The Identification of Three new Biosynthetic Intermediates and One further Biosynthetic Enzyme in the Clavulanic Acid Pathway

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Labelling experiments are described that identify three new compounds, N^2-(2-carboxyethyl)arginine, 5-guanidino-(2-oxoazetidin-1-yl)pentanoic acid, and 3-hydroxy-5-guanidino-2-(2-oxoazetidin-1-yl)pentanoic acid as biosynthetic precursors of proclavaminic acid and hence clavulanic acid in *Streptomyces clavuligerus* ATCC 27064 and a new amidino hydrolase, which hydrolyses 3-hydroxy-5-guanidino-2-(2-oxoazetidin-1-yl)pentanoic acid to proclavaminic acid has been characterised.

As part of our goal to elucidate the biosynthesis of clavulanic acid **1**[1,2] we demonstrated[3] that arginine **2** and not ornithine is the amino acid that is processed into the biosynthetic pathway.

Also we reported[4] on the production of two new arginine derivatives **3** and **4** by the mutant *S. clavuligerus* dclH 65 which is blocked in clavulanic acid biosynthesis. In addition, sequencing of the DNA of the clavulanic acid gene cluster identified an open reading frame, which showed homology to arginase.[5] From these data we can postulate a number of possible biosynthetic sequences prior to proclavaminic acid **5** which are shown in Scheme 1. In this communication we

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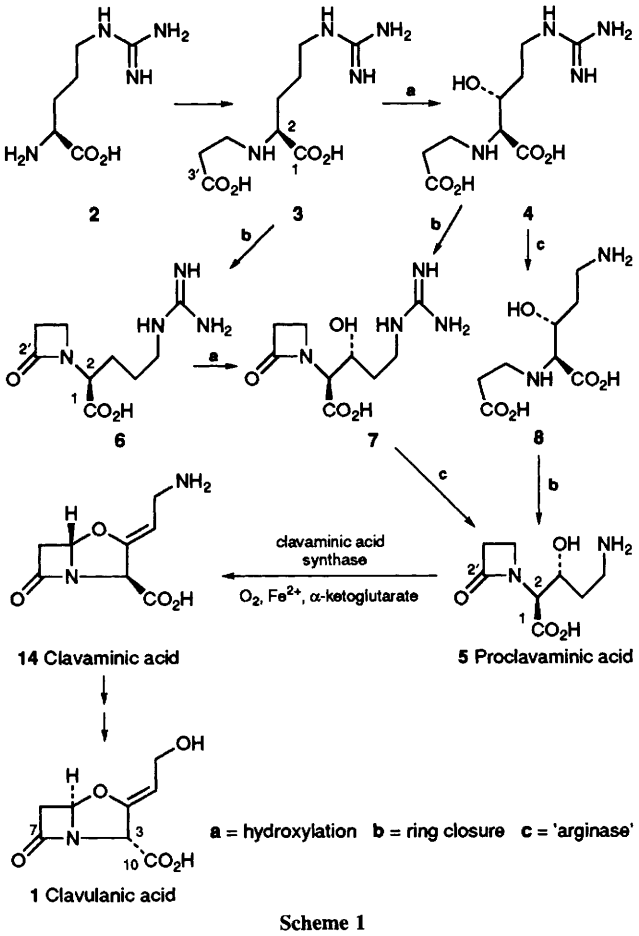
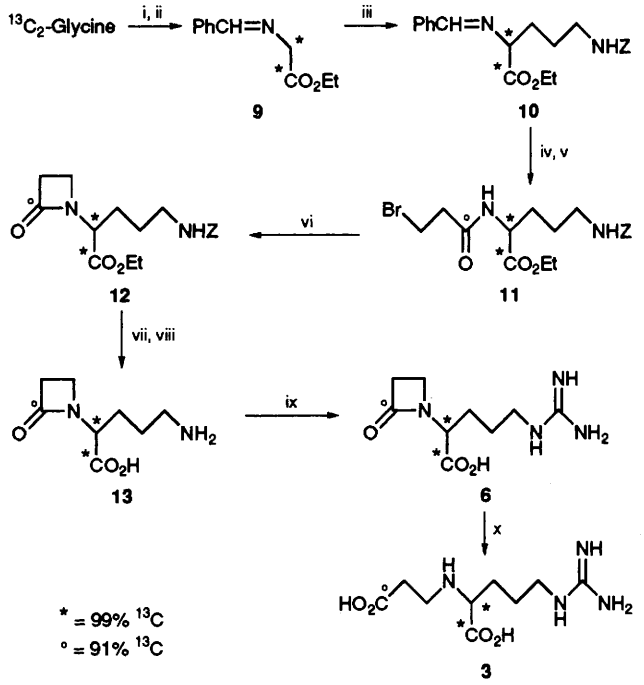


Scheme 1



Scheme 2 *Reagents and conditions*: Z = $PhCH_2OCO^-$: i, EtOH, HCl, heat (99%); ii, PhCHO, $MgSO_4$, Et_3N (96%); iii, lithium diisopropylamide, hexamethylphosphoramide, ICH_2CH_2NHZ, iv, 1 mol dm^{-3} HCl; v, NaOH, $BrCH_2CH_2$ $^{13}COCl$; vi, powdered KOH, tetrabutylammonium bromide, H_2O, sonicate (32% overall from **9**); vii, Na_2CO_3, 40% aq. EtOH;[19] (91%) viii, H_2, Pd-C (10%) (99%); ix, K_2CO_3, aminoiminomethanesulfonic acid (60%); x, 1 mol dm^{-3} HCl (100%).

present the results of feeding ^{13}C-labelled compounds in order to elucidate these early biosynthetic events.

Commencing with **3**, at least three biochemical steps are required to generate proclavaminic acid **5**, *i.e.* the introduction of a hydroxy function (step **a**), formation of the β-lactam ring (step **b**) and hydrolysis of the guanidino group to an amino group (step **c**). To determine which, if any, of compounds **3**, **4**, **6**, **7** and **8** actually lie on the pathway each was synthesised triply labelled with ^{13}C for feeding experiments. In each case the five carbon chain was labelled in the 1 and 2 positions (99% ^{13}C) and also the carbonyl group of the three carbon unit (91% ^{13}C). This labelling strategy was adopted since it was known[6] that spin–spin coupling, between carbons 7, 3 and 10 of clavulanic acid, would be observed in the ^{13}C NMR spectrum if incorporation of all three labelled carbons occurred without bond breakage.

Treatment of DL-*threo*-[1,2,2′-$^{13}C_3$]proclavaminic acid[6]‡ **5** with aminoiminomethanesulfonic acid[7] yielded [1,2,2′-$^{13}C_3$]-**7** which on acid hydrolysis yielded [1,2,3′-$^{13}C_3$]-**4**. Acid hydrolysis of [1,2,2′-$^{13}C_3$]-**5** gave [1,2,3′-$^{13}C_3$]-**8**. The synthesis of [1,2,3′-$^{13}C_3$]-**3** and [1,2,2′-$^{13}C_3$]-**6** was accomplished according to Scheme 2. Following the method of Stork *et al.*[8] $^{13}C_2$-glycine (99% 1,2-^{13}C) was elaborated into the protected [1,2-$^{13}C_2$]-ornithine derivative **10** *via* the benzylidene glycine ester **9**. Mild acid hydrolysis of **10** yielded the amino ester which was acylated with [1-^{13}C]-3-bromopropionyl chloride (91% 1-^{13}C)[6] to yield **11**. Base treatment of **11** gave the β-lactam **12**. Cleavage of the carboxy and amino protecting groups afforded the [1,2,2′-$^{13}C_3$]-**13**[1] which was converted to the target guanidino acid [1,2,2′-$^{13}C_3$]-**6** with aminoiminomethanesulfonic acid.[7] Acid treatment of [1,2,2′-$^{13}C_3$]-**6** yielded [1,2,3′-$^{13}C_3$]-**3**.

The five racemic $^{13}C_3$-labelled compounds **3**, **4**, **6**, **7** and **8** were each fed to an *S. clavuligerus* ATCC 27064 fermentation, in the clavulanic acid production phase, and the clavulanic acid samples produced were isolated as the benzyl ester.[6] The levels of incorporation of the administered compounds were determined by ^{13}C NMR and after silylation, GC/MS of the 9-*O*-silylated derivatives. The observed ^{13}C-incorporations are summarised in Table 1. In the three samples where ^{13}C-enrichment was observed (compounds **3**, **6** and **7**), it was noted that enrichment was specific to carbons 3, 7 and 10 of clavulanate with ^{13}C–^{13}C spin–spin couplings being observed between all three labelled centres (Fig. 1) indicating that bond breakage had not occurred during incorporation. These results lead us to the conclusion that **3**, **6** and **7** are precursors in the biosynthesis of proclavaminic acid **5**, and hence clavulanic acid, and that logically they occur in the pathway in that order. The evidence indicates that **4** does not lie on the biosynthetic pathway and the production of this compound by the blocked mutant dclH 65[4] may result from the hydroxylation of **3** or an ester thereof.

Clearly, the transformation of **7** to **5** could be mediated by an arginase, and the genetic evidence[5] indicates that such an enzyme may occur in the clavulanic acid biosynthetic pathway. Accordingly, we looked for evidence of this enzymatic activity. We found that cell-free preparations of *S. clavuligerus* ATCC 27064 were able to convert one of the enantiomers of racemic **7** quantitatively to proclavaminic acid **5** and urea. The activity was enhanced in the presence of Mn^{2+}, a phenomenon reported for arginases from *Bacillus anthracis*[9] and staphylococcal[9,10] sources. (2*S*)-**6**, prepared from the corresponding (2*S*)-5-amino derivative,[1] was also hydrolysed under these conditions, but much less rapidly than **7**, whereas no hydrolysis could be detected for **3** or **4**. Clearly this enzyme, which we have named proclavaminic acid amidino hydrolase

‡ Satisfactory analytical and/or spectroscopic data were obtained for all new compounds.

Table 1 Summary of ^{13}C-labelled precursor feeding experiments

Precursor	% Incorporation[a] (^{13}C NMR)	% Incorporation[a] (Mass spec.)
3	1.1	1.3
4	ND	ND
6	11.8	12.0
7	5.1	5.3
8	ND	ND

[a] ND = No enrichment detected at any carbon centre. Calculations based on one enantiomer being utilised.

(PAH), is able to discriminate efficiently between the guanidino groups of these early biosynthetic precursors. Since the absolute stereochemistry of natural proclavaminic acid is (2*S*,3*R*), the enantiomer of **7** which is the substrate for PAH will also presumably have the (2*S*,3*R*) stereochemistry.

PAH was purified by conventional techniques until it showed essentially one band on SDS-PAGE electrophoresis, which corresponded to an M_r of 33 000. The pure enzyme did not hydrolyse arginine to ornithine and is therefore different to the arginase previously reported in *S. clavuligerus*.[11] The N-terminal amino acid sequence of PAH correlated with the open reading frame of the amidino hydrolase related-gene in the clavulanic acid genetic cluster.[5] The M_r for the amidino hydrolase calculated from the DNA sequence was 33 374. From the above data we conclude that (2*S*,3*R*)-**7** is probably converted directly to (2*S*,3*R*)-**5** in the biosynthetic pathway to clavulanic acid.

The lower level of incorporation of ^{13}C-labelled **3** compared with **6** and **7** indicates that either the compound is less efficiently transported into the cells, or that the true intermediate is possibly a derivative of **3**, such as a coenzyme A thioester, which might favour ring closure. The result with **3** indicates that the generation of the β-lactam of **6** is by a biochemically unprecedented process, *i.e.* by amide bond formation, whereas the literature indicates that the precursors of the other monocyclic β-lactams, the nocardicins[12] and probably monobactams,[12,13] already have the amide bond formed prior to ring closure. In these compounds ring closure is by displacement of a serine derived hydroxy function with inversion. It is perhaps ironic that clavulanic acid, a medicinally useful β-lactamase inhibitor, appears to be biosynthesised by a mechanism that is formally the reverse of that of β-lactamases! Previous labelling studies have shown that the β-lactam carbons of clavulanic acid are derived from the glycolytic pathway, with pyruvate[14] and D-lactate[15] being specifically incorporated into the β-lactam ring. Hence, the C_3 moieties of **3**, **6** and **7** would be derived from the same pathway. It is known that pyruvate and lactate can be biochemically converted to acrylate[16] and malonic semialdehyde.[17] Enzymatically catalysed Michael addition of arginine to acrylate, or Schiff's base addition with malonic semialdehyde, followed by reduction would yield precursor **3** in either case. The possible derivation of **3** from the reaction of arginine with malonic semialdehyde is of particular interest as it bears a strong resemblance to the biosynthesis of the opine metabolites, where similar Schiff's base formation and subsequent reduction occur.[18]

Our recent paper[2] with Professor Baldwin's group reported that clavaminic acid synthase (CAS), the enzyme which oxidatively cyclises proclavaminic acid **5** to clavaminic acid **14** later in the pathway (Scheme 1), efficiently hydroxylates (2*S*)-**6** to give **7** *in vitro*. Further studies are required to determine whether CAS is responsible for this hydroxylation *in vivo*.

The evidence presented indicates that, the β-lactam ring of proclavaminic acid is constructed in a novel manner involving an amide forming reaction; that the arginine derivatives (2*S*)-**6** and (2*S*,3*R*)-**7** are the biosynthetic precursors of proclavaminic acid **5**, that (2*S*)-**6** is hydroxylated to (2*S*,3*R*)-**7** and that a new enzyme PAH hydrolyses (2*S*,3*R*)-**7** to proclavaminic acid **5**. This work now brings the number of known biosynthetic intermediates in the clavulanic acid pathway to five.[6]

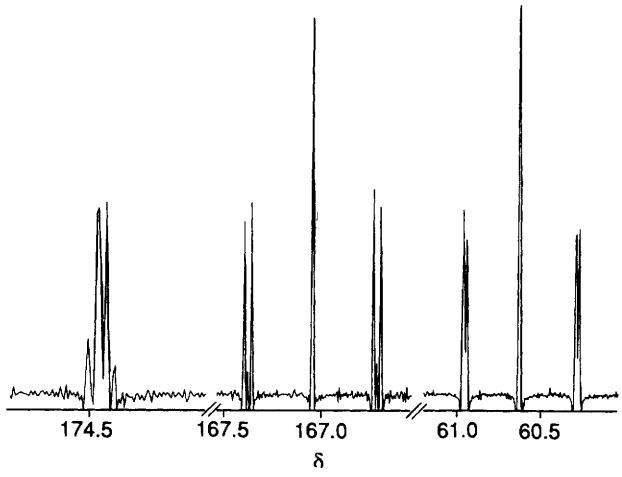


Fig. 1 ^{13}C NMR spectrum of carbons 7, 10 and 3 of benzyl clavulanate from feeding **6**. $J_{7,10}$ 3.8, $J_{3,7}$ 1.9 and $J_{3,10}$ 67.2 Hz.

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