Isolation of Two Novel Intracellular β-Lactams and a Novel Dioxygenase Cyclising Enzyme from *Streptomyces clavuligerus*

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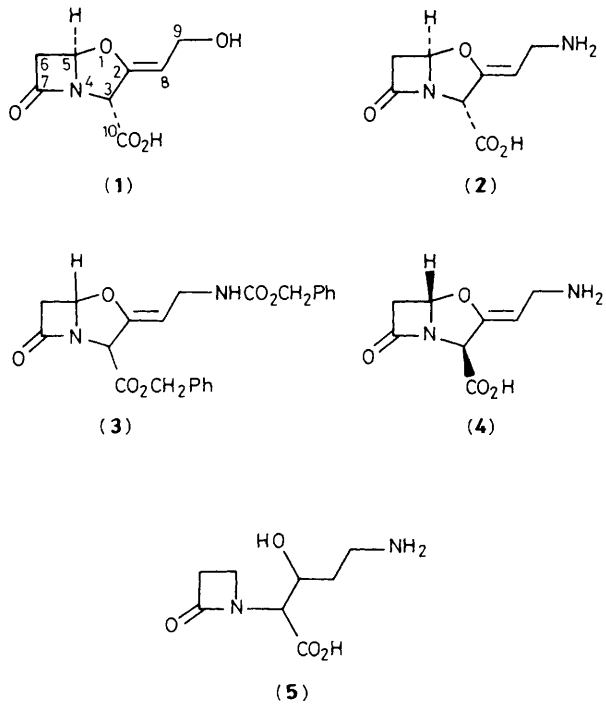
Two novel β-lactams, one monocyclic, the other bicyclic, and a dioxygenase enzyme which converts the former to the latter, have been isolated from the mycelium of the clavulanic acid producing organism *Streptomyces clavuligerus* ATCC 27064.

Clavulanic acid[1] is a commercially important β-lactamase inhibitor. It has a fused bicyclic β-lactam structure (**1**) with the 3*R*,5*R* stereochemistry.[2] Several papers have reported biosynthetic experiments which demonstrate that glycerol,[3] glyceric acid,[4] and β-hydroxypropionic acid[5] are precursors of the β-lactam moiety (C-5, -6, and -7), whereas glutamate[6] and ornithine[7] are precursors of the C-5-moiety (C-10, -3, -2, -8, and -9). These studies were carried out by feeding isotopically labelled precursors to fermentations of *Streptomyces clavuligerus*. We report here the first cell-free studies of clavulanic acid biosynthesis, undertaken with a view to finding late-stage biosynthetic intermediates.

Following other workers' successful application of cell-free techniques to penicillin and cephalosporin biosynthesis,[8,9] we attempted to demonstrate the production of clavulanic acid in ultrasonically disrupted cells of *S. clavuligerus*, ATCC 27064. As many of the enzymes involved in penicillin and cephalosporin biosynthesis are dioxygenases,[8,9] we added the co-substrate α-ketoglutarate, the co-factor Fe^{2+} ion and aerated the reaction mixture. Production of clavulanic acid was sought by bioassay[1] and by h.p.l.c. analysis having first derivatised the samples with imidazole.[10]

No production of clavulanic acid was detected; however, h.p.l.c. analysis gave a peak with a shorter retention-time



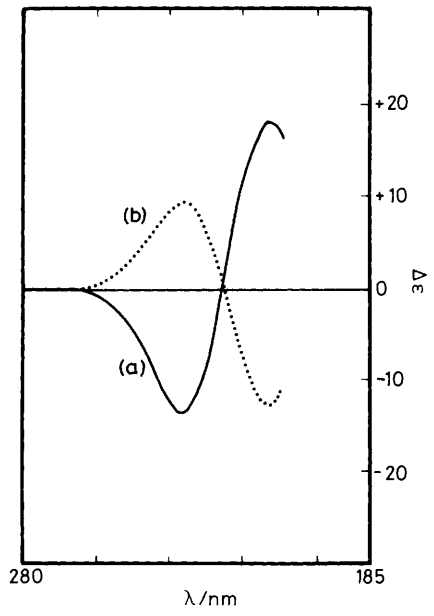


Figure 1. Circular dichroism spectra of natural metabolite and synthetic 9-aminodeoxyclavulanate. (a) Metabolite, (b) 9-aminodeoxyclavulanate; concentration; *ca.* 1 mM, pathlength: 0.2 cm, solvent: H_2O; metabolite θ −72 500 (235 nm), +82 000 (212 nm), 9-aminodeoxyclavulanate θ +64 500 (235 nm), −83 000 (212 nm).

Table 1. β-Lactamase inhibitory activity of the regenerated natural bicyclic metabolite (clavaminic acid) and 9-aminodeoxyclavulanic acid.

Source of β-lactamase	[a]I_{50}/μg ml^{-1} Clavaminic acid	9-Aminodeoxy clavulanate
Staphylococcus aureus Russell	>50	0.05
Escherichia coli K12, RGN 238 (OXA-1)	>50	0.35
Escherichia coli JT4 (TEM-1)	>50	0.38
Proteus mirabilis C889	>50	0.05
Klebsiella pneumoniae E70	>50	0.04

[a] I_{50} is the concentration of compound required to inhibit the rate of hydrolysis of nitrocefin ([S] = 250 μg ml^{-1}) by 50%. The test compounds were incubated with the enzymes for 5 min before addition of substrate.

which increased in height over a period of 20 min. When compared with analogues produced by chemical modification of clavulanic acid, the unknown substance was found to possess the same h.p.l.c. characteristics as 9-aminodeoxyclavulanate (**2**)[11] under a variety of eluting conditions. To confirm the structure of the unknown product it was isolated from a large scale cell-free reaction after derivatisation with benzyl chloroformate followed by benzyl bromide, purified, and examined by ^{1}H and ^{13}C n.m.r., i.r., and mass spectroscopy. The data indicated the material to have the constitution (**3**), by comparison with an authentic sample prepared from clavulanic acid.[12] Catalytic hydrogenolysis of the derivatised metabolite yielded a product with the same h.p.l.c. retention time as the underivatised metabolite. Examination of the hydrogenolysis product by ^{1}H n.m.r., i.r., and mass spectroscopy, indicated that the structure of the metabolite was indistinguishable from (**2**). A comparison of the β-lactamase inhibitory properties of the regenerated natural metabolite with those of (**2**) produced chemically from clavulanic acid is shown in Table 1. The metabolite was devoid of inhibitory activity as opposed to the compound derived from clavulanic acid. The rational explanation for this conflict between the physical and biological data is that the natural metabolite is the enantiomer of (**2**). This was confirmed by comparison of the circular dichroism spectra of the two compounds (Figure 1). Thus structure (**4**) was assigned to the product of the cell-free reaction, with the absolute configuration as indicated. The compound has been named clavaminic acid.

Clavaminic acid is produced during the course of the cell-free reaction and is thus being formed enzymatically from another compound (which we have named proclavaminic acid) present in the cell extract. Proclavaminic acid was isolated in substantially pure form from cell extract by column chromatography methods and reverse phase h.p.l.c. Proclavaminic acid was detected in column eluates by enzymatic cyclisation to clavaminic acid and subsequent h.p.l.c. analysis. The purified proclavaminic acid was examined by ^{1}H and ^{13}C n.m.r., i.r., and fast atom bombardment mass spectrometry. By comparing these data with those of model compounds, the material was assigned structure (**5**). This structure has subsequently been confirmed by chemical synthesis.[13]

The enzyme present in the cell-free extract which cyclises proclavaminic acid to give clavaminic acid was purified using conventional techniques. The purified enzyme showed only one band when examined by sodium dodecylsulphate polyacrylamide gel electrophoresis and corresponded to an M_r of 49 200. When run on a non-denaturing electrophoresis system the molecular weight was determined as 47 000. The isoelectric point was determined as pI 5.65. The Fe^{2+} ion and oxygen were essential for enzyme activity and α-ketoglutarate was the preferred co-substrate, although the enzyme would accept α-ketoadipate. Stoicheiometric cyclisation of proclavaminic acid could only be achieved if two equivalents of α-ketoglutarate were present. We suggest this enzyme be named clavaminic acid synthetase.

We have examined other micro-organisms for the presence of proclavaminic acid, clavaminic acid, and clavaminic acid synthetase, and we have found evidence for them in the other known clavulanic acid producing strains *S. jumonjinensis* ATCC 29864[14] and *S. katsurahamanus* T-272,[15] and also in the cephamycin producer *S. lipmanni* NRRL 3584.[16] Other clavams[17] have been reported which are known to have *S* stereochemistry at C-5 and these too have been isolated from a variety of organisms. However none of these compounds possesses a carboxyl group at C-3. The fact that both proclavaminic acid and clavaminic acid possess the ornithinyl residue suggests that they might be precursors of clavulanic acid, as ornithine is known to be a well incorporated precursor of clavulanic acid.[7] Evidence that this is indeed the case is presented in a subsequent paper.[18]

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