The fate of [2,3,3-²H₃, 1,2-¹³C₂]-d,l-glycerate in clavulanic acid biosynthesis

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The hydrogen at C-2 of glycerate is lost in the biosynthesis of clavulanic acid.

Elusive to experiment and mechanistic understanding has been the biosynthesis of the 4-membered ring of deoxyguanidinoproclavaminic acid 1, the first β -lactam-containing intermediate in the anabolic pathway to clavulanic acid 2.1 These three carbons are known to be derived efficiently from glycerol **3**, accompanied by the loss of H_{C} ^{2,3} Subsequent radiochemical experiments with samples of this C3-carbohydrate bearing tritium stereospecifically at the pro(R) hydroxymethylene established that chiral information was delivered through the entire biosynthetic pathway to clavulanic acid 2 such that the (1R,2S)-hydrogen (\hat{H}_A) of **3** is lost while H_B is incorporated in 2 at C-5.4 Similarly, in the clavaminate synthase-catalysed cyclization/dehydration of proclavaminic acid 5 to clavaminic acid 6 it was determined that formation of the oxazolidine ring involved specific replacement of the 4'S-label (HA) with substrate oxygen (retention of configuration).⁵ Finally, the demonstration that both H_A and H_B are retained from glycerol 3 to proclavaminic acid 5 allowed important deductions to be made about the cryptic formation of the β -lactam ring. Generation of the azetidinone N–C-4' bond takes place with (a)loss of the glycerol oxygen, (b) no net change in oxidation state at this methylene carbon and, (c) overall retention of configuration,⁶ unlike monocyclic β -lactam formation in nocardicin⁷ and, presumably, the monobactams. Lastly, the stereochemical inversion that takes place between clavaminic acid 6 and clavulanic acid 2 must occur with retention of $H_{\rm B}$.

The complete loss of H_C from glycerol **3**, but the retention of both H_A and H_B into proclavaminic acid **5** followed by their stereospecific loss and retention, respectively, into clavulanic acid **2** point to the intervention of conventional glycolytic metabolism, *e.g.* to glycerate **4**, prior to uptake into the β -lactam biosynthetic pathway. Earlier whole-cell studies revealed that [2-3H, 1-1⁴C]-d-glycerate gave efficient incorporation of carbon label into the β -lactam ring (95–100%) of clavulanic acid, but only 4–11% of the tritium could be accounted for in the molecule by chemical degradation, principally at C-6 and C-8.³ Given the inherent inaccuracies of low radioactivity in this technically difficult double label experiment, we have reexamined the fate of the glycerate C-2 hydrogen by a methodologically distinct means.

The experiment to test the retention or loss of H-2 was designed to use two 13C-labels as internal measures of intact and absolute carbon utilization and then multiple deuterium labels to monitor changes at H-2 knowing that one of the glycerate H-3 hydrogens would be cleanly retained in clavulanic acid 2. Thus, (±)-[2,3,3- ${}^{2}H_{3}$, 1,2- ${}^{13}C_{2}$]glycerate **12** (Scheme 2) was prepared as follows. [1,2-13C2]Bromoacetate 7 was converted to its crystalline p-nitrobenzyl (PNB) ester and treated with PPh₃ to form the phosphonium salt 8. Facile ylide formation in D_2O allowed rapid exchange at the C-2 methylene followed by Wittig reaction with deuterioformaldehyde in $D_2O^{8,9}$ in the presence of anhydrous K_2CO_3 yielded *p*-nitrobenzyl [2,3,3-²H₃, 1,2-13C2]acrylate 10 in excellent yield. Sharpless dihydroxylation^{10,11} gave PNB glycerate 11. Hydrogenolysis in the presence of NaHCO3 and reverse-phase HPLC purification (Partisil 10 ODS 3, 25×250 mm, H₂O elution) provided the desired quintuply labelled sodium glycerate **12** in nearly 60% overall yield. ¹³C{¹H} NMR analysis of the product [Fig. 1(*a*)] displayed the expected carboxyl resonance as a clean doublet (${}^{1}J_{CC} = 54.2 \text{ Hz}$) while the corresponding doublet for C-2 was further split into a pair of 1:1:1 triplets owing to the directly bound deuterium (${}^{1}J_{CD} = 22.1 \text{ Hz}$) and broadened slightly by the adjacent C-3 deuteria.

Cultures (2.0 l) of *Streptomyces clavuligerus* (ATCC 27064) were grown as previously described (500 ml/4 l flask).¹² After 48 h, **12** (2.0 mmol/l) was administered in equal portions to the fermentations under sterile conditions. After an additional 96 h, the clavulanic acid produced was isolated by adsorption onto carbon, conversion to its *p*-bromobenzyl ester and purification by silica gel chromatography (40 mg).^{2,13} Careful ¹³C{¹H} NMR analysis of this product showed the natural abundance singlet for the β -lactam carbonyl, C-7, flanked by a doublet (${}^{1}J_{CC} = 38.9$ Hz) indicating 0.80–0.85% intact incorporation of the multiply labeled glycerate carbon skeleton [Fig. 1(*b*)].



Scheme 2 Reagents and conditions: i, PNB–OH, EDC, DMAP, CH₂Cl₂, room temp., 3 h, 95%; ii, PPh₃, THF–CH₂Cl₂, room temp., 24 h; iii, D₂O, room temp., 24 h; iv, D₂CO in D₂O, K₂CO₃, 24 h, 80% for 3 steps; v, Bu'OOH, cat. OsO₄, Et₄NOAc, acetone, 0 °C to room temp., 6 h, 85%; vi, H₂, 10% Pd–C, NaHCO₃, H₂O–THF, room temp., 24 h, then RP–HPLC, 92%

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Centred upfield at δ 46.39, the natural abundance peak for C-6 could be found flanked by upfield α - and α/β -shifted doublets.¹⁴ That marked by (\blacktriangle) corresponds to ¹³C at C-6 paired to ¹³C at C-7 and ²H-label at C-5 (α - and β -heavy isotopes; $\Delta \delta = 0.144$ ppm). This species represents 80% fully intact incorporation of 12. The second, smaller doublet (\bullet) is due to doubly ¹³C-labelled molecules, but having ¹H at C-5 (α-heavy isotope; $\Delta \delta = 0.019$ ppm). While this 20% of incorporated precursor seems high, it is an artifact⁴ of cummulative isotope effects¹⁵ that discriminate against ²H-labelled molecules, but let the very small proportion of [1H,13C]-labelled precursor and intermediate molecules pass more swiftly along the pathway. These observations notwithstanding, notably absent is a 1:1:1 triplet corresponding to deuterium directly bound to C-6 and a concommitant β -isotope shift detectable in the doublet for C-7. In an attempt to establish an upper bound for incorporation of deuterium at C-6, a ²H{¹H} NMR spectrum was recorded (data not shown). Deuterium label at C-5 was easily observed and, while some minor secondary incorporation at C-9 could be



Fig. 1 Partial ¹³C{¹H} NMR spectra at 100 MHz of (*a*) $[2,3,3-^{2}H_{3}, 1,2-^{13}C_{2}]$ -d,l-glycerate **12** in D₂O, referenced to DSS; (*b*) biosynthetically enriched *p*-bromobenzyl clavulanate in CDCl₃, referenced to SiMe₄, 21 000 transients

detected, none was seen at C-6. Within the limits of the NMR methods used, we place the possible level of deuterium incorporation at C-6 from each of these NMR experiments at < 0.05%.

We are led to conclude from these data that ²H-2 is lost from 12 upon incorporation into clavulanic acid. There is a caveat, however remote, that H-2, unlike H-3, is labile among the intermediates of glycolysis despite the use of a triglyceridebased medium to favour gluconeogenesis.^{3,12} The possibility exists that the metabolic flux among these intermediates is so great relative to the rate of entry into the biosynthetic pathway that the C-2 deuterium has exchanged for hydrogen before detectable incorporation at C-6 can occur. This proviso aside, the loss of H-2 from glycerate imposes new limitations on the mechanism of β -lactam formation in clavulanic acid biosynthesis, a process whose full understanding has thus far resisted experimental enquiry.

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