Incorporation of β-Hydroxypropionate into the β-Lactam Residue of Clavulanic Acid

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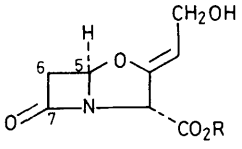
β-Hydroxypropionate is shown by radiochemical techniques to be incorporated specifically into the C-3 residue of the β-lactam portion of clavulanic acid.

Since the discovery in 1976 of the clinically important clavulanic acid (**1a**)[1] produced by *Streptomyces clavuligerus*, its biosynthesis and in particular the biosynthetic origin of the β-lactam segment (C-5—7) have been under intensive investigation. Early studies by Elson *et. al.*[2] indicated that glycerol is incorporated intact into the C-3 segment; however, very recently, Townsend showed that glycerate is a better precursor.[3] We now report a high and specific incorporation of radiolabelled β-hydroxypropionic acid (**2**) into the β-lactam segment of (**1**). Our choice of the potential precursor was

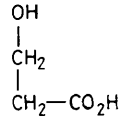
Table 1. Incorporation of radiolabelled β-hydroxypropionates into *p*-bromobenzyl clavulanate (**1b**) and detection of labels in the C-5—7 segment.

	Precursor[a]			Clavulinate		C-5—7 segment	
Expt.	Label	Amount/mmol	^{14}C spec. act.[b] (^{3}H/^{14}C ratio)	% ^{14}C Spec. inc. into (**1b**)	^{3}H/^{14}C in (**1b**) (% ^{3}H retained)	% ^{14}C retained	^{3}H/^{14}C ratio (% ^{3}H retained)
1	[1-^{14}C]	2.5	88.0	5.7 ± 1.2		92.1 ± 2	
2	[2-^{14}C]	2.0	82.5	5.0 ± 1.1		97.1 + 2	
3	[2-^{3}H,2-^{14}C]	2.0	82.5 (5.70)	6.3 + 1.3	5.45 (95.6 ± 2)	95.0 ± 2	3.11 (57 ± 2)

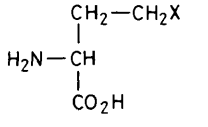
[a] Precursor is sodium β-hydroxypropionate. [b] μCi/mmol.



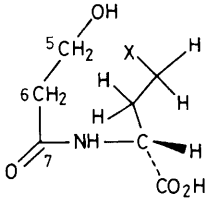
(1) a; R = H
b; R = *p*-$BrC_6H_4CH_2$



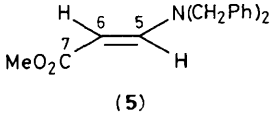
(2)



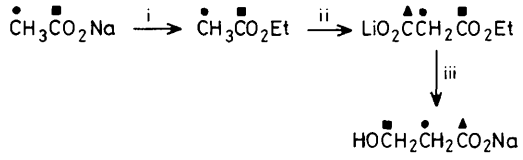
(3) a; X = CO_2H
b; X = CH_2NH_2



(4) a; X = CO_2H
b; X = CH_2NH_2



(5)



Scheme 1. ■, ●, ▲, possibilities for ^{14}C labelling. *Reagents:* i, $(EtO)_3P{:}O$; ii, a, LDA, b, $^{\blacktriangle}CO_2$; iii, a, $LiBH_4$, b, H^+, c, NaOH.

based on the following observations: (a) the oxidation levels of the carbon atoms in β-hydroxypropionate are the same as those of the corresponding atoms in clavulanic acid; (b) β-hydroxypropionic acid is known to be a product of glycerol metabolism,[4] which is in full agreement with the earlier described results on glycerol incorporation.

The synthesis of the appropriately labelled β-hydroxypropionates from readily available labelled sodium acetate or carbon dioxide[5] (Scheme 1) involves the formation of ethyl acetate, its deprotonation by lithium di-isopropylamide (LDA), and quenching with CO_2 to give the lithium monoethyl malonate. This is then treated with $LiBH_4$, which selectively reduces the ester end of the malonate monoester. Labelling at C-2 is obtained by hydrogen exchange with tritiated water at the monoethyl malonate stage.

The incorporation experiments were carried out using Aharonowitz's medium,[6] containing aspargine and starch, rather than the usual glycerol-based medium.[7] The fermentations of *S. clavuligerus* (ATCC—27064) were carried out at 28 °C in 21 shaken flasks each containing 500 ml of the above media. To ensure the necessary aerobic requirements, sterile air was flushed through the flasks at a rate of 200 ml/min. Radiolabelled precursors were administered after 30 h incubation, at the beginning of the production stage,[8] in concentrations indicated in Table 1 to a total volume of 1.5 l. After a further 40 h the cells were filtered off and clavulanic acid isolated from the supernatant liquid as its *p*-bromobenzyl ester (**1b**).[7] The fraction of radioactivity in the β-lactam carbon atoms was determined by degradation of (**1b**) with dibenzylamine in methanol to give the crystalline methyl *trans*-3-(*N,N*-dibenzylamino)acrylate (**5**).[9]

The high retention of the ^{14}C label in the fragment (**5**) is consistent with an intact incorporation of β-hydroxypropionate specifically into the C_3 unit C-5—7 of clavulanic acid. The virtually unchanged ^{3}H:^{14}C ratio in the clavulanic acid produced in experiment 3 suggests that no tritium is lost from the C-2 methylene group at any stage of the biosynthesis. The retention of only 57% of the tritium in the corresponding degradation product (**5**) can be explained by exchange with the medium. This was checked by carrying out the degradation in MeOD and monitoring the proton content of the resulting (**5**) by ^{1}H n.m.r. spectroscopy. The signal for the vinyl proton corresponding to H-6 of (**1**) was reduced in intensity by 60%. On this basis, if allowance is made for the isotope effect reducing removal of tritium, it is reasonable to conclude that virtually all the tritium in the clavulanic acid produced in experiment 3 resides at C-6.

We now propose that the first step in the building of the skeleton of clavulanic acid involves the coupling of β-hydroxypropionate with an appropriate C_5 amino acid such as glutamate (**3a**) or ornithine (**3b**) to form the hypothetical 'dipeptide' intermediate (**4**). This closely resembles the classical Arnstein tripeptide which is known to undergo enzymatic oxidative ring closure in the biosynthesis of penicillin.[10] In the clavulanic acid case the complete retention of ^{3}H at C-6 in experiment 3 argues against any enzyme-free intermediate in which C-5 of (**4**) is at the aldehyde level of oxidation, because rapid exchange would occur. We therefore propose that a concerted oxidation and ring closure is involved in the later stages, as has been proposed by Baldwin for penicillin.[11]

We thank Professor Y. Aharonowitz and Mrs. V. Kuper for preliminary experiments and advice on growing *S. clavuligerus*, Dr. J. Staunton for discussions, Glaxo Research Ltd., and Beecham Pharmaceuticals for samples of lithium and

potassium clavulanate, and the Bat Sheva de Rothschild Fund for the Encouragement of Research for financial support (A. L. G. is a Fellow of the Bat Sheva Foundation, 1984—86).

Received, 4th August 1985; Com. 1138

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