# β-Lactam synthetase: implications for β-lactamase evolution

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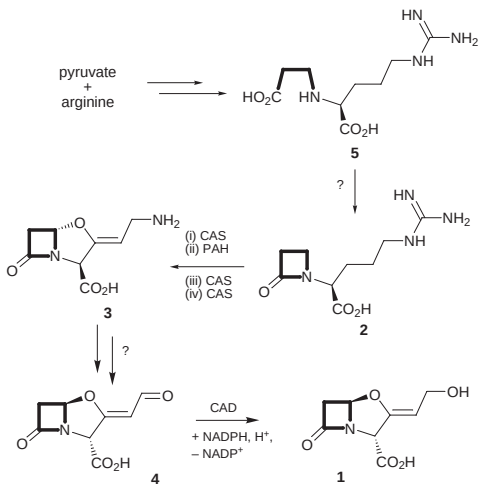
*Received (in Cambridge, UK) 27th August 1998, Accepted 15th September 1998*

**A synthetase enzyme catalyses formation of the monocyclic β-lactam ring during clavulanic acid biosynthesis.**

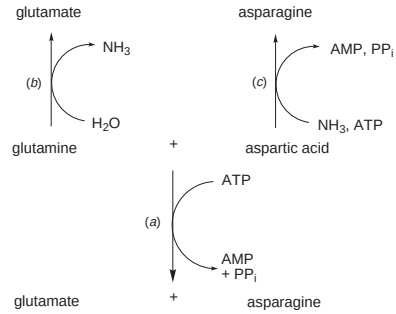
The most important inhibitor of serine β-lactamases is clavulanic acid **1**,[1] which is produced by some *Streptomyces spp*.[2] Whilst the central portion (*i.e*. **2** to **3**) of the clavulanic acid **1** biosynthesis pathway is established, significant gaps remain (Scheme 1). Nearing the end of the pathway the process by which the stereochemistry of the ring junction and C-3 positions of clavulanic acid are inverted to give the aldehyde **4** is unknown. At the beginning of the pathway, the intermediates leading to monocyclic β-lactam **2** are unclear. Labelling experiments have demonstrated that likely direct primary metabolic precursors of the 3 and 5-carbon portions of the clavulanic acid skeleton are pyruvate[3,4] and arginine.[5] Further studies have indicated that **5** is an intermediate in the pathway.[6]

Our sequencing studies demonstrated that within the biosynthesis gene cluster[7] of **1** an open reading frame (ORF3) displays a *ca*. 25% sequence identity over 434 of its 513 residues with Asn synthetase B (AS-B), the first 50 amino acids showing no homology. AS-B catalyses the ATP dependent synthesis of Asn from Asp and Gln; *in vitro* Gln can be replaced with $NH_3$ (Scheme 2).[8] We speculated that the ORF3 gene encoded the enzyme responsible for the conversion of **5** to **2**. Instead of catalysing the intermolecular formation of an amide bond as for AS-B, we envisaged that a synthetase catalysed the cyclisation of **5** to **2**, which in one sense may be viewed as a 'reverse' β-lactamase.

The ORF3 gene was PCR amplified from a cosmid containing the genetic information for the biosynthesis of **1** and sub-cloned into the pET-24a(+) vector,‡ which expressed




**Scheme 1** Outline of the biosynthetic pathway leading to clavulanic acid. CAS = clavaminate acid synthase, PAH = proclavaminate amidinohydrolase, CAD = clavulanic acid dehydrogenase. Each CAS catalysed step is coupled to the conversion of 2-oxoglutarate and dioxygen to succinate.

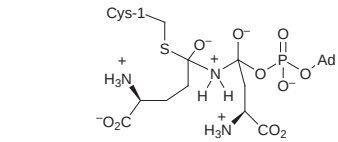



**Scheme 2** Reactions catalysed by *E. coli* asparagine synthetase B (AS-B): (*a*) *in vivo* reaction, and (*b*) and (*c*) reactions also catalysed *in vitro*.

native recombinant enzyme in *Escherichia coli* BL21(DE3) at *ca*. 10% of total soluble cell protein. The recombinant ORF3 protein was purified by standard methodologies. N-Terminal sequencing, Western blot, and SDS-PAGE analyses demonstrated the expression and purification of the desired 56 kDa protein.§ Synthetic standards of **5** and **2** were prepared by published methods[9] and used to devise HPLC assays for these compounds. Crude extracts of the recombinant *E. coli* containing the ORF3 gene product and purified protein product were tested for the ability to catalyse the conversion of synthetic **5** to β-lactam **2**. The most sensitive assay involving the derivatisation of guanidino side chains based on the work of Kai *et al*.[10] showed clear conversion of **5** to **2**, when using either crude or purified recombinant protein, but only in the presence of $Mg^{2+}$, enzyme and ATP. The production of **2** was confirmed by preparative HPLC leading to its isolation. Spectroscopic characterisation of **2** showed it to be identical to the authentic synthetic material [$\delta_H$(500MHz; $D_2O$) 1.48–1.55 (2H, m, $CH_2CH_2CH_2N$), 1.65–1.73 and 1.76–1.80 (2 × 1H, 2 × m, $CH_2CH_2CHN$), 2.82–2.89 (2H, m, $NCH_2CH_2CO$), 3.13 (2H, t, *J* 5, $CH_2CH_2CH_2N$), 3.29–3.31 and 3.35–3.38 (2 × 1H, 2 × m, $NCH_2CH_2CO$), 3.99 (1H, dd, *J* 5 and 10, $CHCO_2H$); *m/z* (ESI) 229 ($MH^+$)]. The ORF3 product catalysing the conversion of **5** to **2** was named β-lactam synthetase 1 [BLS (1)].

AS-B possesses an N-terminal cysteine residue and is a member of the family of Ntn (N-terminal nucleophilic) amidotransferases.[11] Studies on AS-B suggest that the N-terminal cysteine attacks the carbonyl of the amide of Gln, in order to increase the nucleophilicity of the amide nitrogen for reaction with the acceptor carbonyl.[12] The β-carboxylate of Asp is activated by reaction with ATP to form β-Asp-AMP and pyrophosphate ($PP_i$). The timing of nitrogen transfer from the activated glutamine is uncertain, but may proceed *via* a complex to which AMP is still bound **6** or an imide intermediate **7**.[13]

Amongst Ntn enzymes, BLS has an unusual N-terminus lacking an N-terminal cysteine (or serine/threonine), and is probably incapable of activating the nitrogen of the Gln amide for nucleophilic attack. We propose BLS activates the β-amino acid **5** by reaction with ATP to form an acyl adenylate **9** (Scheme 3). Instead of being attacked by Gln this intermediate undergoes cyclisation to **2** and AMP. The N-terminal residue of



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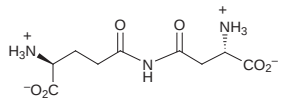

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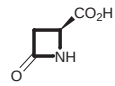

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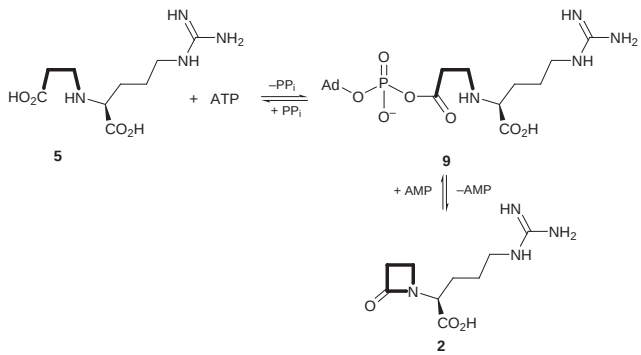
BLS is Gly, implying an Ntn type mechanism is not operating in BLS catalysis, unless the N-terminal amino group acts as the general base presumably required to deprotonate the β-amino group of **5**. The proposed BLS mechanism leads to the possibility that the transfer of $NH_3$ from Gln to Asp during AS-B catalysis also proceeds *via* a β-lactam, **8**. Whatever, the structural and mechanistic relationships between AS-B enzymes and BLS suggest a close evolutionary relationship. Studies aimed at converting AS-B into a β-lactam synthetase and *vice versa* have been initiated.

An open reading frame (*car*A) encoding for an enzyme with sequence similarities to AS-B/BLS has also been found within the biosynthetic gene cluster encoding for enzymes making 1-carbapenem-3-carboxylic acid.[14,15] The particular step catalysed by the CarA enzyme is unknown, but it has been proposed that it catalyses the formation of the β-lactam ring.[15,16] Our results support this proposal, but suggest the CarA enzyme catalyses an ATP/synthetase type reaction rather than a β-lactam cyclisation reaction proceeding *via* a thiol ester intermediate.[17,18]

The only other β-lactam forming enzyme to be so far described is isopenicillin N synthase,[19] which catalyses the oxidation of the peptide L-δ-(α-aminoadipoyl)-L-Cys-D-Val to give isopenicillin N, the first β-lactam formed in the biosynthesis of penicillins. Thus, β-lactam formation during the biosynthesis of clavams, which is driven by the hydrolysis of ATP, occurs *via* a very different mechanism to that during penicillin formation, which is driven by the reduction of dioxygen.

Since AS-B and related enzymes are widespread, and relatively minor modifications of them might lead to BLS type enzymes, BLS activity may have evolved before the less common and seemingly more complex secondary metabolic machinery required for penicillin and cephalosporin biosynthesis. Simple β-lactams such as **8** might have been (and may still be?) present in metabolism before other β-lactams which are more well known because of their antibacterial activity. β-Lactamases may have initially evolved to hydrolyse these monocyclic β-lactams, suggesting they may be ancient enzymes, and it is possible the chromosomal location of some β-lactamase genes reflects this.



**Scheme 3**

Analogous modifications of other Ntn enzymes may result in the formation of enzymes catalysing intramolecular cyclisation rather than intermolecular reactions. Thus, for example, modification of Ntn glutamine synthetases would lead to enzymes catalysing the formation of pyroglutamate from glutamate or other γ-lactams from appropriate γ-amino acid precursors.

Note: After acceptance of our manuscript we became aware of the work of Bachmann *et al*.,[20] which also demonstrates that ORF3 encodes for a BLS involved in clavam biosynthesis.

Dedicated to Professor Jack E. Baldwin on the occasion of his 60th birthday.

## Notes and references

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‡ Automated fluorescence DNA sequencing verified the sequence of the first 500bp.

§ Edman degradation of the recombinant protein gave the amino acid sequence GAPVLPAAFGFLASARTGGG, identical with that predicted for the native protein.


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*Communication 8/06713I*