Evidence that Arginine is a Later Metabolic Intermediate than Ornithine in the Biosynthesis of Clavulanic Acid by *Streptomyces clavuligerus*

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The feeding of radiolabelled ornithine and arginine to mutants of *Streptomyces clavuligerus* auxotrophic for arginine has shown that ornithine has to be converted to arginine prior to incorporation into clavulanic acid.

Papers[1,2] from these laboratories have described the isolation of proclavaminic acid **1** and clavaminic acid **2** and their identification as biosynthetic precursors of the commercially important β-lactamase inhibitor[3] clavulanic acid **3**. Results of feeding experiments using the labelled five carbon α-amino acids δ-hydroxynorvaline, [4,5] glutamic acid,[6] β-hydroxyornithine,[7] ornithine[5,8,9] and arginine[5,9] have indicated that the urea cycle amino acids ornithine and arginine are the most efficiently incorporated into clavulanic acid.[5,9] This evidence indicates that an amino acid from the urea cycle provides the carbon atoms of positions 2, 3, 8, 9 and 10 of clavulanic acid, and hence the same carbon atoms of clavaminic acid **2** and carbon atoms 1–5 of proclavaminic acid **1**.

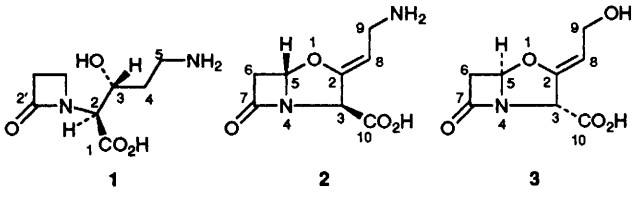
Ornithine and arginine are interconvertable by the urea cycle (Scheme 1) and arginase has been shown to be functioning during *S. clavuligerus* fermentation.[9] By considering the efficiency of incorporation of labelled ornithine and arginine into clavulanic acid, Townsend and Ho[5] and Romero *et al.*[9] concluded that ornithine is probably the amino acid from the urea cycle that is processed into the clavulanic acid pathway. This and the two following communications demonstrate that it is arginine that is used in the clavulanic acid biosynthetic pathway, and describe the results of experiments which clarify the nature of the biosynthetic steps between the urea cycle and proclavaminic acid.

Spores of *S. clavuligerus* were subjected to mutagenesis using far ultraviolet radiation and arginine auxotrophs were selected. Two auxotrophs, argB-2 and argC-2, were identified, each lacking one of the critical enzymes for converting ornithine to arginine, respectively argininosuccinate synthase and ornithine carbamoyl transferase (see Scheme 1). Both auxotrophs produced clavulanic acid when grown in complete media. If the biosynthetic pathway to proclavaminic acid **1**, and hence clavulanic acid **3**, leaves the urea cycle at ornithine both argB-2 and argC-2 should be capable of incorporating labelled ornithine into clavulanic acid. If proclavaminic acid is biosynthesised from arginine the two auxotrophs would not be able to incorporate labelled ornithine into clavulanic acid.

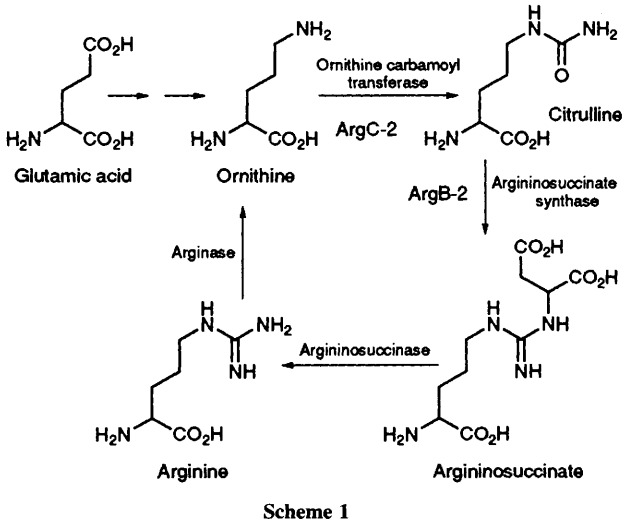
Cells from fermentations[10] of the two mutants and the parent strain were treated separately with [U-^{14}C]ornithine or [U-^{14}C]arginine and incubated for 4 h. The supernatants were analysed by TLC eluting with a system which separated ornithine, arginine and clavulanic acid.‡ Autoradiography indicated that both of the radiolabelled amino acids had been incorporated into clavulanic acid in the parent strain and labelled arginine was incorporated into clavulanic acid in both argB-2 and argC-2. However, ornithine did not appear to be incorporated into clavulanic acid by argB-2 and argC-2. Owing to the difficulty of accurate quantitation of this result by TLC the experiment was repeated on a scale such that the clavulanate could be isolated and counted in order to put an upper limit on the level of ornithine incorporation.

[U-^{14}C]ornithine was fed separately to fermentations of argB-2 and argC-2 during the clavulanic acid production phase. The clavulanic acid isolated as the *p*-bromobenzyl ester from each of these fermentations was repeatedly recrystallised. In both cases the radioactive content was so low (approximately twice background) that crystallisation was stopped before constant specific activity was achieved. Incorporations calculated from the final activities were 0.03% for argB-2 and 0.06% for argC-2 which, when compared with 10.6%[8] of ornithine into clavulanic acid by *S. clavuligerus* ATCC 27064, indicates the incorporation of ornithine into clavulanic acid is blocked in the arginine auxotrophs.

Thus, cells specifically blocked in the synthesis of arginine from ornithine are unable to use ornithine for the production of clavulanic acid, while the unblocked strain is able to use either ornithine or arginine. The logical explanation of these data is that arginine is taken into the clavulanic acid biosynthetic pathway and ornithine is not a direct precursor but proceeds *via* the urea cycle and arginine. The conversion of the guanidino function of the arginine moiety to the amino group of **1** thus occurs in secondary metabolism indicating that the early precursors in the clavulanic acid pathway will be guanylated. Consequently, an amidino hydrolase must operate later in the pathway in order to arrive at proclavaminic acid **1**. Data from the sequencing of the DNA of the clavulanic acid gene cluster has identified an open reading frame which shows homology to arginase.[11] The stage at which this conversion occurs, and evidence as to the nature of the intermediates



1 **2** **3**



Scheme 1

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‡ Polygram Silica G eluting with butanol : ethanol : water [2 : 1 : 1]

between arginine and proclavaminic acid **1**, is presented in the following communications.

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