STUDIES ON THE BIOSYNTHESIS OF CLAVULANIC ACID. I

INCORPORATION OF ^{13}C-LABELLED PRECURSORS

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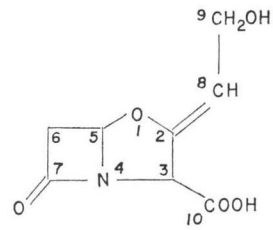
The biosynthesis of clavulanic acid was investigated by feeding ^{13}C-labelled precursors to *Streptomyces clavuligerus* fermentations. The resulting samples of clavulanic acid were isolated as the benzyl ester and were examined by ^{13}C NMR spectroscopy for ^{13}C-enrichment. The results showed that the carbon skeleton of 1,3-$^{13}C_2$-glycerol was incorporated intact into the three β-lactam carbons of clavulanic acid. Studies with 1-^{13}C-acetate, 2-^{13}C-acetate and 1,2-$^{13}C_2$-acetate indicated that the remaining five carbons of clavulanic acid were probably derived from α-ketoglutarate. 1-^{13}C-Propionate and 3-^{13}C-propionate were not metabolised *via* the same route as glycerol, but were probably converted to succinate, *via* methylmalonyl CoA, and hence *via* the tricarboxylic acid cycle to the clavulanic acid precursors.

Clavulanic acid[1] is a novel fused β-lactam produced by *Streptomyces clavuligerus* ATCC 27064. It is a potent inhibitor of β-lactamases from a wide range of Gram-positive and Gram-negative bacteria and also possesses weak antibacterial activity[2,3,4].

Fig. 1. Clavulanic acid



While the compound bears a superficial resemblance to the penicillins, cephalosporins and cephamycins, closer inspection indicates that the "tripeptide theory" of biosynthesis cannot be readily applied to this structure. The absence of a 6-amino group and aminoadipyl side chain, and the presence of an oxazolidine rather than a thiazolidine or dihydrothiazine ring all point to a different route of biosynthesis. However, intriguingly, *S. clavuligerus* ATCC 27064 also produces penicillin N, 7-(5-amino-5-carboxyvaleramido)-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid, cephamycin C[5,6] and deacetoxycephalosporin C[7], suggesting that some parts of the biosynthetic pathways to these compounds may be common; possibly the final ring closure sequences may be performed by the same enzyme (s).

In this paper we report on the possible origins of the carbon skeleton of clavulanic acid, based on data obtained from ^{13}C-labelled precursor feeding and ^{13}C-NMR studies.

Materials and Methods

Chemicals

^{13}C-Labelled precursors were purchased from Prochem, British Oxygen Co. Ltd., Deer Park Road, London SW19 3UF, U. K.; 1,3-$^{13}C_2$-glycerol was synthesised from 1,3-$^{13}C_2$ diethylmalonate by oxidising with lead tetraacetate followed by reduction with lithium aluminum hydride:[8]

All precursors were approximately 90 atom % ^{13}C at the site of label. Other chemicals used were of Analar grade.

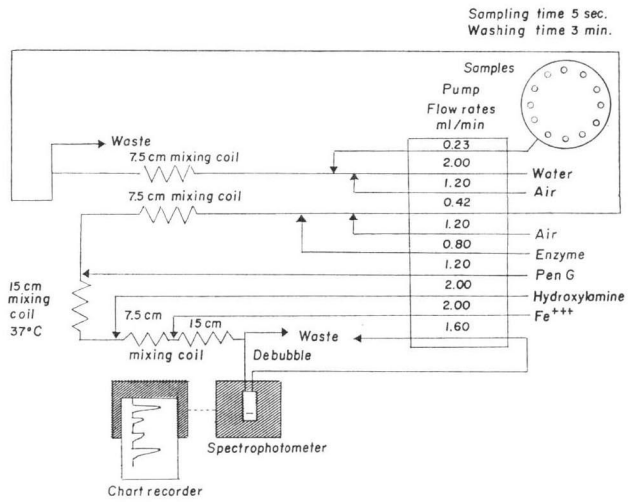
Culture

The organism used in these studies was *Streptomyces clavuligerus* IT2, a reisolate of *S. clavuligerus* ATCC 27064. The organism was grown in a 2-liter bench fermenter (Biolafitte, Life Science Laboratories Ltd., Leagrave, Luton, Bedfordshire, U. K.) at 26°C, aerated at 0.75 liters sterile air per minute. The pH was not controlled. The medium consisted of 1.0% w/v triglyceride (Prichem P224, Prices Ltd., Bromborough, Bebington, Cheshire, U. K.), 1.5% w/v soya bean flour (Arkasoy 50, British Arkady Co. Ltd., Old Trafford, Manchester, U. K.), 0.1% w/v potassium dihydrogen orthophosphate in deionised water, total volume 1.5 liters. The medium was adjusted to pH 7.0 before autoclaving. A spore inoculum was used ($\sim 10^8$ colony forming units). Samples of culture were removed periodically during growth and assayed for clavulanic acid content using an automated enzyme inhibition assay (see below). Labelled compounds were added during the clavulanic acid production phase. In the case of ^{13}C-bicarbonate, aeration was stopped and stirring speed reduced for 30 minutes after addition to prevent $^{13}CO_2$ being lost by sparging.

Automated enzyme inhibition assay

The ability of clavulanic acid to inhibit β-lactamases[2,3] was used as the basis for an automated assay. Standard solutions of sodium clavulanate tetrahydrate or culture supernatants were mixed with an R-factor mediated β-lactamase from *Escherichia coli* W3110[9] (obtained from Microbiological Research Establishment, Porton, Wiltshire, U. K.), and incubated with penicillin G (5.0 mg/ml added concentration). Residual penicillin G in the reaction mixture (which is dependent on the concentration of clavulanic acid in the reaction mixture) was determined by reacting with hydroxylamine and ferric ammonium sulphate,[10] and measuring the optical density at 490 nm. The β-lactamase reagent and penicillin G were made up in 0.05 M tris buffer pH 7.0. The concentration of β-lactamase was adjusted to just destroy the penicillin when no inhibitor was present.

Fig. 2. Automated assay for clavulanic acid
All autoanalyser equipment Technicon or equivalent.



Isolation and purification

Clavulanic acid was isolated as the benzyl ester using the method of READING (to be published; see also[1]). When the clavulanic acid titre in the culture had ceased to rise, (110~140-hour growth), the mycelium was removed by centrifugation and the culture supernatant freeze dried. The freeze dried solids were treated with benzyl bromide in dimethylformamide, and the resulting benzyl clavulanate extracted into ethyl acetate. The ethyl acetate fraction was evaporated to low bulk and further purified by column chromatography using Sephadex LH20 (Pharmacea), (solvent system, cyclohexane - chloroform, 1: 1); followed by silica gel column chromatography, (gradient elution with ethyl acetate: cyclohexane). Purity of the products was judged by t.l.c. (Merck, silica gel 60; solvent system, cyclohexane - ethyl acetate 4: 6, benzyl clavulanate Rf 0.35) using 2% triphenyltetrazolium chloride in 50% aqueous methanol, and dilute aqueous potassium permanganate as detecting spray reagents. The ^{13}C-NMR also indicated any major impurities in the benzyl clavulanate samples.

^{13}C-NMR spectroscopy

Proton decoupled ^{13}C-NMR spectra were obtained using a Varian CFT 20 spectrometer with an 8-

mm probe. All spectra were obtained for approximately 0.5 M solutions in chloroform *d* at approx. 30°C. Tetramethyl silane was used as an internal standard. All spectra were run with identical spectrometer parameters.

Spectra of the ^{13}C enriched samples were compared with a standard spectrum of benzyl clavulanate to give a ratio for the level of ^{13}C at each carbon with reference to the natural abundance level. The benzyl methylene was used as an internal natural abundance reference in the enriched samples.

Measurements of the peak heights were used in most cases to obtain the relative ^{13}C levels; in order to obtain reliability in the peak heights a negative exponential weighting function (−0.05 sec) was applied to the free induction decay spectrum before transformation. This process ensures adequate digitisation. In cases where ^{13}C-^{13}C spin-spin coupling was observed, owing to the use of a doubly labelled precursor, integration was used to obtain the relative ^{13}C levels. Integration was also used, in these cases, to estimate the proportion of precursor incorporated intact.

A comparison was made between the two methods of obtaining the relative ^{13}C levels; results were found to lie within the experimental error limits of ±10%. The quoted spin-spin coupling constants are accurate to ±1 Hz.

The assignments of the chemical shifts for the benzyl clavulanate carbons were made with reference to those reported by HOWARTH *et al*[4] for methyl clavulanate, except that the shifts at 174 and 167 p.p.m. can now be definitely assigned to C-7 and C-10 respectively (see Discussion).

Results

^{13}C-NMR spectroscopy of the benzyl clavulanate samples derived from ^{13}C-precursor fed fermenta-

Table 1. ^{13}C-Enrichments of benzyl clavulanate carbons

Precursor	Amount added (mg)	Time of addition	Enrichment ratio at carbon*								
			5	6	7	9	8	2	3	10	Benz CH_2
$CH_3{}^{13}COO^-$	4×62.5	Throughout antibiotic production phase	1.02	0.91	1.74	1.68	0.99	1.24	0.98	1.67	1.00
	250	Mid-production phase	1.00	0.95	1.51	1.55	1.01	1.19	1.06	1.48	1.00
$^{13}CH_3COO^-$	150	Mid-production phase	1.57	1.84	1.64	1.18	1.71	1.59	1.63	1.85	1.00
	2×125	Early and late production phase	1.42	1.33	1.61	1.08	1.67	1.58	1.52	1.63	1.00
$^{13}CH_3{}^{13}COO^-$	2×125	Early and late production phase	1.37	°1.67	°1.72	□1.79	□1.69	1.64	△1.64	△1.54	1.00
$CH_3CH_2{}^{13}COO^-$	250	Mid-production phase	0.90	1.13	1.50	1.11	0.98	0.96	1.13	1.89	1.00
	2×112	Early and late production phase	1.04	0.92	1.71	1.01	1.04	1.29	0.99	1.63	1.00
$^{13}CH_3CH_2COO^-$	4×62.5	Throughout production phase	1.36	1.51	0.95	1.05	0.95	1.14	1.22	1.02	1.00
	242	Late production phase	1.40	1.28	1.56	1.07	1.07	1.74	1.32	1.42	1.00
$HO^{13}CH_2$-$CHOH^{13}CH_2OH$	150	Mid-production phase	†3.60	0.97	†5.03	1.01	1.00	1.34	1.04	1.37	1.00
$H^{13}CO_3^-$	2×125	Early and late production phase	0.92	0.89	1.40	0.90	0.94	1.12	1.08	1.47	1.00
Chemical shift ppm			88.0	46.4	174.3	57.3	100.5	152.4	60.7	167.0	67.9

* See Fig. 1 for numbering of carbons.

°, □, △, † ^{13}C–^{13}C couplings observed between these pairs of carbons.

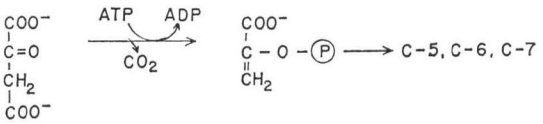
tions showed some ^{13}C enrichment in every case. When 1,2-$^{13}C_2$-acetate was used as precursor, ^{13}C-^{13}C spin-spin couplings were observed between C-6 and C-7 ($^1J_{^{13}C-^{13}C}=38.4$ Hz), C-8 and C-9 ($^1J_{^{13}C-^{13}C}=47.3$ Hz) and C-3 and C-10 ($^1J_{^{13}C-^{13}C}=67.0$ Hz). Integration of these peaks and their satellites indicated that approximately 38% of the enrichment at C-6 and C-7 was due to incorporation of intact acetate carbons, whereas this figure was 75% for C-8 and C-9 and 47% for C-3 and C-10. When 1, 3-$^{13}C_2$-glycerol was used as precursor, coupling was observed between C-5 and C-7 ($^2J_{^{13}C-^{13}C}=8.6$ Hz). Integration indicated that approximately 70% of the enrichment at these carbons was due to incorporation of the intact glycerol carbon skeleton.

Details of the ^{13}C-enrichment of the benzyl clavulanate samples, derived from the various precursors, are shown in Table 1.

Discussion

Of the data in Table 1, the incorporation of 1, 3-$^{13}C_2$-glycerol was the most striking. The relatively large enrichments at C-5 and C-7 accompanied by ^{13}C-^{13}C coupling indicated that the carbon skeleton of the β-lactam ring was derived from glycerol, without any intermediate rearrangement of the three carbons. The coupling of C-7 with C-5 also indicated that the assignment of the shift δ 174 ppm as C-7 was correct. The only other significant enrichments using this precursor were at C-2 and C-10. These were relatively much lower and no coupling could be detected.

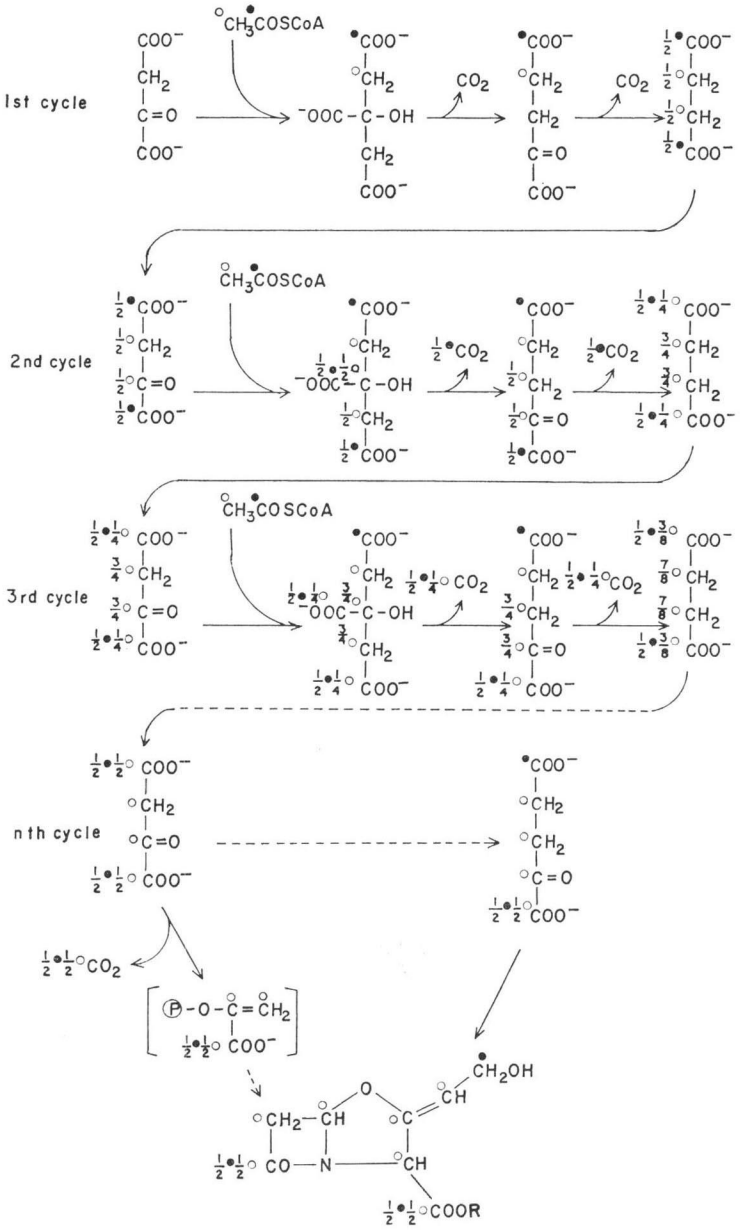
Adding 1-^{13}C-acetate or 2-^{13}C-acetate to duplicate fermentations gave good reproducibility in the labelling patterns of the resultant benzyl clavulanate. The benzyl clavulanate derived from 2-^{13}C-acetate was labelled to a significant level in 7 out of the 8 carbons. This obviously indicated extensive metabolism of the acetate, rather than a simple joining together of C_2 units. Also, using both these precursors, label appeared in the β-lactam carbon skeleton. As the *S. clavuligerus* was grown aerobically in a medium containing a high proportion of triglyceride, which would be catabolised to acetate, it was likely that any acetate fed to the culture would be metabolised *via* the tricarboxylic acid (TCA) cycle. Also, as the medium was low in carbohydrate, it was possible that one or more of the several shunts from the TCA cycle back to the glycollytic pathway might be operating to generate C_3 compounds, and hence carbons 5, 6 and 7 of clavulanic acid, *e. g.*:



The TCA cycle would also provide a five carbon skeleton (α-ketoglutarate) which might be a precursor of carbons 9, 8, 2, 3 and 10 of clavulanic acid.

To test these hypotheses the theoretical pattern of labelling of clavulanic acid derived *via* the above routes was worked out (Fig. 3) and compared with the actual patterns observed. It must be borne in mind that in the TCA cycle citrate is metabolised as an asymmetric molecule,[11] whereas succinate and fumarate are not. Therefore, any asymmetry of label in α-ketoglutarate would be lost in the subsequent stages of the cycle. Also, any label remaining in oxalacetate would be available for recycling by addition of a further acetate unit. It must be noted that the γ and δ carbons of α-ketoglutarate are derived from a labelled acetate unit, and although the label is subsequently distributed in a population of succinate molecules, this carbon-carbon bond remains intact. Therefore, when 1, 2-$^{13}C_2$ acetate is fed as precursor it would be predicted that ^{13}C-^{13}C spin-spin coupling would be observed between C-8 and C-9, C-6 and C-7, and C-3 and C-10. However, the couplings between C-6 and C-7, and C-3 and C-10 should indicate only half the incorporation of intact acetate carbon skeleton compared with C-8 and C-9. Comparison of Fig. 3 with Table 1 shows that there was a good qualitative agreement between the observed patterns of enrichment and the theoretical enrichments, when 1-^{13}C-acetate and 2-^{13}C-acetate were used as precursors. However, quantitatively, the enrichments of C-7 and C-10 were too high. When 1, 2-$^{13}C_2$-

Fig. 3. Theoretical distribution of label from acetate through the tricarboxylic acid cycle (Only essential stages shown)



acetate was the precursor the observed couplings were in agreement with the theory.

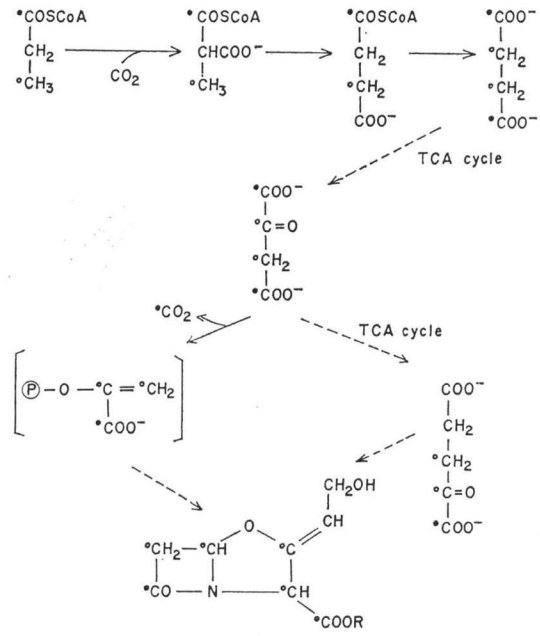
The enrichments obtained using 1-^{13}C-propionate and 3-^{13}C-propionate indicated that this molecule was not being metabolised *via* the same route as glycerol, as significantly large amounts of label appeared in the oxazolidine ring of clavulanic acid. Also, with 3-^{13}C-propionate, more than one atom of the β-lactam ring became labelled. Clearly, also, propionate was not being metabolised to acetate, as no label appeared at C-8 or C-9 in any sample. A possible explanation of the observed labelling is that the propionate was being metabolised *via* methylmalonyl CoA[12] to succinyl CoA and hence *via* the TCA cycle (Fig. 4). This would result in label from 1-^{13}C-propionate being incorporated in C-7 and C-10

as observed in both fermentations. Label from 3-^{13}C-propionate would be incorporated in C-5 and C-6, and C-2 and C-3. This was observed in one fermentation, but in a second experiment when the label was added late in the antibiotic production phase the carbonyls C-7 and C-10 were also labelled, suggesting that a different metabolic route may have been operating.

As carboxylation by carbon dioxide was implicated, ^{13}C-bicarbonate was added to a *S. clavuligerus* IT2 fermentation. This resulted in labelling of C-7 and C-10 as would be predicted from Fig. 4. The conversion of propionate to pyruvate and subsequent carboxylation to oxalacetate[12] or malate[12] would not result in the observed distribution of label from 3-^{13}C-propionate, as no symmetrical intermediates are involved.

The uptake of carbon dioxide into methylmalonyl CoA would also offer an explanation for the elevated quantity of label in C-7 and C-10 in benzyl clavulanate samples derived from 1- or 2-^{13}C-acetate. ^{13}C-Carbon dioxide would be liberated during the metabolism of these compounds *via* the TCA cycle, which could then re-enter the cycle *via* succinyl CoA.

Fig. 4. Theoretical distribution of label from propionate through the methylmalonate pathway



In summary, it would appear that the precursor of C-5, C-6 and C-7 of clavulanic acid is a 3-carbon compound which can be derived from glycerol or the later stages of the TCA cycle. Probably it is an intermediate of the glycollytic pathway, or derived from such an intermediate. C-9, C-8, C-2, C-3 and C-10 are derived from the carbon skeleton of α-ketoglutarate, probably *via* glutamate, the terminal carboxyl being reduced to alcohol. It seems unlikely that the penicillin and cephalosporin precursor, δ-(α-aminoadipyl)cysteinyl-valine, is involved. While cysteine can be derived from glycollytic intermediates, valine is not derived from the TCA cycle; a complicated rearrangement of the valine carbon skeleton would be required to give the observed patterns of labelling of clavulanic acid.

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