The Roles of Clavaminic Acid and Proclavaminic Acid in Clavulanic Acid Biosynthesis

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Clavulanic acid samples isolated from fermentations of *Streptomyces clavuligerus* ATCC 27064 fed with ^{13}C-clavaminic acid or ^{13}C-proclavaminic acid were found to be appropriately labelled, indicating that the title compounds are biosynthetic precursors of clavulanic acid.

In a previous paper[1] we described the isolation of two ornithine-containing metabolites, clavaminic acid (**1**) and proclavaminic acid (**2**), from the mycelium of the clavulanic acid (**3**) producer, *Streptomyces clavuligerus* ATCC 27064. We also isolated an enzyme which converted (**2**) into (**1**). As ornithine is known[2] to be a biosynthetic precursor of clavulanic acid, we suspected that (**1**) and (**2**) might be late-stage intermediates in the biosynthetic pathway. We therefore attempted to show that clavaminic acid (**1**) could be converted to clavulanic acid (**3**) in a cell-free system.

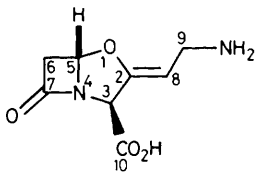
When clavaminic acid (**1**) was added to ultrasonically disrupted *S. clavuligerus* ATCC 27064 mycelium in the presence of pyridoxal phosphate, pyruvate, and NADPH, a small production (0.3% yield) of clavulanic acid (**3**) was observed. An α-keto acid was found to be necessary, with glyoxalate, pyruvate, or α-ketobutyrate being preferred by the enzyme system. Attempts to improve the yield of clavulanic acid have not so far been successful, possibly due to instability of the converting enzyme system. In order to give stronger evidence that the two novel metabolites are precursors of clavulanic acid we resorted to isotopic labelling and feeding experiments.

The two diastereoisomers (**2a**) and (**2b**) of proclavaminic acid were prepared in ^{13}C-labelled form as shown in Scheme 1. Samples of ^{13}C-labelled (**2a**) were also converted into the corresponding labelled clavaminic acids (**1**) by treatment with a partially purified preparation of clavaminic acid synthetase.[1] The reaction mixtures were purified to remove the remaining unnatural enantiomer of (**2a**). Labelled samples of (**1**), (**2a**), and (**2b**) were separately fed to *S. clavuligerus* fermentations

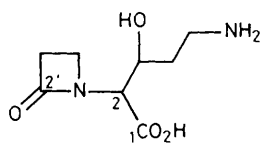
Table 1. Summary of ^{13}C-labelled precursor feeding experiments.

		Position of ^{13}C-label in		
Experiment	Precursor	Precursor	Benzyl clavulanate	% incorporation
1	(**1**)	3 and 10	3 and 10	6.5
2	(**1**)	3, 7 and 10	3, 7 and 10	4.3
3	(**2a**)	1 and 2	1 and 2	3.5[a]
4	(**2b**)	1 and 2	ND[b]	ND[b]

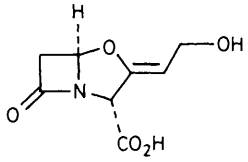
[a] Assuming that only one enantiomer is incorporated. [b] ND = No labelling detected.



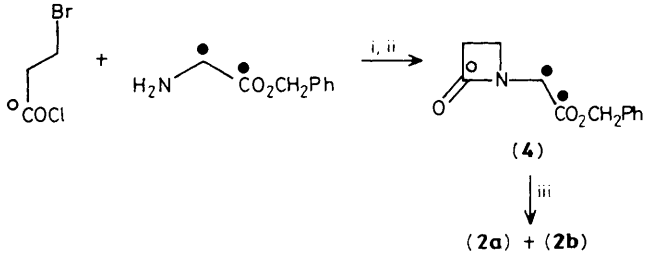
(3S,5S)-(1)



(2) †



(3R,5R)-(3)



Scheme 1. *Reagents and conditions:* i, tetrahydrofuran–H_2O; ii, tetrabutylammonium bromide–NaOH; iii, as in ref. 3. ● ^{13}C (99 atom %), ○ ^{12}C or ^{13}C (91 atom %).

during the clavulanic acid production phase. The resulting samples of clavulanic acid were isolated as the benzyl ester[4] and examined by ^{13}C n.m.r. The observed ^{13}C-labelling patterns of the benzyl clavulanate samples are summarised in Table 1. In the three samples where ^{13}C-enrichment was observed, ^{13}C–^{13}C spin-spin couplings were observed between all labelled centres indicating that bond breakage had not occurred during incorporation. The benzyl clavulanate derived from Experiment 1 was shown to be enantiomerically pure by conversion into clavulanic acid by hydrogenolysis and coupling to *R*-phenylalanine methyl ester under standard acylation conditions. The resulting clavulanyl-*R*-phenylalanine methyl ester was examined by h.p.l.c. and found to contain no (<1%) (3*S*,5*S*)-clavulanate. As the ^{13}C n.m.r. shifts for C-7, C-3 and C-10 of the benzyl ester of (**1**) are almost identical to those of the benzyl ester of (**3**) it was important to show that the apparent labelling of benzyl clavulanate was not due to contamination with the highly enriched precursor or derivative thereof. Examination of the benzyl clavulanate from Experiment 2 by ^1H n.m.r. showed no shift corresponding to a 9-aminodeoxyclavulanate species (detection limit <1%). The benzyl clavulanate was further converted into the 9-O-methyl ether,[5] which was examined by gas chromatography–mass spectrometry. This confirmed that the ^{13}C-enriched species was derived from (**3**) rather than from (**1**). The extent of incorporation of precursors (**1**) and (**2a**) into clavulanic acid was not as high as one might expect for late-stage biosynthetic intermediates. However, when the fermentation broth from Experiment 3 was examined just prior to derivatisation it was found that the majority of (**2a**) was still present in the extracellular fluid. It was calculated that virtually all of the (**2a**) which had been taken up by the cells had been incorporated into clavulanic acid. On the basis of the above experimental data we conclude that (**1**) and (**2a**) are precursors of clavulanic acid.

The penicillin–cephalosporin pathway has been the most extensively studied β-lactam biosynthetic pathway to date. Clavulanic acid biosynthesis differs from this in that, so far, no free monocyclic intermediate has been found in the former pathway. In addition, the α-centre of the valyl moiety of penicillin is inverted from *S*- to *R*-stereochemistry early in the pathway (when L-valine is incorporated into the tripeptide precursor)[6] whereas the equivalent inversion in the clavulanate pathway occurs between clavaminic acid and clavulanic acid (the C-5 centre also inverting). Elucidation of the mechanism of the inversion of these two centres in clavaminic acid is an intriguing problem which we are currently studying, along with the mechanism of β-lactam ring closure. Many of the enzymes in the penicillin–cephalosporin pathway are Fe^{2+} ion dependent dioxygenases and one marked similarity between the two pathways is that clavaminic acid synthetase[1] also belongs to this class of enzymes.

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† Diastereoisomers are (**2a**) and (**2b**). (**2a**) is the diastereoisomer which contains the enantiomer corresponding to natural proclavaminic acid.[1]

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