Evidence that the Immediate Biosynthetic Precursor of Clavulanic Acid is its *N*-aldehyde Analogue

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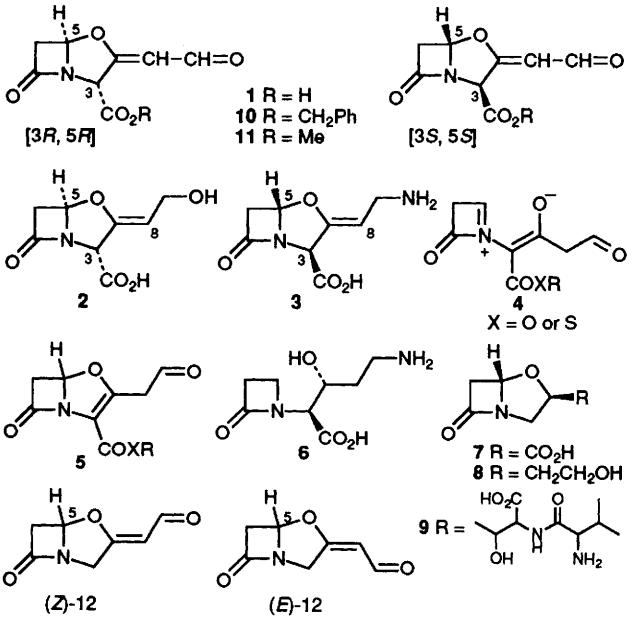
(3*R*, 5*R*) Clavulanate-9-aldehyde **1** has been detected in *Streptomyces clavuligerus* and an NADPH dependent dehydrogenase capable of reducing **1** to clavulanic acid **2** has been isolated from this organism.

In earlier communications we have described the biosynthesis of the clinically important β-lactamase inhibitor clavulanic acid **2** from arginine to the (3*S*,5*S*) bicyclic β-lactam clavaminic acid[1] **3** in *Streptomyces clavuligerus*. No intermediates have yet been described after clavaminic acid, though by inspection it is clear that inversion of the stereochemistry at C-3 and C-5, and substitution of a hydroxy for the 9-amino function are necessary to transform **3** to **2**. Inversion of the C-3 and C-5 stereochemistry of clavulanates to give mixtures of the (3*R*,5*R*) and (3*S*,5*S*) enantiomers has previously been observed in chemical reactions. A mechanism for this has been proposed[2] which proceeds *via* the intermediacy of the iminium ion **4** to the clavem **5**, which could isomerise to give the clavulanate system. As the endocyclic double bond of the clavem is conjugated to the carboxylate, a second conjugating group such as an ester or aldehyde is required in the exocyclic side-chain in order to promote the isomerisation of the double bond into the exocyclic position. We have already published evidence[3] that the hydrogen at C-8 is exchanged in the biosynthesis of **2** from **3** which indicates that such an isomerism may occur in nature. The 9-oxygen of clavulanate is derived from molecular oxygen,[4] indicating that the 9-amino function of **3** is probably removed by oxidative cleavage yielding the 9-aldehyde **1**. Thus, **1** can be considered a logical biosynthetic intermediate between **3** and **2**. Additionally, it has been suggested[5] that **1** could decarboxylate and hence be the biosynthetic progenitor of the (5*S*) non-carboxylated clavam metabolites. This hypothesis is supported by the fact that proclavaminic acid **6**, a known precursor of clavulanic acid, is also a precursor of the (5*S*) clavams **7** (produced by *S. clavuligerus*), **8** and **9** (produced by *S. antibioticus*).[5] We now report the occurrence of (3*R*,5*R*) **1** in *S. clavuligerus* and the purification of an NADPH dependent enzyme which reduces **1** to clavulanic acid. We also report that **1** readily decarboxylates and that a synthetic ester of **1** is able spontaneously to invert its stereochemistry from (3*R*,5*R*) to (3*S*,5*S*).



Synthetic aldehyde free acid **1** was prepared by brief hydrogenation of the benzyl ester[6] **10** over palladium on charcoal. Spectroscopic analysis (NMR and IR) of the reaction mixture indicated that the product was a 2:1 mixture of the (*E*) and (*Z*) geometrical isomers of **1**. NMR and HPLC analyses showed that an aqueous solution of **1** at neutral pH decomposed with a half-life of approximately one hour at room temperature. Consequently, the acid was further characterised by esterification with diazomethane to form **11**, which gave analytical data indistinguishable from those obtained by A. G. Brown *et al.*[6]

Synthetic **1** was efficiently extracted at 4 °C into methyl acetate from an equal volume of water at acidic pH, so this procedure was applied to culture broths of the clavulanic acid producer *S. clavuligerus* SC 2 (a reisolate of ATCC 27064), and of a clavulanic acid deficient mutant *S. clavuligerus* dcII 111. HPLC analysis of the concentrated extracts showed in each case a peak with the same retention time as synthetic **1** and which possessed the same UV chromophore. Quantitation of these peaks indicated original concentrations of **1** of 4 mg l^{-1} in *S. clavuligerus* dcII 111 broth and 0.4 mg l^{-1} in *S. clavuligerus* SC 2. When the methyl acetate extract from *S. clavuligerus* dcII 111 was allowed to stand at room temperature, it decarboxylated to give (*E*)-**12** and (*Z*)-**12**. (*E*)-**12** was purified by chromatography over Sephadex LH20 followed by semi-preparative HPLC over silica. The NMR, IR, UV, HPLC and mass spectral data for this compound were indistinguishable from those of (*E*)-**12** prepared from synthetic **1**. However, addition of the enantioselective solvating reagent *S*-2,2,2-trifluoro-1-(9-anthryl)ethanol to the NMR solutions indicated that synthetic (*E*)-**12** was a mixture of enantiomers at C-5, in the ratio *R*:*S*, 2:1, whereas only a single enantiomer could be detected in natural (*E*)-**12** (limit of detection *ca.* 10%). Synthetic (*E*)-**12** had been prepared from benzyl (3*R*,5*R*) clavulanate and partial racemisation must have occurred in the aldehydic benzyl ester **10**. The C-5 stereochemistry of natural (*E*)-**12** corresponded to that of the major enantiomer of the synthetic material and it was hence concluded that the former has the (5*R*) stereochemistry. Circular dichroism spectroscopy corroborated this conclusion; the natural and synthetic decarboxylated products both gave a positive inflection with a maximum at 237.4 nm (MeCN), but the intensity of absorption of the natural material was 2.4 times that of the synthetic. From this data we conclude that the aldehyde **1** exists *in vivo* as the (3*R*,5*R*) enantiomer [the (3*R*,5*S*) (3*S*,5*R*) diastereoisomer is not, apparently, thermodynamically favoured in clavulanate derivatives and has not been reported], and that the C-5 position does not racemise either in the free acid form, or when decarboxylated. Further evidence that natural **1** has the (3*R*,5*R*) stereochemistry was provided by its biological properties, as clavulanates with the (5*R*) stereochemistry possess β-lactamase inhibitory activity,[7] whereas those with the (5*S*) stereochemistry do not.[8] Synthetic **1** was found to possess β-lactamase inhibitory activity in the chromogenic cephalosporin assay,[9] as did the supernatant broth from *S. clavuligerus* dcII 111. This inhibited TEM 1 β-lactamase to the same extent as a solution of 375 mg l^{-1} of clavulanic acid; however, unlike the clavulanic acid

solution, the activity of the broth decayed with a halflife of approximately one hour, indicating that the broth contained a clavulanate with the (5*R*) stereochemistry with a rate of decay the same as that of **1**.

S. clavuligerus SC 2 was examined for its ability to reduce **1** to clavulanic acid. Synthetic **1** was incubated with a broken cell suspension[10] of *S. clavuligerus* SC 2. The β-lactamase inhibitory properties of the reaction mixture were compared with standard solutions of clavulanic acid by KAG bioassay.[11] As **1** decayed during the course of this assay, the results indicated only the β-lactamase inhibitory activity of clavulanic acid. The reduction was rapid in the presence of NADPH, but failed when this was replaced by NADH or when the broken cell suspension was absent or denatured by boiling. The enzyme responsible for the reduction of **1** to **2** was purified from cells of *S. clavuligerus* SC 2 disrupted by sonication. The soluble cell contents were treated with protease inhibitors and freed of nucleic acid, then applied to a Pharmacia Red-Sepharose resin which was washed with buffer and an NADH solution before desorption by a solution of NADPH. The enzyme was detected in the eluate by its ability to convert synthetic **1** to clavulanic acid. Analysis of the active fraction by sodium dodecylsulfate polyacrylamide gel electrophoresis showed a single band of molecular mass 28 000. Sequencing gave the 20 *N*-terminal amino acids (Fig. 1). An open reading frame, which commences with a DNA sequence corresponding to the *N*-terminal amino acids, has been located in the clavulanic acid gene cluster,[12] and shows homology to known NADPH dependent dehydrogenases from both prokaryotes and eukaryotes.[13] This enzyme was named clavulanic acid dehydrogenase.

The partial racemisation of synthetic **12** described above was attributed to racemisation of the aldehyde benzyl ester **10** from which it was made. This phenomenon was more closely examined by optical rotation and NMR spectroscopy using the enantioselective solvating reagent (*S*)-2,2,2-trifluoro-1-(9-anthryl)ethanol. Freshly prepared **10** gave an optical rotation $[\alpha]^{20}_D$ + 53.9 (*c* 2%, CH_2Cl_2) and NMR analysis indicated a 93 : 7 mixture of enantiomers. When a neutral chloroform solution of **10** was allowed to stand for three days at ambient temperature, then purified over silica, it gave a rotation $[\alpha]^{20}_D$ + 16.4 (*c* 2%, CH_2Cl_2) and an NMR spectrum which showed a mixture of enantiomers in the ratio 65 : 35. The ester had thus spontaneously racemised. As discussed above, the intermediate **4** has been proposed to provide a mechanism for this inversion. We suggest that a biological ester of **1** (oxygen ester or coenzyme A thioester) may mediate a similar inversion in the biosynthetic transformation of the (3*S*,5*S*) stereochemistry of **3** to the (3*R*,5*R*) stereochemistry of **2**.

The experiments above are evidence that the (3*R*,5*R*) aldehyde **1** is the ultimate biosynthetic precursor to clavulanic acid **2**, and that the former is reduced in *S. clavuligerus* by an NADPH dependent dehydrogenase. The conjugated aldehyde function provides a mechanism for rendering the double bond exocyclic after stereochemical inversion at C-3 and C-5 of a suitable clavulanate ester. Additionally, **1** readily decarboxylates, but, as naturally occurring **1** possesses the (5*R*) stereochemistry, it is less certain that this molecule is involved in the biosynthesis of the (5*S*) clavams, there being no evidence for the natural occurrence of the (5*S*) **1**.

S A L Q G K V A L I T G A S S G I G E

Fig. 1 The *N*-terminal amino acid sequence of CAD

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