The origin of the β-lactam carbons of clavulanic acid

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Pyruvate is the most likely primary metabolic source of the three β-lactam carbons of clavulanic acid.

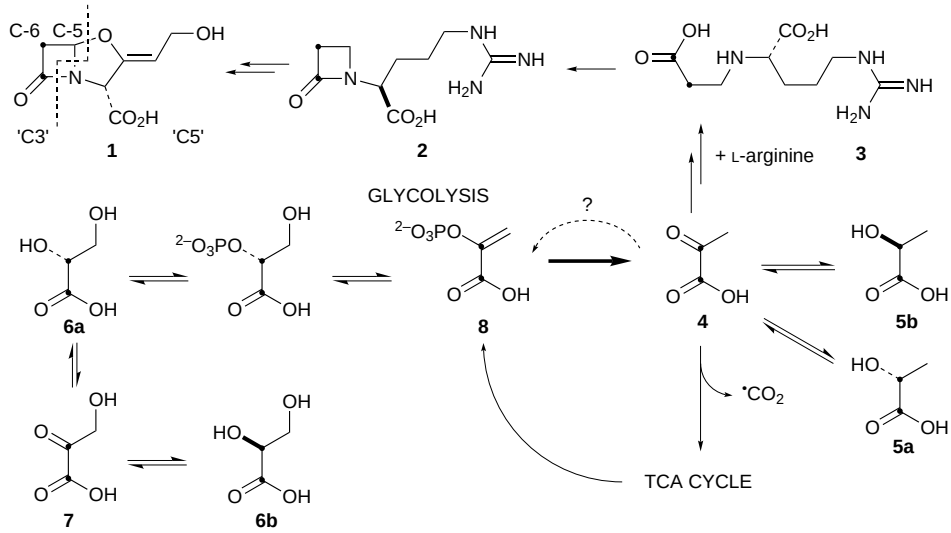
Clavulanic acid **1** is a commercially important inhibitor of β-lactamases, and is produced *via* fermentation of *Streptomyces clavuligerus*. It is known that the primary metabolic precursor of the five-carbon unit (Scheme 1) of clavulanic acid is arginine,[1] Pioneering studies by Elson *et al.*[2] and Townsend *et al.*[3] have demonstrated that the precursor of the three-carbon unit is a three-carbon primary metabolite (both pyruvate[2] and glycerate[3] have been proposed) although no conclusive evidence has yet been presented. Monocyclic β-lactam **2** has been identified as an intermediate in clavulanic acid **1** biosynthesis (Scheme 1) and there is evidence[4] that it is precursed by the acyclic compound **3**, but the pathway from primary metabolism to these putative intermediates is unknown.

Feeding experiments using labelled precursors have apparently demonstrated that glycerol,[3,5] propionate,[5] β-hydroxypropionate,[6] pyruvate,[3] and D- and L-glycerates[3] are specifically incorporated into the β-lactam ring of **1**. It has been reported that both L- and D-[2-^{3}H, 1-^{14}C]glycerate are specifically incorporated into **1** to similar levels, but that only in the case of the D-isomer is any ^{3}H incorporated at C-6 of **1**. A recent study[3b] using racemic [1,2-$^{13}C_2$, 2,3,3,-$^{2}H_3$]glycerate, however, reported that no incorporation of the C-2 label into C-6 of **1** was observed. Other possible 3-carbon precursors such as malonic acid semi-aldehyde, β-hydroxypropionate or acrylate cannot be ruled out by these previous studies.

Herein we report studies using optically active ^{13}C and ^{2}H labelled lactates and glycerates which imply that pyruvate **4** and not glycerate is the primary metabolic precursor of the three β-lactam carbons of **1**.† 1,2-^{13}C labelled species were used in order to generate doublets in the ^{13}C NMR spectra separating the signals arising from labelled material from the relatively intense singlet of the unlabelled material, allowing accurate incorporation levels to be determined. Retention levels of ^{2}H were analysed by the observation of splitting or isotopic shifts of the doublets.

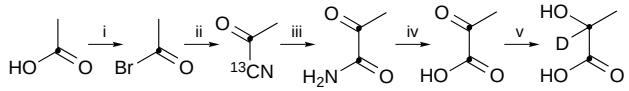
The L- and D-[1,2-$^{13}C_2$, 2-^{2}H]lactates **5a**,**b** were synthesised[7,8] in 23% yield (and > 98% isotopic purity) from [1-^{13}C]acetic acid (Scheme 2). Notably Tris-HCl buffer was used for the enzyme catalysed reductions of pyruvate to L- and D-lactates rather than phosphate buffer[9] which resulted in a mixture of lactates with ^{1}H and ^{2}H at C-2. Synthesis of DL-[1,2-$^{13}C_2$, 2,3,3-$^{2}H_3$]glycerate[10]‡ was achieved from [$^{2}H_2$]formaldehyde (Scheme 3) in an overall yield of 80% and > 98% isotopic purity. DL-[1,2-$^{13}C_2$, 1,1-$^{18}O_2$]glycerate was synthesised in a similar fashion. Resolution of the racemic labelled glycerate was achieved by HPLC on a preparative C-18 column using the method of Horikawa *et al.*[11] In the lactate and glycerate syntheses, hydrolysis of the intermediate nitriles to the acids by literature conditions[12,13] was low yielding. Use of strong acid ion exchange resin however resulted in good yields and trivial product purification.

The labelled precursors were fed to shake flask cultures of a *Streptomyces clavuligerus* wild-type re-isolate (SC2) in a minimal medium.[5] Clavulanic acid **1** was isolated as its benzyl ester after gel and silica chromatography and analysed by ^{13}C NMR spectroscopy acquired under fully quantitative conditions. It was found that optimisation of the aquisition parameters was required in order to obtain spectra which were suitable for integration due to the large pertubations in signal intensity caused by the ^{13}C and ^{2}H isotope effect on spin relaxation rates. Samples were also examined by ^{2}H NMR spectroscopy to corroborate results. The spectra recorded for **1** derived from the labelled lactates indicated that whilst both D- and L-lactate **5a**,**b** were specifically incorporated into **1** to similar levels (1.2 and 1.9% respectively), in neither case had any retention of ^{2}H at C-6 of **1** occurred. This implies that lactate **5a**,**b** must pass *via* pyruvate **4** before incorporation into **3**. The spectra recorded for

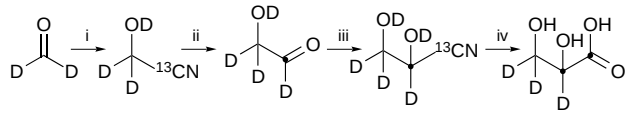


Scheme 1 Proposed biosynthesis of clavulanic acid **1** (^{2}H labels not shown)

the L- and D-glycerates **6a**,**b** similarly showed no evidence for retention of ^2H at C-6 of **1**. In contrast, 80% of the clavulanic acid **1** derived from either D- or L-labelled glycerate was labelled with ^2H at C-5. These results are consistent with results reported by Pitlik and Townsend using racemic material.[3b] The total carbon incorporation for D-glycerate **6a** was 1.9% whilst that for L-glycerate **6b** was only 0.3%. The ^2H labelling of C-5 of **1** is in good agreement with previous results.[3,14,15] The intact incorporation of the 1,2-^{13}C labels from lactate and glycerate together with the lack of incorporation of the C-2 ^2H label from either substrate indicate that neither is the primary metabolic precursor for the β-lactam carbons of **1**. Instead the results imply that pyruvate is the precursor. Oxidation of lactate **5a**,**b** to pyruvate **4** will result in the loss of the 2-^2H label whilst retaining the carbon skeleton. Glycolysis of glycerate **6a**,**b** to pyruvate **4** will similarly remove the 2-^2H label and result in some loss of the 3-^2H label through enolisation. The low level of incorporation of L-glycerate **6b** probably reflects the differences between metabolism of L- and D-glycerate in *S. clavuligerus*. In mammalian systems it is known that L-glycerate may be epimerised to D-glycerate *via* oxidation to hydroxypyruvate[16] **7**. The low incorporation of ^{18}O (19%) into the C-7 carbonyl of **1** from DL-[1,2-$^{13}C_2$, 1,1-$^{18}O_2$]glycerate is



Scheme 2 Synthesis of L- and D-[1,2-$^{13}C_2$, 2-^2H]lactates **5a**,**b**. *Reagents and conditions*: i, PBr_3; ii, $Cu^{13}CN$; iii, HCl, Et_2O then H_2O; iv, DOWEX-50 (H^+), H_2O, 80 °C; v, D- or L-lactate dehydrogenase, NAD^+, yeast alcohol dehydrogenase, Tris-HCl, CD_3CD_2OD, pH 8.5, 25 °C.



Scheme 3 Synthesis of DL-[1,2-$^{13}C_2$, 2,3,3-$^2H_3$]glycerate **6a/b**. *Reagents and conditions*: i, $K^{13}CN$, pH 8.5, 25 °C, D_2O; ii, Pd–$BaSO_4$, D_2, pH 1.7, D_2O; iii, $K^{13}CN$, pH 8.5, 25 °C, D_2O; iv, AMBERLITE IR-120 (H^+), D_2O, 95 °C.

Table 1 Incorporation data for labelled lactate and glycerate

Precursor	Carbon[e] incorporation (%)	Retention at C-6[d] (%)	Retention at C-5[d] (%)
L-[1,2-$^{13}C_2$, 2-^2H]lactate[a] **5b**	1.9	< 2	n/a
D-[1,2-$^{13}C_2$, 2-^2H]lactate[a] **5a**	1.2	< 2	n/a
DL-[1,2-$^{13}C_2$, 2,3,3-$^2H_3$]glycerate **6a**/**b**	1.6	< 1	80
L-[1,2-$^{13}C_2$, 2,3,3-$^2H_3$]glycerate[b] **6b**	0.3	< 5	80
D-[1,2-$^{13}C_2$, 2,3,3-$^2H_3$]glycerate[c] **6a**	1.9	< 2	80
DL-[1,2-$^{13}C_2$, 1,1-$^{18}O_2$]glycerate[c]	1.8	(C-7 retn: 19% ^{18}O)	

[a] > 98% ee. [b] > 90% ee. [c] > 95% ee. [d] As a proportion of carbon incorporation; levels measured by ^2H NMR were within 5%. [e] Adjusted to allow for the amount of **1** present before addition of labelled species, in order to allow for more reliable comparison of labelling results between different fermentation batches.

an additional indication of the metabolic conversions that this species must undergo before incorporation into **2**. Cell extracts from fermentations of labelled lactate, analysed by ^{13}C-INADEQUATE NMR, showed that an accumulation of [1,2-$^{13}C_2$]alanine could be detected indicating conversion of lactate **5a**,**b** to pyruvate **4**. It is conceivable that lactate is converted to glycerate before assimilation into **3**, however, metabolism *via* the TCA cycle will break the 1-2 ^{13}C–^{13}C bond and is thus ruled out. Additionally the direct conversion of pyruvate **4** to phosphoenol pyruvate **8** has not been reported for *Streptomyces* ssp. and would not explain the loss of all the 2-^2H label (and 20% of the 3-^2H labels) in the labelled glycerate fermentions which imply oxidation of C-2 to the ketone. We conclude that the most likely primary metabolic source of the β-lactam carbons of clavulanic acid **1** is pyruvate **4** not glycerate **6a**,**b** or lactate **5a**,**b**.

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Footnotes

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‡ The same material was prepared using an alternative route by Pitlik and Townsend [ref. 3(*b*)].

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