The fate of [2,3,3-$^2H_3$, 1,2-$^{13}C_2$]-d,l-glycerate in clavulanic acid biosynthesis

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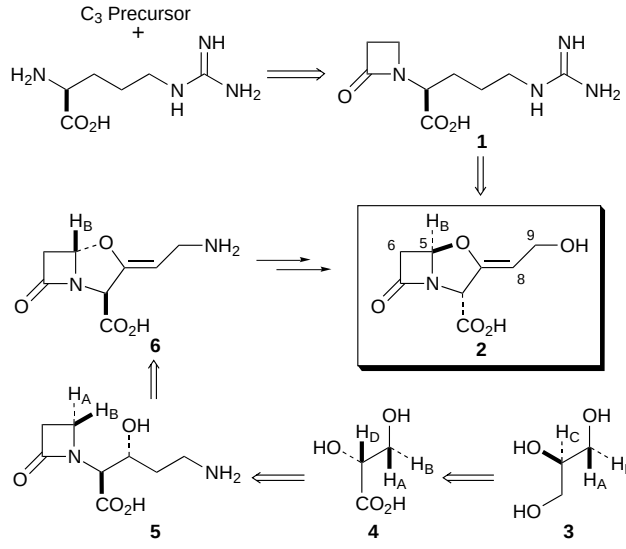
The hydrogen at C-2 of glycerate is lost in the biosynthesis of clavulanic acid.

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 C_3-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 (1*R*,2*S*)-hydrogen (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 (H_A) 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 nocardicin[7] 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_B.

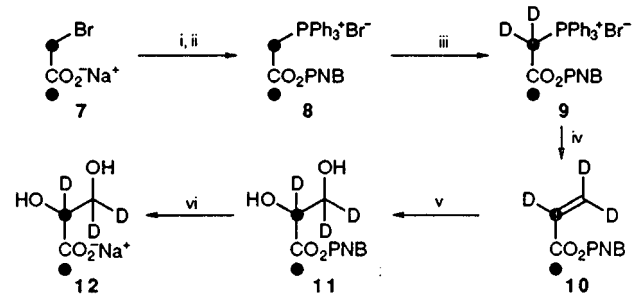
The complete loss of H_C from glycerol **3**, but the retention of both H_A and H_B into proclavaminic acid **5** followed by their stereospecific loss and retention, respectively, into clavulanic acid **2** point to the intervention of conventional glycolytic metabolism, *e.g.* to glycerate **4**, prior to uptake into the β-lactam biosynthetic pathway. Earlier whole-cell studies revealed that [2-^{3}H, 1-^{14}C]-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 re-examined 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 ^{13}C-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-$^2H_3$, 1,2-$^{13}C_2$]glycerate **12** (Scheme 2) was prepared as follows. [1,2-$^{13}C_2$]Bromoacetate **7** was converted to its crystalline *p*-nitrobenzyl (PNB) ester and treated with PPh_3 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-$^2H_3$, 1,2-$^{13}C_2$]acrylate **10** in excellent yield. Sharpless dihydroxylation[10,11] gave PNB glycerate **11**. Hydrogenolysis in the presence of $NaHCO_3$ and reverse-phase HPLC purification (Partisil 10 ODS 3, 25 × 250 mm, H_2O elution) provided the desired quintuply labelled sodium glycerate **12** in nearly 60% overall yield. $^{13}C\{^1H\}$ NMR analysis of the product [Fig. 1(*a*)] displayed the expected carboxyl resonance as a clean doublet ($^1J_{CC}$ = 54.2 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 ($^1J_{CD}$ = 22.1 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 $^{13}C\{^1H\}$ NMR analysis of this product showed the natural abundance singlet for the β-lactam carbonyl, C-7, flanked by a doublet ($^1J_{CC}$ = 38.9 Hz) indicating 0.80–0.85% intact incorporation of the multiply labeled glycerate carbon skeleton [Fig. 1(*b*)].



Scheme 1



Scheme 2 *Reagents and conditions*: i, PNB–OH, EDC, DMAP, CH_2Cl_2, room temp., 3 h, 95%; ii, PPh_3, THF–CH_2Cl_2, room temp., 24 h; iii, D_2O, room temp., 24 h; iv, D_2CO in D_2O, K_2CO_3, 24 h, 80% for 3 steps; v, Bu^{t}OOH, cat. OsO_4, Et_4NOAc, acetone, 0 °C to room temp., 6 h, 85%; vi, H_2, 10% Pd–C, $NaHCO_3$, H_2O–THF, room temp., 24 h, then RP–HPLC, 92%

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 (▲) corresponds to ^{13}C at C-6 paired to ^{13}C at C-7 and ^{2}H-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 (●) is due to doubly ^{13}C-labelled molecules, but having ^{1}H at C-5 (α-heavy isotope; $\Delta\delta$ = 0.019 ppm). While this 20% of incorporated precursor seems high, it is an artifact[4] of cummulative isotope effects[15] that discriminate against ^{2}H-labelled molecules, but let the very small proportion of [^{1}H,^{13}C]-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 $^{2}H\{^{1}H\}$ 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 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%.

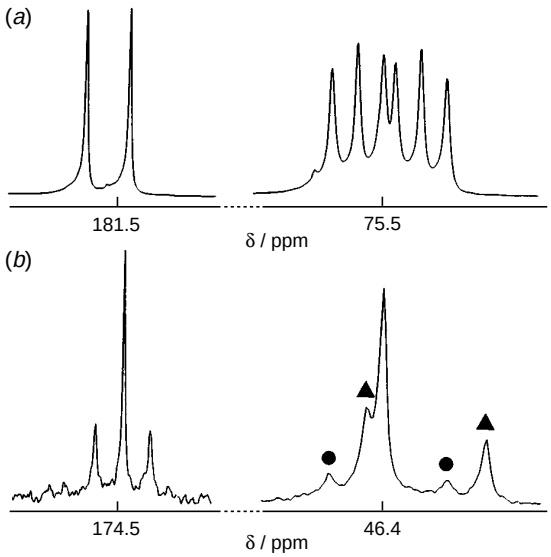


Fig. 1 Partial $^{13}C\{^{1}H\}$ NMR spectra at 100 MHz of (*a*) [2,3,3-$^{2}H_3$, 1,2-$^{13}C_2$]-d,l-glycerate **12** in D_2O, referenced to DSS; (*b*) biosynthetically enriched *p*-bromobenzyl clavulanate in $CDCl_3$, referenced to $SiMe_4$, 21 000 transients

We are led to conclude from these data that ^{2}H-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 triglyceride-based 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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