T. M. Hoette, R. K. Strong, B. R. Byers and K. N. Raymond, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 18499–18503.
7 (*a*) J. Y. Lee, B. K. Janes, K. D. Passalacqua, B. F. Pfleger, N. H. Bergman, H. Liu, K. Håkansson, V. S. Ravindranadh, C. A. Courtney, S. Cendrowski, P. C. Hanna and D. H. Sherman, *J. Bacteriol.*, 2007, **189**, 1698–1710; (*b*) B. F. Pfleger, J. Y. Lee, R. V. Somu, C. C. Aldrich, P. C. Hanna and D. H. Sherman, *Biochemistry*, 2007, **46**, 4147–4157; (*c*) D. Oves-Costales, N. Kadi, M. J. Fogg, L. Song, K. S. Wilson and G. L. Challis, *J. Am. Chem. Soc.*, 2007, **129**, 8416–8417; (*d*) D. Oves-Costales, N. Kadi, M. J. Fogg, L. Song, K. S. Wilson and G. L. Challis, *Chem. Commun.*, 2008, 4034–4036; (*e*) A. T. Koppisch, K. Hotta, D. T. Fox, C. E. Ruggiero, C. Y. Kim, T. Sanchez, S. Iyer, C. C. Browder, P. J. Unkefer and C. J. Unfeker, *J. Org. Chem.*, 2008, **73**, 5759–5765; (*f*) B. F. Pfleger, Y. Kim, T. D. Nusca, N. Maltseva, J. Y. Lee, C. M. Rath, J. B. Scaglione, B. K. Janes, E. C. Anderson, N. H. Bergman, P. C. Hanna, A. Joachimiak and D. H. Sherman, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 17133–17138; (*g*) D. T. Fox, K. Hotta, C. Y. Kim and A. T. Koppisch, *Biochemistry*, 2008, **47**, 12251–12253.
8 D. P. Bloxham, D. C. Parmelee, S. Kumar, R. D. Wade, L. H. Ericsson, H. Neurath, K. A. Walsh and K. Titani, *Proc. Natl. Acad. Sci. U. S. A.*, 1981, **78**, 5381–5385.
9 N^1-(3,4-dihydroxybenzoyl)-spermidine is a potential intermediate in petrobactin biosynthesis. We previously reported that we could detect no activity with this spermidine analogue as a substrate for AsbA.[7c] Here we employed lower concentrations of this compound than in our previous experiments. High concentrations of the compound appear to inhibit the enzyme, as suggested by the decreased amounts of **11** produced with higher concentrations of N^1-(3,4-dihydroxybenzoyl)-spermidine. We employed our previously reported[7d] AMP release assay to measure the relative rates of product formation using concentrations of ATP and citric acid presumed to be well above the K_m, with different concentrations of N^1-(3,4-dihydroxybenzoyl)-spermidine. In all cases the measured rates were comparable to the background rate of AMP formation resultingfrom ATP hydrolysis and much lower than the rates observed using spermidine as a substrate. These data provide further support for our previous conclusion[7c,d] that N^1-(3,4-dihydroxybenzoyl)-spermidine is not a significant intermediate in petrobactin biosynthesis.
10 R. Chenevert, B. Ngatcha, T. Beatrice, Y. Rose, S. Yannick and D. Goupil, *Tetrahedron: Asymmetry*, 1998, **9**, 4325–4329.
11 R. J. Bergeron, M. Xin, R. E. Smith, M. Wollenweber, J. S. McManis, C. Ludit and K. A. Abboud, *Tetrahedron*, 1997, **53**, 427–434.