Clavulanic acid biosynthesis; the final steps

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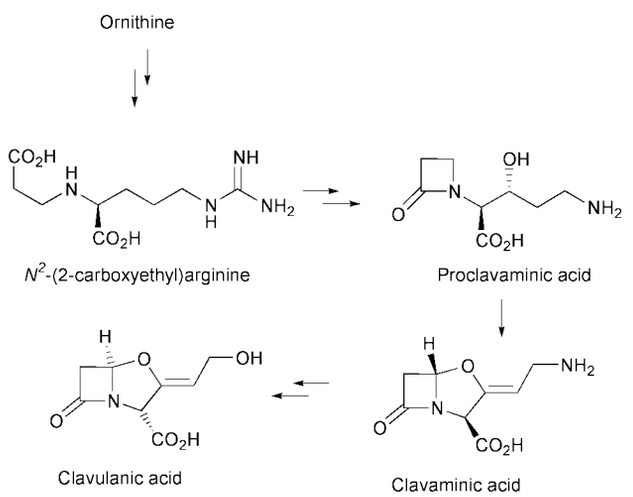
The chemically unstable anabolite (3*R*,5*R*)-clavulanate-9-aldehyde **1** and an NADPH dependent dehydrogenase have been detected in the broth of *Streptomyces clavuligerus*. The purified enzyme was shown to make clavulanic acid by reduction of the aldehydic moiety of synthetic **1** to the allylic alcohol of clavulanic acid **2**. A DNA sequence corresponding to the enzyme's *N*-terminal amino acid sequence was located within the clavulanic acid biosynthetic gene cluster. We have named this novel enzyme clavulanic acid dehydrogenase (CAD). In an attempt to determine the origin of **1**, fermentations of *S. clavuligerus* were fed ornithine labelled with stable isotopes in the carboxy group. The results of these experiments are discussed.

Introduction

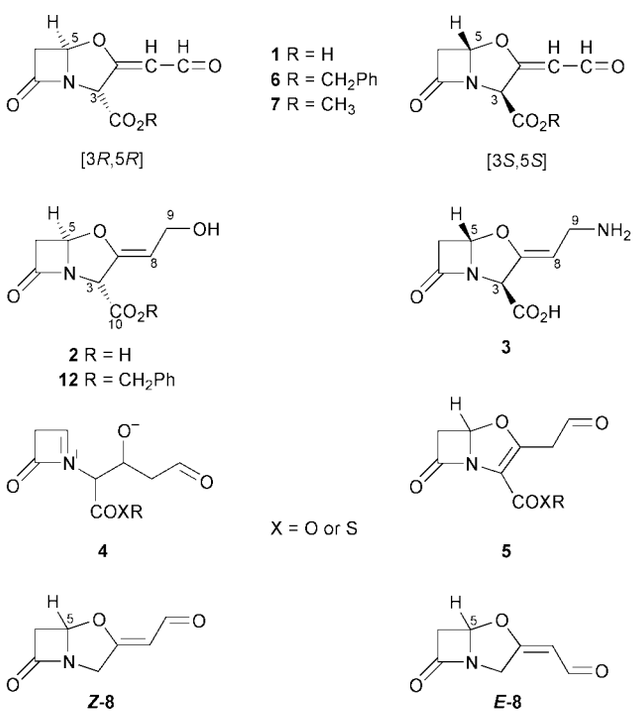
Clavulanic acid **2** is a potent β-lactamase inhibitor which is used clinically in conjunction with the penicillin amoxicillin to treat bacterial infections that resist penicillin monotherapy by the production of β-lactamases. It is produced by submerged culture of *Streptomyces clavuligerus* and all the intermediates of the biosynthetic pathway (Scheme 1) have been identified



Scheme 1 The biosynthetic pathway of clavulanic acid.

from the acyclic intermediate N^2-(2-carboxyethyl)arginine to the bicyclic β-lactam clavaminic acid **3**.[1] However, investigation of the subsequent steps, in which the C-9 amino group of clavaminic acid is transformed to a C-9 hydroxy and the (3*S*,5*S*) stereochemistry is inverted to give the (3*R*,5*R*) stereochemistry of clavulanic acid, has been hampered by the apparent instability of the biosynthetic intermediates.

We have proposed a biochemical mechanism for the stereochemical inversion, which was suggested by the base catalysed inversion of both chiral centres of clavulanate esters that have unsaturation at the 9-carbon.[2] It is apparent that formation of the betaine **4** will eliminate the stereochemistries present at C-3 and C-5 of clavaminic acid **3** and that subsequent cyclisation of



4 to the clavem **5** would permit the alternative stereochemistry to be formed at C-5. Migration of the endocyclic double bond of **5** to the exocyclic position recreates stereochemistry at C-3 and such tautomerisation is consistent with the loss of a labelled hydrogen from the C-8 position which is observed during clavulanic acid biosynthesis.[3] The derivation of the C-9 oxygen of clavulanic acid from molecular oxygen[4] suggests that the amino function of clavaminic acid is oxidised to form a C-9 aldehyde and this allylic aldehyde could provide the unsaturation required for stereochemical inversion. Clavulanate-9-aldehyde **1** consequently fulfils the necessary biochemical criteria and is proposed as the rational candidate for the final intermediate in the clavulanic acid biosynthetic pathway. However, formation of the betaine **4** requires that an ester moiety is present at the carboxy to assist the initial proton abstraction from C-3. An ester of the aldehyde **1** is, therefore, the most probable penultimate biosynthetic precursor of clavulanic acid at which inversion of the stereochemistries at C-3 and C-5 occurs.

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In this paper, we give a full account of the findings outlined in our communication,[5] together with experiments in which ornithine labelled in the carboxy group was fed to *S. clavuligerus* fermentations in order to provide evidence for an ester intermediate in the biosynthesis of clavulanic acid.

Results and discussion

Synthesis and properties of clavulanate-9-aldehyde 1

In order to initiate the investigation, we prepared a standard sample of the aldehyde free acid **1** by chemical means. Brief hydrogenation of the previously reported aldehyde benzyl ester[6] **6** over palladium on charcoal gave predominately the required acid. Spectroscopic analysis (NMR and IR) of the crude reaction mixture indicated that the product was a 2 : 1 mixture of the *E*- and *Z*- geometrical isomers and the ultraviolet spectrum gave a maximum at 262 nm corresponding to the allylic aldehyde chromophore. The crude product **1** was purified by collection of the reversed phase HPLC eluate fraction that contained this chromophore.

Both NMR and HPLC analysis showed that an aqueous solution of **1** adjusted to neutral pH decomposed with an approximate half-life of one hour at room temperature. Consequently, further purification of the acid was not attempted but its identity was established beyond doubt by derivatisation of the unpurified material with diazomethane to form the methyl ester **7**. Analysis of the purified methyl ester gave data indistinguishable from that obtained for the same compound prepared by a different route by Brown *et al.*[6]

Discovery of naturally occurring clavulanate-9-aldehyde 1

The chemical instability of the synthetic aldehyde **1** precludes conventional experiments designed to establish its intermediacy in the clavulanic acid biosynthetic pathway. For example, a successful synthesis of **1** containing stable isotopes would be expected to be problematic and the resulting material would decompose substantially before entering the producing organism resulting in little production of labelled clavulanic acid. Nor would a labelling experiment prove that the aldehyde was a constituent of the clavulanic acid biosynthetic pathway, as an adventitious dehydrogenase might be capable of the reduction of the aldehyde to clavulanic acid.

We therefore examined a variety of mutant *Streptomyces clavuligerus* cultures for their ability to produce **1**. Thin layer chromatography of the culture broths showed that a high-titre clavulanic acid producing strain and a blocked mutant (dcl I 111) contained detectable levels of a compound with the same mobility as the synthetic aldehyde **1** and which gave the same blue colour on visualisation with Schiff reagent. Since the synthetic aldehyde free acid **1** was efficiently extracted at 4 °C into methyl acetate at acidic pH, this extraction procedure was applied to the culture broths of two strains – the clavulanic acid producer *S. clavuligerus* SC 2 (a reisolate of ATCC 27064), and the clavulanic acid deficient mutant *S. clavuligerus* dcl I 111. HPLC analysis of the concentrated extracts showed in each case a peak which eluted 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* dcl I 111 broth and 0.4 mg l^{-1} in *S. clavuligerus* SC 2.

These results established the presence of the aldehyde in a producing culture of *S. clavuligerus* and its accumulation to a steady state concentration determined by its rate of production and chemical decay in a culture blocked at a late stage of biosynthesis. However, they provided no information on the stereochemistry of the aldehyde at the 3 and 5 carbons and no opportunity for confirmatory evidence of the structure. Consequently we sought a stable derivative of **1** which was amenable to purification and full chemical characterisation. Reduction of the aldehyde function with a range of reducing reagents was unsuccessful as were attempts to form stable Schiff base derivatives. Esterification of the carboxy group was considered to be unsuitable for the determination of stereochemistry due to the racemisation of these derivatives, which will be addressed in greater detail later. However, when a solution of the synthetic aldehyde **1** was allowed to stand at room temperature, it was found to decarboxylate with the formation of the clavam derivatives *E*-**8** and *Z*-**8** as the principal products and these compounds proved sufficiently stable chemically and at the C-5 stereocentre, for rigorous purification.

The methyl acetate extract from *S. clavuligerus* dcl I 111 was allowed to stand at room temperature and the decarboxylated product *E*-**8** was purified by size exclusion and silica gel chromatography. This simplistic isolation of low levels of *E*-**8** from the plethora of compounds present in the broth was facilitated by the transition of the aldehyde **1** from a free acid to a neutral molecule after the extraction, as this distinguished it from the other extracted species. The structure of the extracted *E*-**8** was confirmed by NMR, IR, UV, HPLC and mass spectral data which were indistinguishable from those of a sample of *E*-**8** prepared from synthetic **1**.

The stereochemistry of the natural *E*-**8** was determined by comparison of its NMR spectrum in the presence of enantiomeric solvating reagent with that of *E*-**8** derived from clavulanic acid, the stereochemistry of which has been unequivocally assigned by X-ray diffraction.[6] Addition of *S*-2,2,2-trifluoro-1-(9-anthryl)ethanol to the NMR solutions indicated that synthetic *E*-**8** was a mixture of enantiomers at C-5, in the ratio *R*–*S*, 2 : 1, whereas only a single enantiomer could be detected in the naturally derived *E*-**8** (limit of detection *ca.* 10%). Synthetic *E*-**8** had been prepared from benzyl (3*R*,5*R*)-clavulanate and partial racemisation was assumed to have occurred in the aldehydic benzyl ester **6**. The C-5 stereochemistry of naturally derived *E*-**8** 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 (CH_3CN), but the intensity of absorption of the natural material was 2.4 times that of the synthetic. From these 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 thermodynamically disfavoured in clavulanate derivatives and has not been reported], and that the C-5 position does not racemise either in the free acid form, nor when the acid decarboxylates.

The biochemical properties of the naturally derived aldehyde **1** confirmed the assignment of the (5*R*) stereochemistry. Clavulanates with the (5*R*) stereochemistry possess β-lactamase inhibitory activity[7] whereas those with the (5*S*) stereochemistry do not.[8] The chromogenic cephalosporin assay[9] showed synthetic **1** to possess strong β-lactamase inhibitory activity, so this test was applied to the supernatant from a culture broth of the clavulanic acid deficient mutant *S. clavuligerus* dcl I 111. The supernatant fluid inhibited TEM 1 β-lactamase to the same extent as a solution of 375 mg l^{-1} of clavulanic acid; however, unlike a clavulanic acid solution, the activity of the broth solution decayed with an approximate half life of one hour (Fig. 1). This indicated that the broth contained a β-lactamase inhibitor with the same activity and rate of decay as that of synthetic (3*R*,5*R*) clavulanate-9-aldehyde **1**.

Discovery and properties of CAD

Clavams produced by *Streptomycetes*, such as clavam-2-carboxylate **9** and valclavam **10**, possess the 5*S* stereochemistry[1] and the clavulanate-9-aldehyde we have described is unique

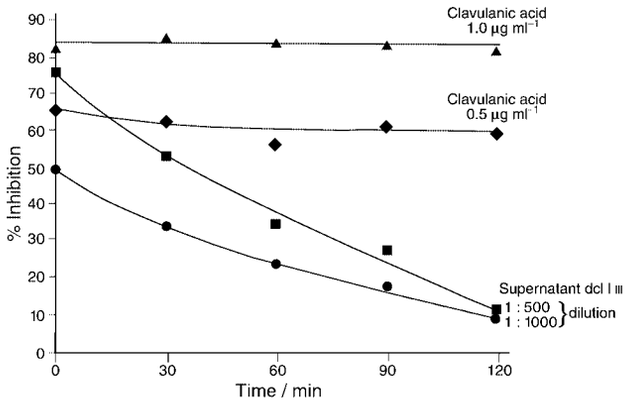
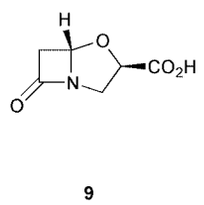


Fig. 1 The β-lactamase inhibitory activity of *S. clavuligerus* dcl I 111 broth supernatant and clavulanic acid against time (Chromogenic Cephalosporin).

amongst naturally occurring clavams in sharing the 5*R* stereochemistry with clavulanic acid. The coincidence of the stereochemistries, the similarity in chemical structure and the presence of clavulanate-9-aldehyde **1** in the supernatant of a clavulanic acid producing strain, implies that these two metabolites emanate from the same biosynthetic pathway and that one may be the precursor of the other. In this case we would expect to find an enzyme produced by *S. clavuligerus* capable of converting one to the other and, since we considered it probable that the more stable metabolite was the final product, the mycelia of *S. clavuligerus* were examined for the ability to reduce clavulanate-9-aldehyde to clavulanic acid.



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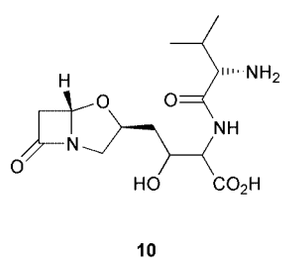

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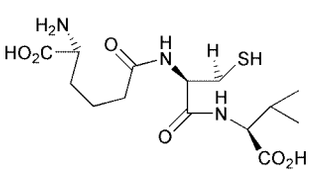

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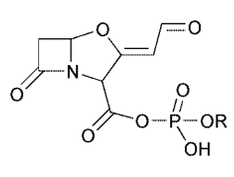

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Synthetic **1** was incubated with a broken cell suspension[10] of *S. clavuligerus* SC 2 which had been washed in order to remove endogenous clavulanic acid and the β-lactamase inhibitory properties of the reaction mixture were compared with standard solutions of clavulanic acid by KAG bioassay.[11] In the KAG assay, a β-lactamase producing strain of *Klebsiella aerogenes* is grown overnight on an agar plate containing a penicillin. The β-lactamase destroys the penicillin and the bacterium grows, except in an area of the plate into which a β-lactamase inhibitor has been introduced, where there is no growth. Since the aldehyde **1** decayed during the course of the assay, the results indicate the activity of a stable β-lactamase inhibitor. The conversion of the aldehyde to a stable β-lactamase inhibitor was rapid in the presence of NADPH but failed in its absence or when it was replaced by NADH (Table 1) or when the broken cell suspension was absent or denatured by boiling (Table 2). The product was identified as clavulanic acid by comparison of the reaction mixture with a standard sample by chromatography followed by visualisation on a KAG plate

Table 1 Efficacy of NADH and NADPH as co-enzymes in the reduction of clavulanate-9-aldehyde **1** to clavulanic acid **2** by a broken cell suspension (enzyme) of *S. clavuligerus*

			Clavulanic acid **2**/μg l^{-3}	
Enzyme	Aldehyde **1**	Co-enzyme	t_0	t_{60}
+	−	—	<2.4	<2.4
−	+	—	6.7	6.7
+	+	—	8.0	7.0
+	−	NADPH	<2.4	<2.4
+	+	NADPH	28.8	27.2
+	+	NADH	6.2	6.6
−	+	NADH	7.0	7.0

Table 2 Inactivation of *S. clavuligerus* broken cell suspension (enzyme) by heat treatment

			Clavulanic acid **2**/μg l^{-3}	
Enzyme	Aldehyde **1**	Co-enzyme	t_0	t_{60}
+	−	—	<2.4	<2.4
−	+	—	5.0	6.0
+	+	—	4.4	5.6
+	−	NADPH	<2.4	<2.4
+	+	NADPH	25.6	22.4
−	+	NADPH	4.8	4.8
Boiled	+	NADPH	6.6	6.6
Boiled	−	NADPH	<2.4	<2.4

and also by HPLC. A tenfold reduction in the amount of the broken cell suspension added to the reaction mixture made no difference to the speed or endpoint of the reduction suggesting high activity of the enzyme *in vivo*.

Preparation of the clavulanate-9-aldehyde **1** for these studies was complicated by the instability of the aldehyde benzyl ester **6** on purification over silica gel.[6] Oxidation of benzyl clavulanate gave a mixture of the aldehyde benzyl ester and the starting material, and incomplete separation of these components resulted in contamination of the aldehyde with approximately 1% of clavulanic acid. Consequently all samples in the Tables 1 and 2 which contain the aldehyde gave a constant zone of inhibition equivalent to about 5 μg ml^{-1} of clavulanic acid which is independent of the reduction of the aldehyde. The overall yield of the hydrogenation and enzymatic reduction with respect to the aldehyde benzyl ester employed was 10%. However, the yield of the hydrogenation reaction has been shown to be approximately 30% and produces the aldehyde **1** as a mixture of the *E* and *Z* isomers in the ratio 2 : 1. Consequently, if the *Z* stereochemistry product can only be produced from the *Z* isomer of the aldehyde, the yield of clavulanic acid relative to the available aldehyde precursor may approach quantitative.

The unstable aldehyde **1** is therefore reduced to the stable β-lactamase inhibitor, clavulanic acid **2** by an enzyme which is present in the mycelia of *S. clavuligerus* and which is dependent for its action on the reducing co-factor NADPH. The rapidity of the reduction, which was complete on mixing of the reagents, suggests that the equilibrium lies on the side of the reduced product and attempts to oxidise clavulanic acid to the aldehyde under the same conditions but in the presence of an excess of $NADP^+$ were not successful. It is therefore logical to conclude that the action of the dehydrogenase *in vivo* is to reduce clavulanate-9-aldehyde **1** to clavulanic acid **2**.

Isolation of CAD

The enzyme responsible for the reduction of the aldehyde **1** to clavulanic acid **2** was purified in a single fractionation step using a dye resin with a specific affinity for NADPH dependent

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

Fig. 2 The *N*-terminal amino acid sequence of CAD.

dehydrogenases. *S. clavuligerus* SC 2 cells were disrupted by sonication and the soluble cell contents were treated with protease inhibitors and freed of nucleic acid. The active fraction was applied to a Pharmacia Red-Sepharose resin which was washed with buffer and an NADH solution before desorption by a solution of NADPH. The purified enzyme was detected in the eluate by its ability to convert synthetic **1** to clavulanic acid. Analysis of the active fraction by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a band of molecular weight 28 kDa and sequencing of this band gave the initial twenty *N*-terminal amino acids (Fig. 2) of the protein. An open reading frame, which commences with a DNA sequence corresponding to the *N*-terminal amino acid sequence, has been located in the clavulanic acid gene cluster, and shows homology to known NADPH dependent dehydrogenases from both prokaryotes and eukaryotes.[12] The molecular weight of the protein predicted from the sequence of the open reading frame is 26.323 kDa

The non-producing *S. clavuligerus* mutant strain dcl I 111 has been shown to possess a disruption in the DNA encoding the dehydrogenase by complementation experiments in which the biosynthesis of clavulanic acid was restored by the insertion of a plasmid containing the undisrupted dehydrogenase sequence.[12] It may thus be concluded that the accumulation of the aldehyde in dcl I 111 and the inability of this strain to produce clavulanic acid are due to its production of an inactive form of the dehydrogenase enzyme which is unable to convert the aldehyde intermediate in the biosynthetic pathway to clavulanic acid. We have named the enzyme which reduces clavulanate-9-aldehyde, clavulanic acid dehydrogenase (CAD).

Spontaneous racemisation of benzyl clavulanate-9-aldehyde 6

The partial racemisation of synthetic **8** noted above, was attributed to racemisation of the aldehyde benzyl ester **6** 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 **6** gave an optical rotation $[\alpha]_D^{20}$ +53.9 ($c = 2\%$, dichloromethane), and NMR analysis indicated a 93 : 7 mixture of enantiomers. A neutral chloroform solution of **6** was allowed to stand for three days at ambient temperature and periodic NMR analysis showed the ratio of the signals due to each enantiomer to be changing. Purification of the product over silica, showed that the specific rotation of **6** had fallen to $[\alpha]_D^{20}$ +16.4 and the NMR spectrum showed a mixture of enantiomers in the ratio 65 : 35. The ester had thus spontaneously racemised. An intermediate of the type **4** has already been proposed to provide a mechanism for this inversion and we have suggested that an ester or a thioester of **1** may mediate a similar inversion in the biosynthetic transformation of the (3*S*,5*S*) stereochemistry of clavaminic acid to the (3*R*,5*R*) stereochemistry of clavulanic acid.

Investigation of oxygen exchange by mass spectrometry

A thioester intermediate has previously been proposed in the biosynthesis of bicyclic β-lactams. Delderfield *et al.*[13] demonstrated the involvement of a thioester intermediate in penicillin biosynthesis and Baldwin *et al.*[14] showed that the exchange of an oxygen in the carboxy moiety, which is axiomatic for the exclusive operation of this mechanism, occurs during the synthesis of the tripeptide intermediate, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) **11**. Consequently, if thioesterification occurs in clavulanic acid biosynthesis, an intermediate prior to the putative betaine **4** will be incorporated into clavulanic acid with the exchange of one oxygen of its carboxy moiety. Of the early intermediates, ornithine is the most extensively incorporated into clavulanic acid[15] and its carboxy oxygens may be conveniently labelled with [^{18}O] by the method of Murphy and Clay.[16]

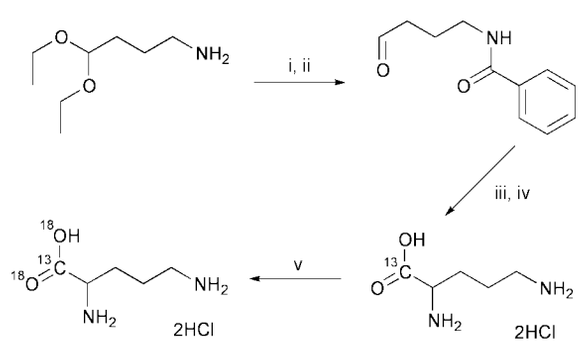
Ornithine was heated with acidified $H_2{}^{18}O$ and, after trituration, was shown to be a mixture of [$^{18}O_2$]ornithine and [^{18}O,^{16}O]ornithine dihydrochlorides in the ratio 91 : 9 by IR, ^{13}C NMR and elemental analysis. Addition of [$^{16}O_2$]ornithine to the ^{13}C NMR solution resulted in a third resonance 2.5 Hz downfield from that assigned to the ^{18}O labelled carboxy signal indicating that undetectable amounts of [$^{16}O_2$]ornithine remained after exchange. Further confirmation of the labelling was obtained by the formation of the benzyl 2*N*,5*N*-bis(benzyloxycarbonyl) ester which, on analysis by mass spectrometry, was found to be a mixture of the [$^{18}O_2$] and [^{18}O,^{16}O] isotopes in the ratio 86 : 14.

The labelled ornithine was fed to cultures of *S. clavuligerus* in early production phase and the resulting clavulanic acid was isolated[10] as its benzyl ester **12**. ^{13}C and ^{1}H NMR and IR spectroscopy showed the sample to be indistinguishable from benzyl clavulanate containing natural isotopic abundance, but after trimethylsilylation of the C-9 hydroxy, repeated gas chromatography/mass spectrometry showed that the signals representing mass ions two (*m*/*z* 362) and four (*m*/*z* 364) units greater than the molecular ion were enhanced in intensity over the equivalent ions in benzyl clavulanate containing natural isotopic abundance. The variation in intensity of any of the signals over five determinations was less than 5% of the average. The difference between the intensity of the enriched signals and those of natural abundance, expressed as a percentage of the total benzyl clavulanate signal was [^{18}O] 3.8% and [$^{18}O_2$] 3.1%.

Since these results apparently showed the incorporation of ornithine both with and without exchange of an oxygen atom, a confirmatory feeding experiment was performed using (*RS*)–[1-^{13}C,$^{18}O_2$]ornithine dihydrochloride **13**. The incorporated [^{18}O] atoms could then be observed in the ^{13}C NMR spectrum by the upfield isotopic shift of the resonance due to the clavulanate 10-[^{13}C] to which they were attached.

Investigation of oxygen exchange by NMR

RS-[1-^{13}C]ornithine dihydrochloride (92% ^{13}C) consisting of 87% [$^{18}O_2$] and 13% [^{18}O,^{16}O] was synthesised by a Strecker reaction[17] followed by acid catalysed exchange with $H_2{}^{18}O$ as before (Scheme 2). When this material was fed to *S. clavuligerus*



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Scheme 2 *Reagents*: i, Benzoyl chloride, pyridine; ii, 2 M H_2SO_4; iii, NH_4Cl, $Na^{13}CN$; iv, conc. HCl reflux; v, $H_2{}^{18}O$, HCl

and the resulting clavulanic acid was isolated as the benzyl ester **12**, NMR showed an enrichment of ^{13}C at the clavulanate carboxy equivalent to 6.3%. Data point compression beneath this signal revealed four resonances which were assigned as 10-^{13}C atoms bearing [^{18}O] and [^{16}O] atoms in the four possible configurations shown in Fig. 3. The upfield shift of the 10-[^{13}C]

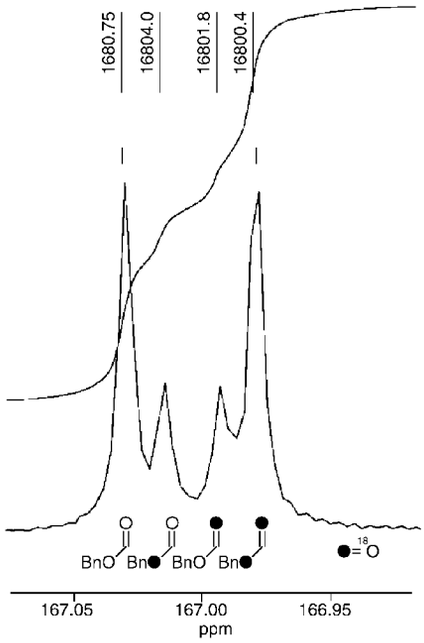

Fig. 3 [^{18}O] Enriched benzyl clavulanate; NMR of 10-^{13}C

resonances due to substitution of ^{16}O by ^{18}O is in close agreement with the literature.[18] Enhancement and comparison of the intensity of these signals with those of benzyl clavulanate containing natural abundance 10-[^{13}C] and run under identical conditions, gave incorporations of [^{18}O] 2.1% and [$^{18}O_2$] 3.9%. Clearly the ornithine had again been incorporated into the clavulanic acid both with and without the loss of oxygen from the carboxy.

The retention of a high proportion of the [$^{18}O_2$] carboxylate species in clavulanic acid produced from [$^{18}O_2$]ornithine rules out the possibility of a simple ester or thioester as a late stage intermediate in clavulanic acid biosynthesis since, by currently accepted biosynthetic mechanisms, this would cause the loss of one [^{18}O] to give only a [^{16}O,^{18}O] labelled product. However, the production of clavulanate containing both [$^{18}O_2$] and [^{16}O,^{18}O] labelling of the carboxylate species could be explained by the intermediacy of a mixed anhydride such as **14**. This could be hydrolysed either at the carboxy centre with the loss of an [^{18}O] atom or at the phosphorus with retention of both of the [^{18}O] atoms and the simultaneous operation of these mechanisms would produce the labelling pattern which we have observed in clavulanic acid. The hydrolysis of a phosphate mixed anhydride at both the phosphorus and carbon centres has been proposed as a candidate mechanism for the partial loss of oxygen observed in a reaction catalysed by ACV synthetase from the penicillin biosynthetic pathway.[19]

Conclusion

We have shown that clavulanate-9-aldehyde **1** is co-produced with clavulanic acid by fermentations of *S. clavuligerus* in the production phase. The stereochemistry of the aldehyde **1** is, uniquely for naturally occurring clavams, the same as clavulanic acid and the similarity in the structure of **1** and clavulanic acid allows a simple reduction to convert one to the other. An NADPH dependent dehydrogenase which mediates the rapid reduction of the aldehyde has been discovered and the isolated enzyme was shown to be encoded by an open reading frame within the clavulanic acid biosynthetic gene cluster. Disruption of this open reading frame results in a mutant strain (dcl I 111) which is incapable of the biosynthesis of clavulanic acid and produces an elevated titre of the aldehyde. For these reasons we propose that clavulanate-9-aldehyde **1** is the final anabolite in the biosynthetic pathway of clavulanic acid.

The benzyl ester of the synthetic aldehyde **1** spontaneously racemised to give a mixture of the (3*R*,5*R*) and (3*S*,5*S*) enantiomers and this suggested that an ester intermediate may be involved in the biosynthesis of clavulanic acid. However, the fate of stable oxygen isotopes in ornithine fed to producing strains of *S. clavuligerus* indicates that a mixed anhydride is a more likely candidate for this late-stage intermediate than a thioester.

Experimental

IR spectra were recorded on a Perkin-Elmer 983 spectrophotometer or FTIR. ^{1}H and ^{13}C NMR spectra were recorded either on a Bruker AM 250, DPX 250 or AM 400 and ^{13}C spectra were routinely proton decoupled. Mass spectra were recorded on a VG 7070F spectrometer using electron impact (EI) or chemical ionisation (CI). For fast-atom bombardment (FAB) and high resolution spectra, a VG ZAB IF double-focusing instrument was used. Analytical TLC was carried out on Merck pre-coated silica gel 60 F_{254} glass plates which were visualised with UV light and/or iodine vapour or, in the case of clavams, with TTC spray (4% triphenyltetrazolium chloride in methanol diluted with an equal volume of 1 M NaOH). Column chromatography was carried out on Merck (9385) silica 60. Anhydrous magnesium sulfate was used for drying organic solutions. CD spectra were recorded on a Jasco J40C spectrometer and optical rotations were measured on a Perkin-Elmer 141 polarimeter and are given in 10^{-1} deg cm^2 g^{-1}

Cultures of *Streptomyces clavuligerus* were obtained by re-isolation of the commercially available ATCC 27064 (*S. clavuligerus* SC2) and from a collection of clavulanic acid decreased mutants generated within SmithKline Beecham (dcl mutants). These were fermented in the same manner as previously described.[20] Clavulanic acid production by clavulanic acid dehydrogenase was assayed by the KAG[11] assay. *Klebsiella aerogenes*, NCTC 11228 (ATCC 29665), was grown on DST agar containing 5μg ml^{-1} penicillin G and assay samples were placed in 8 mm diameter wells or on dried filter discs. Chromatographic tapes (Whatman No. 1) were dried and applied directly to the plates. The agar plates were incubated at 37 °C overnight and the concentration of clavulanic acid estimated from the diameter of the zone of inhibition of bacterial growth in comparison with the zone produced by standard samples.

Sodium 3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylate 1 (R = Na)

Benzyl 3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylate **6** (50 mg, 0.17 mmol)[6] was dissolved in tetrahydrofuran (5 ml), treated with 0.2 M NaH_2PO_4 which had been adjusted to pH 6.9 with sodium hydroxide (5 ml), and hydrogenated at ambient temperature and pressure for ten minutes with 10% palladium on carbon (40 mg). The temperature was maintained below 10 °C while the reaction mixture was filtered and evaporated to dryness and the residue was immediately redissolved in D_2O to give a solution of sodium 3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylate **1** (R = Na), δ_H (250 MHz, D_2O) *inter alia* *E* isomer 3.25 (1H, d, *J* 17.4, 6β-H), 3.65 (1H, dd, *J* 17.4 and 2.9, 6α-H), 5.73 (1H, d, *J* 1.2, 2-H), 5.79 (1H, dd, *J* 8.3 and 1, 8-H), 5.94 (1H, d, *J* 2.7, 5-H), 9.55 (1H, d, *J* 8.3, 9-H), *Z* isomer 3.29 (1H, d, *J* 17.3, 6β-H), 3.65 (1H, dd, *J* 17.4 and 2.9, 6α-H), 5.27 (1H, s, 2-H), 5.50 (1H, d, *J* 8.2, 8-H), 6.00 (1H, d, *J* 2.7, 5-H) and 9.70 (1H, d, *J* 8.2, 9-H). The *E* and *Z* isomers were assigned on the relative positions of their 8-proton resonance[21] and were in the ratio *E*–*Z* (2 : 1). Material prepared by the above hydrogenation with water substituting for the phosphate solution and adjustment of the pH after the reaction to 6.5 with 0.1 M NaOH gave **1** (R = Na) (yield

35% from NaOH consumed), $\lambda_{max}(H_2O)$ 262 nm; ν_{max}(KBr) 3400, 1792 (br), and 1640 (br) cm^{-1}; thin layer chromatography eluted with butan-1-ol–ethanol–water (4 : 1 : 1) and visualised with Schiff's reagent gave a blue zone (R_f: 0.52). Addition of a known weight of fumaric acid to the crude product solution followed by NMR analysis indicated a yield of 20% by comparison of the intensity of the fumarate protons with the intensity of the aldehydic protons of **1** (R = Na). When this solution was allowed to stand at room temperature for 3.5 h, the product was seen to have decomposed to give a yield of 3.2%. Analytical HPLC of **1** (R = Na) over a Waters C_{18} Microbondapak column eluted at 2 ml min^{-1} with 0.1 M NaH_2PO_4, which had been adjusted to pH 3.2 with sodium hydroxide, gave a peak with a retention time of 6.1 min when the eluent was monitored by an ultra violet detector at 260 nm.

HPLC purification of 1 (R = Na). Compound **1** (R = Na), prepared by hydrogenation of the benzyl ester **6**, was evaporated to a small volume, adjusted to pH 4.0 with 0.5 M orthophosphoric acid and made up to 2 ml with water. This solution was chromatographed over a Waters C_{18} Radpak cartridge supported in a Waters Z module and eluted at 4 ml min^{-1} with 0.1 M NaH_2PO_4 adjusted to pH 4.0 with sodium hydroxide. A fraction coincident with the elution of a strong chromophore at 260 nm was collected between 3.8 and 5.0 min. Evaporation of this fraction followed immediately by NMR gave a spectrum consistent with **1**.

Spontaneous decomposition of 1 (R = Na). A solution of **1** (R = Na) in 0.2 M NaH_2PO_4 adjusted to pH 6.9 with a solution of sodium hydroxide was allowed to stand at room temperature. HPLC analysis showed the solution to contain 85% of the original **1** after 35 minutes and 63% after 53 minutes.

Methyl 3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylate 7 from 1

An aqueous solution of sodium 3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate **1** (R = Na) prepared from the benzyl ester **6** (100 mg, 0.35 mmole) by hydrogenation, was acidified to pH 2.5 under a layer of ether (5 ml) at 4 °C and stirred with the addition of aliquots of diazomethane every five minutes. After twenty minutes, excess diazomethane was removed in a stream of nitrogen and ethyl acetate (30 ml) was added. The organic layer was separated, dried with magnesium sulfate and chromatographed rapidly over silica gel in ethyl acetate–hexane (1 : 1) to give the title compound as a mixture of geometric isomers *E*–*Z* (1.8 : 1) (12 mg, 16% from the benzyl ester **6**). The spectral data obtained for these isomers was indistinguishable from that obtained in the previous preparation.[6]

Extraction of 1 from *S. clavuligerus* broth

Baffled 500 ml conical flasks containing 60 ml of fermentation medium were inoculated with 2 ml of an *S. clavuligerus* dcl I 111 culture from a seed fermentation. The culture was fermented for 50 hours and the broth was rapidly cooled to 2 °C. The mycelia were separated by centrifugation, and the supernatant was acidified to pH 3.2 with 5 M HCl and extracted with an equal volume of methyl acetate. The methyl acetate extract (MAE) was maintained at less than 10 °C and was used immediately in the following experiments.

HPLC assay for 1 in *S. clavuligerus* broth

A sample of the methyl acetate extract (MAE) (10 ml) was concentrated by evaporation to an aqueous residue, re-adjusted to pH 3.2 and made up to a known volume of about 0.5 ml. This sample was immediately compared with a fresh, chemically prepared sample of **1** by HPLC and gave a peak equivalent to 4 μg ml^{-1} of **1** in the dcl I 111 culture broth and 0.4 μg ml^{-1} in *S. clavuligerus* SC2 broth.

Isolation of (5*R*,*E*)-3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo-[3.2.0]heptane *E*-8 from *S. clavuligerus* dcl I 111 broth

The methyl acetate extract (MAE) (1.15 l) was allowed to stand at room temperature for four hours when HPLC showed no further increase in the peaks due to the decarboxylated products **8**. The solution was then evaporated to a small volume, the residue diluted with chloroform (200 ml) and the resulting aqueous phase (50 ml) discarded. The organic phase was washed with 1 M NaH_2PO_4 adjusted to pH 6.5, dried with magnesium sulfate and evaporated to dryness. This residue was chromatographed over Sephadex LH 20 (Pharmacia) in ethyl acetate–hexane (1 : 1) and evaporated to give an oil (40 mg oil containing 2.0 mg *E*-**8**; purity 5.0%). This material (30 mg) was chromatographed over a Waters μ-Porasil Radpak cartridge eluted at 2 ml min^{-1} with chloroform–hexane–ethanol (3 : 6 : 0.1) (*E*-**8** retention time 6.0 minutes) and subsequently chromatographed over the same matrix in dichloromethane–hexane–ethanol (3 : 6 : 0.1) to give (5*R*,*E*)-3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane *E*-**8** (0.8 mg); CD $\Delta\varepsilon_{237.4}$ 14.6 (CH_3CN) (Found M^+ 153.0438. Calc.for $C_7H_7NO_3$: M^+ 153.0426); $\lambda_{max}(CH_3CN)$ 249 nm (ε 14187 dm^3 mol^{-1} cm^{-1}); ν_{max}(KBr) 1797, 1668, 1637 and 1592 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 3.12 (1H, d, *J* 16.7, 6β-H), 3.54 (1H, ddd, *J* 0.9, 2.8 and 16.6, 6α-H), 3.92 (1H, d, *J* 17.5, 2α-H), 5.01 (1H, dd, *J* 1.6 and 17.5, 2β-H), 5.66 (1H, d, *J* 2.7, 5-H), 5.82 (1H, dt, *J* 1.7 and 3.6, 8-H) and 9.50 (1H, *J* 4.9, 9-H). Irradiation of the 8-H signal caused a collapse of coupling at the 9-H signal, loss of the smaller coupling at the 2β-H and sharpening of the 2α-H signal. Irradiation of the 2α-H signal caused loss of the smallest coupling at 6α-H. Addition of *R*-2,2,2-trifluoro-1-(9-anthryl)ethanol (1000%) caused no bifurcation of the signal due to the 2-β proton. Addition of the latter NMR solution to a solution comprising a 2 : 1 (*R*–*S*) mixture of enantiomers, showed a relative reduction in the intensity of the 2-β proton of the (5*S*)-enantiomer; *m*/*z* 153 (M^+, 9%), 125 (M^+-CO, 6.3%), 111 (M^+-CH_2CO, 18.9%), 55 (100%).

3-Oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane 8 from synthetic 1

A solution of **1** prepared by hydrogenation of **6** (90 mg, 0.31 mmol) in tetrahydrofuran (90 ml) was allowed to stand at room temperature for four hours when HPLC showed no further change in the intensity of the peaks. The reaction mixture was evaporated to dryness and the residue redissolved in ethyl acetate (50 ml), washed with a saturated aqueous solution of $NaHCO_3$, 0.1 M HCl and a saturated aqueous solution of NaCl, dried with $MgSO_4$, and evaporated to an oil (50 mg). Semi-preparative HPLC of this oil over a Waters μ-Porasil Radpak cartridge eluted at 2 ml min^{-1} with chloroform–hexane–ethanol (3 : 6 : 0.1) gave *E*-3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane *E*-**8** (8.3 mg, 49%) as an oil, CD $\Delta\varepsilon_{237.4}$ 6.1 (CH_3CN); $\lambda_{max}(CH_3CN)$ 249 nm (ε 13 399 dm^3 mol^{-1} cm^{-1}); ν_{max}(KBr) 1794, 1669, 1638 and 1591 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 3.19 (1H, d, *J* 16.7, 6β-H), 3.59 (1H, ddd, *J* 0.9, 2.8 and 16.6, 6α-H), 3.99 (1H, d, *J* 17.5, 2α-H), 5.06 (1H, dd, *J* 1.5 and 17.4, 2β-H), 5.72 (1H, d, *J* 2.8, 5-H), 5.87 (1H, dt, *J* 1.8 and 3.6, 8-H) and 9.57 (1H, d, *J* 5.0, 9-H); addition of *R*-2,2,2-trifluoro-1-(9-anthryl)ethanol (1000%) caused separation of the signals due to the 2-β proton of each enantiomer, and irradiation at the 8 proton chemical shift caused both sets of 2-β protons to lose their smaller coupling. The two sets of 2-β proton signals were in the ratio 2 : 1; *m*/*z* 153 (M^+, 23%), 125 (M^+-CO, 12%), 111 (M^+-CH_2CO, 24%) and 55 (M^+-98, 100%). Further elution gave *Z*-3-oxoethylidene-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane *Z*-**8** containing 5% of the *E* isomer

(2.3 mg, 14%), ν_{max}(KBr) 1792 and 1660 (br) cm^{-1}; δ_H (250 MHz, $CDCl_3$) 3.25 (1H, d, *J* 16.8, 6β-H), 3.52 (1H, ddd, *J* 0.8, 2.8 and 16.9, 6α-H), 3.85 (1H, d, *J* 16.8, 2α-H), 4.68 (1H, dd, *J* 0.8 and 17.0, 2β-H), 5.23 (1H, dt, *J* 1.0 and 7.9, 8-H), 5.81 (1H, d, *J* 2.8, 5-H) and 9.96 (1H, d, *J* 7.8, 9-H); *m/z* 153 (M^+, 24%) 125 (M^+-CO, 11%), 111 (M^+-CH_2CO, 29%) and 55 (M^+-98, 100%).

β-Lactamase inhibition by 1 (R = Na)

A solution of **1** (R = Na) was tested as a β-lactamase inhibitor by the automated chromogenic substrate assay of Reading and Farmer.[22] Taking the yield of **1** from the hydrogenation reaction to be 35%, gave an I_{50} with pre-incubation of enzyme and inhibitor of 0.01 μg ml^{-1} and without pre-incubation of 1 μg ml^{-1}.

β-Lactamase inhibition by *S. clavuligerus* dcl I 111 supernatant

The β-lactamase inhibitory activity of dcl I 111 supernatant was estimated by the chromogenic cephalosporin assay of Reading and Farmer.[22] A solution of nitrocephin (250 mg ml^{-1}) and RTEM JT4 β-lactamase from *E. coli* (5×10^{-3} BCD) in 0.05 M NaH_2PO_4 adjusted to pH 7.3 was incubated at 37 °C. The optical absorption of this reaction mixture at 482 nm was read in a 1 cm cuvette every minute for nine minutes to obtain an initial rate of β-lactamase activity. Standard reactions containing aliquots of a solution of clavulanic acid (final concentrations: 1.0 and 0.5 μg ml^{-1}) in phosphate buffer were assayed in the same manner. Test reactions comprised known final dilutions of the supernatant of dcl I 111 cultures which had been incubated for 43 hours. The clavulanic acid stock solution and the dcl I 111 supernatant were allowed to stand at room temperature, and were assayed for their initial rate of β-lactamase activity every half hour.

The percentage inhibition of each reaction was calculated from the β-lactamase activity of the inhibited (*Vi*) and uninhibited (*Vo*) reactions by the formula: % Inhibition = (*Vo* − *Vi*)/*Vo* × 100, and plotted on a graph against time.

Enzymatic reduction of 1 to clavulanic acid 2

An aqueous solution of **1** (R = Na) (0.1 ml, nominally 10 mM) obtained by hydrogenation of **6**, was added to washed *S. clavuligerus* SC2 cells (0.5 ml) which had been sonicated after 48 hours growth.[10] The suspension was treated with 10 mM NADPH (0.1 ml), made up to 1 ml with 50 mM MOPS buffer at pH 7.0 and incubated in stoppered tubes at 26 °C. Samples (0.2 ml) were removed at timed intervals, treated with methanol (0.2 ml) for 5 minutes and centrifuged. The clear supernatants were made up to 1.6 ml with MOPS buffer and assayed on discs by the KAG method.[11] An undiluted reaction mixture was applied to Whatman No. 1 chromatography tapes which were developed in either butan-1-ol–acetic acid–water (12 : 3 : 5) or butan-1-ol–ethanol–water (7 : 1 : 2), dried, and visualised by application to a KAG bioassay plate. The assay was repeated using the conditions in the Tables 1 and 2 with the results indicated. Standard clavulanic acid solutions were developed and visualised similarly.

Isolation of clavulanic acid dehydrogenase (CAD)

An *S. clavuligerus* SC2 culture in clavulanic acid production phase was harvested by centrifugation. The cell pellet was sonicated in 50 mM MOPS buffer (pH 7.5) containing 10% v/v glycerol, 1 mM dithiothreitol (DTT) the protease inhibitors 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine-HCl, and 10 μM L-*trans*-(epoxysuccinyl)leucylamido(4-guanidino)-butane (buffer).

Cell debris was removed by centrifugation and the supernatant treated with streptomycin sulfate (1% final concentration) and left at 4 °C for 30 min. The nucleic acid precipitate was removed by centrifugation and the supernatant loaded onto a column packed with 10 ml of Red-Sepharose (Pharmacia) which had been equilibrated with buffer over one hour. The column was washed with buffer, and non-specifically bound material was eluted with a solution of 10 mM NADH (10 ml). The column was washed with buffer until the absorption (280 nm) of the eluate had returned to baseline levels and eluted at 10 ml h^{-1} with buffer containing NADPH which increased in concentration from 0 to 10 mM over 8 h.

Fractions were collected over ten minute intervals and assayed for CAD activity. Aliquots of each column fraction (25 μl) were incubated with the MOPS buffer (40 μl), 10 mM NADPH (10 μl) and the synthetic aldehyde **1** (25 μl; approx. 1 mg ml^{-1}) at 21 °C for 10 minutes then diluted with 300 μl of cold buffer. These samples (90 μl) were added to wells in KAG agar plates and compared with duplicate samples from which NADPH had been omitted. SDS-PAGE analysis[23] of the fraction containing clavulanic acid dehydrogenase activity showed a band at 28 kDa. An electroblot of the band on an Immobilon-P membrane (Millipore) was sequenced and found to possess the *N*-terminal amino acid sequence given in Fig. 2.

Racemisation of benzyl 3-oxoethylidene-7-oxo-4-oxa-1-aza-bicyclo[3.2.0]heptane-2-carboxylate 6

A solution of the title compound[6] **6** as a mixture of geometrical isomers *E*–*Z* (2 : 1) (162 mg, 0.56 mmol) in chloroform (8 ml) was allowed to stand at ambient temperature away from strong light for three days. The solution was evaporated to dryness, chromatographed over silica gel in ethyl acetate–hexane (1 : 1), and evaporated to give the title compound in the ratio of geometric isomers *E*–*Z* (1.7 : 1) (53 mg, 33%), $[\alpha]_D^{20}$ +16.4 (*c* = 2% in dichloromethane); addition of *R*-2,2,2-trifluoro-1-(9-anthryl)ethanol (1000% by weight) to the NMR solution (250 MHz, $CDCl_3$) caused two sets of signals to appear for the 6-β, 6-α and 8 protons of both the *E* and *Z* isomers and for the 2 proton of the *E* isomer. Irradiation of specific protons demonstrated coupling between the 5 and 6 and the 8 and 9 protons for all these signals. The intensity of the signals indicated a 65 : 35 mixture of enantiomers. When the title compound was freshly prepared the geometric isomer ratio was *E*–*Z* (1.6 : 1), $[\alpha]_D^{20}$ +53.9 (*c* = 2% in dichloromethane), and addition of *R*-2,2,2 trifluoro-1-(9-anthryl)ethanol to the NMR solution showed a 93 : 7 mixture of enantiomers.

(*S*)-[$^{18}O_2$]Ornithine dihydrochloride

Following the method of Murphy and Clay,[16] a closed vial containing *S*-ornithine hydrochloride (200 mg, 1.19 mmol) and $H_2{}^{18}O$ (0.45 ml, 22.5 mmol; 97% ^{18}O, Aldrich) through which gaseous HCl had been bubbled for 30 s (*c* 4 M), was heated to 65 °C for four days. The cooled solution was triturated with 1,4-dioxane (70 ml) and the resulting white crystals washed with ether to provide a mixture of *S*-[$^{18}O_2$]ornithine dihydrochloride and *S*-[^{18}O,^{16}O]ornithine dihydrochloride (226 mg, 91%) (Found: C, 28.93; H, 6.78; N, 13.30; Cl, 34.08. $C_5H_{14}N_2Cl_2{}^{18}O_{1.9}{}^{16}O_{0.1}$ requires C, 28.75; H, 6.75; N, 13.41; Cl, 33.95%); ν_{max}(KBr) 3000 (br), 1708, 1595 and 1493 cm^{-1}; δ_H (250 MHz, D_2O) 1.72–2.19 (4H, m, 3-H_2 and 4-H_2), 3.10 (2H, t, *J* 7.3, 5-H_2), 4.11 (1H, t, *J* 6.2, 2-H); δ_C (100.614 MHz, D_2O) 23.6 (4-C), 27.7 (3-C), 39.6 (5-C), 53.3 (2-C), 172.44 (1-C$^{18}O_2$) and 172.48 (1-C^{18}O,^{16}O; intensity 10% of 172.44 resonance). Addition of [$^{16}O_2$]ornithine to this solution resulted in a third signal (1-C$^{16}O_2$) 2.5 Hz downfield from the (C^{18}O,^{16}O) resonance; *m/z* 137 [$M^{18}O_2$ +H]$^+$.

Benzyl (*S*)-[$^{18}O_2$]-2,5-bis(benzyloxycarbonylamino)pentanoate

Benzyloxycarbonyl chloride (0.014 ml, 0.1 mmol) in THF (5 ml) was added to a solution of *S*-[$^{18}O_2$]ornithine dihydrochloride (9 mg, 0.044 mmol) dissolved in water (5 ml) over

Table 3 The difference in normalised average areas for the ^{18}O enriched and natural abundance ions

m/z	From [$^{18}O_2$]ornithine	Natural abundance	Difference
362	1.67	1.67	0
364	0.166	0.124	0.042
366	0.0375	0.003	0.035

45 min while the temperature was maintained below 5 °C and the pH within the range 12–13 by the addition of a solution of NaOH.

The reaction mixture was evaporated to dryness, redissolved in DMF (10 ml) and reacted with benzyl bromide (15.5 mg, 0.1 mmol) for six days. Evaporation of the bulk of the DMF left a residue which was triturated with ethyl acetate. The ethyl acetate was decanted, the trituration of the solid residue repeated and the ethyl acetate extracts combined and evaporated to dryness. Recrystallisation (diisopropyl ether) gave the product as white rosettes of needles (10 mg, 46%), ν_{max}(KBr) 3330, 1691 (br), 1533, 751 and 697 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.40–1.96 (4H, m, 3-H_2 and 4-H_2), 3.08–3.25 (2H, m, 5H_2), 4.37–4.48 (1H, m, 2-H), 4.65–4.80 (1H, m, NH), 5.08 (2H, s) and 5.10 (2H, s) and 5.15 (2H, m) (CH_2Ph), 5.30–5.40 (1H, m, NH) and 7.25–7.45 (15H, m, Ph); δ_C (100 MHz, $CDCl_3$) 25.79 (4-C), 29.96 (3-C), 40.43 (5-C), 53.59 (2-C), 66.70, 67.08 and 67.24 (C=O), 128.12–128.67 (2,3,4 and 5 phenyl), 135.20, 136.19, 136.56 (1-phenyl), 155.88, 156.37 (HNCO_2) and 171.93 (CO_2); *m/z* 512 ([M$^{18}O_2$+NH_4]$^+$, 86%), 510 ([M^{18}O,^{16}O+NH_4]$^+$, 14%).

Feeding of *S*-[$^{18}O_2$]ornithine dihydrochloride to *S. clavuligerus* fermentations

A sterile-filtered aqueous solution of *S*-ornithine dihydrochloride containing isotope labels in the ratio 86% [$^{18}O_2$] and 14% [^{18}O,^{16}O] (29.8 mg, 0.14 mmol) was fed to *S. clavuligerus* SC2 fermentations (180 ml) during production phase and the resulting clavulanate was isolated[10] and purified as the benzyl ester **12**. ^{13}C and ^{1}H NMR and IR spectroscopy showed the sample to be indistinguishable from benzyl clavulanate containing natural isotopic abundance.

The benzyl clavulanate was treated with a mixture of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide–pyridine (1 : 1) for 30 minutes at room temperature and immediately injected onto a BP1 capillary column (25 metre) which, after 2 min at 120 °C, was heated to 280 °C at 8 °C minute^{-1}. The eluate from the capillary was monitored by a Finnigan MAT TSQ70 mass spectrometer operating in isobutane chemical ionisation mode. The areas beneath the signals due to the molecular ions *m/z* 362 [M$^{16}O_2$+H]$^+$, 364 [M^{18}O,^{16}O+H]$^+$ and 366 [M$^{18}O_2$+H]$^+$, were determined by selected ion recording and averaged for five separate analyses. Identical analyses were performed for benzyl clavulanate of natural isotopic abundance and the maximum variation within each set of five determinations was less than 5% of the average. The difference in normalised average areas for the ^{18}O enriched and natural abundance ions were determined and are shown in Table 3.

RS-[1-^{13}C]Ornithine

A procedure modified from Teplan and Marton[17] was followed: 4,4-diethoxybutylamine (3 g, 18.6 mmol) dissolved in pyridine (45 ml) was treated with benzoyl chloride (2.16 ml, 186 mmol) in a dry atmosphere below 10 °C. After 2 hours, water (60 ml) and ethyl acetate (400 ml) were shaken with the reaction mixture and the ethyl acetate layer was separated, extracted with a saturated aqueous salt solution, evaporated to dryness and azeotroped with toluene to give an oil (4.5 g).

The oil (2 g, 7.5 mmol) was dissolved in THF (50 ml), cooled in an ice bath and stirred with 2 M H_2SO_4 (10 ml) for 0.75 h. An ether extract (250 ml) of the reaction mixture was washed with a saturated aqueous solution of salt (15 ml), added to ethanol (20 ml) and evaporated to a volume of 10 ml. Ethanol (10 ml) and water (20 ml) were added and the cooled solution was adjusted to pH 10.7 with an ammonium hydroxide solution.

Na^{13}CN (172 g, 3.44 mmol; 92% ^{13}C) and NH_4Cl (452 mg, 8.45 mmol) were added and the solution stirred at 4 °C for 10 min before the temperature was raised to 70 °C for 3 hours. The mixture was cooled in ice, treated with concentrated HCl (20 ml) and stirred at room temperature for three days. The solution was boiled under reflux for 0.75 h and then cooled and evaporated to dryness. Further concentrated HCl (20 ml) was added and the solution boiled under reflux for three hours and evaporated to dryness. Acidic hydrolysis and evaporation of the solution were twice repeated, following which the residue was azeotroped with water (50 ml) to give a solid.

A solution of the solid was chromatographed over a 3.5 × 1 inch column of Dowex 50W 8X ion exchange resin in the (H^+) form, which was eluted with 1 M NH_4OH. Fractions containing ninhydrin positive material were bulked, adjusted to pH 3.8 with HCl and evaporated to dryness. The residue was triturated with ethanol and the resulting solid was recrystallised from water (5 ml) to which boiling ethanol (70 ml) had been added, giving a white solid (210 mg, 36% with respect to Na^{13}CN), ν_{max}(KBr) 2976 (br), 2106, 1625 and 1500 (br) cm^{-1}; δ_H (250 MHz, D_2O) 1.60–2.00 (4H, m, 3-H_2 and 4-H_2), 3.00 (2H, t, *J* 7.4, 5-H_2), 3.74 (1H, q, *J* 6.0, 2-H); δ_C (100 MHz, D_2O) 21.9 (4-C), 26.5 (3-C), 38.0 (5-C), 53.3 (d, *J* 53.5, 2-C), 173.2 (1-C); *m/z* (FAB, thioglycerol) MH$^+$ 134.

(*RS*)-[1-^{13}C,$^{18}O_2$]Ornithine dihydrochloride 13

RS-[1-^{13}C]Ornithine (168 mg, 0.99 mmol) was dissolved in $H_2{}^{18}O$ (0.25 ml, 12.5 mmol, 97% ^{18}O) which had been acidified by the passage of gaseous HCl for 30 seconds while atmospheric moisture was excluded by a blanket of argon. The sealed reaction vessel was heated to 70 °C for three days when the solvent was removed under vacuum and the residue dried in a desiccator to give a foam (194 mg). ^{13}C NMR revealed that the ornithine had been substituted with ^{18}O in the following pattern: [$^{18}O_2$] 75%, [^{18}O,^{16}O] 22% and [$^{16}O_2$] 2.7%. Exchange with $H_2{}^{18}O$ (0.1 ml, 5 mmol, 97% ^{18}O) was repeated to give a buff coloured hygroscopic solid (172 mg, 83%), δ_H (400 MHz, D_2O) 1.70–2.07 (4H, m, 3-H_2 and 4-H_2), 3.04 (2H, t, *J* 7.5, 5-H_2), 4.00 (1H, q, *J* 6.1, 2-H); δ_C (100 MHz, D_2O) 23.7 (4-C), 27.8 (3-C), 39.8 (5-C), 53.5 (d, *J* 58.2, 2-C), 172.933 (87%, [^{13}C,$^{18}O_2$]) and 172.967 (13%, [^{13}C,^{18}O,^{16}O]).

Feeding of (*RS*)-[1-^{13}C,$^{18}O_2$]ornithine dihydrochloride 13 to *S. clavuligerus* fermentations

RS-[1-^{13}C]ornithine dihydrochloride containing isotope labels in the ratio 87% [$^{18}O_2$] and 13% [^{18}O] (29.4 mg, 0.14 mmol) was fed to *S. clavuligerus* SC2 fermentations (180 ml) as before and the resulting clavulanate was isolated and purified as the benzyl ester, benzyl 3-(β-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate **12**, δ_H (400 MHz, $CDCl_3$) 3.07 (1H, dd, *J* 16.7 and 0.6, 6β-H), 3.48 (1H, dd, *J* 16.7 and 2.9, 6α-H), 4.14–4,27 (2H, m, 9-H), 4.88 (1H, dt, *J* 7.0 and 1.3, 8-H), 5.09 (1H, m, 2-H), 5.18 and 5.22 (2H, ABq, 12.2, CH_2Ph), 5.70 (1H, dd, *J* 2.8 and 0.5, 5-H) and 7.30–7.42 (5H, m, Ph); δ_C (100.614 MHz, $CDCl_3$) 46.39 (6-C), 57.30 (9-C), 60.57 (d, *J* 67, 3-*C*^{13}C), 60.59 (2-C), 67.66 (*C*H_2Ph), 88.00 (5-C), 100.39 (8-C), 128.41, 128.72 and 134.78 (Ph), 153.38 (3-C), 166.979 (10-^{13}C,$^{18}O_2$), 166.993 (10-^{13}C,[=^{18}O]), 167.015 (10-^{13}C,[-^{18}O]), 167.029 (10-^{13}C,$^{16}O_2$) and 174.25 (m, 7-C).

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