Evidence for Distinct Mechanisms of Monocyclic β-Lactam Biosynthesis

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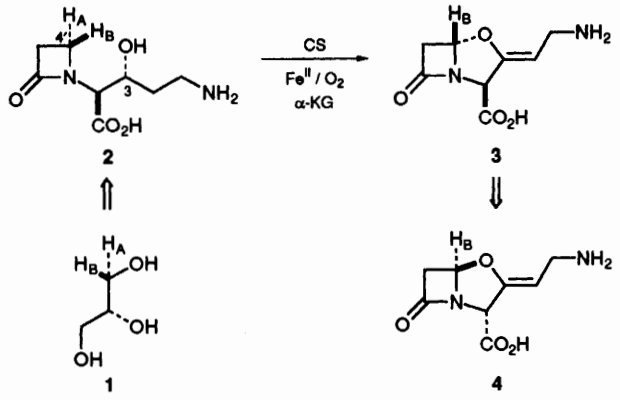
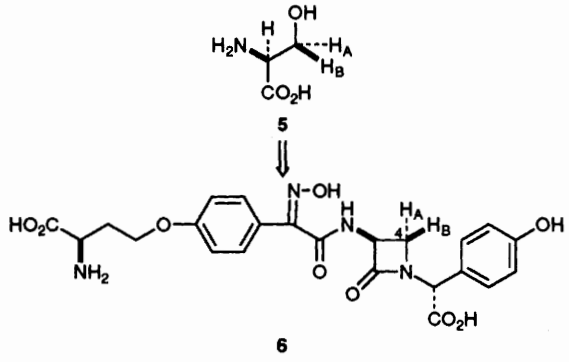
The hydroxymethylene oxidation state of primary metabolic precursors is maintained in the biosynthesis of the β-lactam ring of proclavaminic acid as it is in nocardicin A, but the net stereochemical course of reactions to give the azetidinone N–C-4 bond is the opposite.

The biosynthesis of clavulanic acid **4** proceeds through the intermediacy of the monocyclic β-lactam proclavaminic acid **2** (Scheme 1).[1] Stereochemical experiments with radiolabelled samples of glycerol **1** established specific incorporation of the (1*R*,2*R*) hydrogen (H_B) into clavulanate.[2] Similarly, proclavaminic acid **2** bearing diastereotopic deuterium labels at C-4′ underwent oxidative cyclization/desaturation to clavaminic acid **3** catalysed by clavaminate synthase (CS) in which the 4′(S) label (H_A) was specifically replaced by substrate oxygen.[3,4] From these data it may be deduced that the N–C-4′ bond of proclavaminic acid is formed with retention of configuration, provided the identitity of H_A remains unchanged between **1** and **2**. In the biosynthesis of nocardicin A **6** the hydroxymethylene of L-serine **5** becomes C-4 of the β-lactam ring in an overall process involving no change in oxidation state and clean stereochemical inversion (Scheme 2).[5,6] In this paper, we demonstrate that both *pro*-(*R*) hydroxymethylene hydrogens of glycerol (**1**,-CH_AH_BOH) are retained in **2** through a mechanism of β-lactam formation that must differ from that of the nocardicins.



Scheme 1

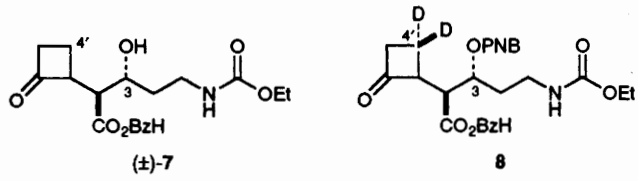


Scheme 2

[1,3-^{3}H]- and [1,3-^{14}C]-glycerol were combined and a portion was diluted with radioinactive material and derivatized as its tris(*p*-nitrobenzoate).[7] Recrystallization to constant specific radioactivity gave a $^3H/^{14}C$ ratio of 13.08. Cultures (1.5 dm^{-3}) of *Streptomyces clavuligerus* (ATCC 27064) were grown as previously described in 15 500 cm^3 Erlenmeyer flasks.[8] At 50 h of growth, 1.6 mmol of doubly labelled glycerol was administered equally among the fermentations under sterile conditions. After an additional 21 h, the cells were collected by centrifugation and a portion (77 g) was ruptured by sonication in 125 cm^3 of 20 mmol dm^{-3} phosphate buffer, pH 7.0. The cell debris was removed by centrifugation, the supernatant was filtered through an Amico PM30 membrane and the ultrafiltrate was lyophilized to dryness. The residue was dissolved in the minimum amount of water, unlabelled racemic proclavaminic acid (100 mg)[9] was added, and the mixture was treated with ethyl chloroformate in acetone (pH 8.5) and that portion soluble in ethyl acetate at pH 2 was esterified with diphenyldiazomethane. Derivatized D,L-proclavaminic acid was reisolated by silica gel chromatography and crystallized to constant specific activity as (±)-**7** (m.p. 70–71 °C, $^3H/^{14}C$ = 6.68).†

The result of this trapping experiment showed a gratifying 51% retention of tritium label relative to the internal ^{14}C-standard consistent with the incorporation of both glycerol *pro*-(*R*) hydroxymethylene hydrogens (**1**, $-CH_AH_BOH$) into proclavaminic acid **2**. The result, however, could be fortuitous owing to secondary (and probably unequal for each isotope) utilization of label in the C_5 portion of the molecule derived by way of the urea cycle amino acids ornithine/arginine.[10] To secure the selective incorporation of both H_A and H_B from **1** into **2**, 5 mmol of [$^2H_5$]glycerol was

† All new compounds gave satisfactory spectral and analytical data.



administered to 2.0 dm^3 of *S. clavuligerus* essentially as above, and the proclavaminate produced was diluted, derivatized and reisolated as (±)-**7**. While diastereotopic, the C-4′ hydrogens are not well resolved in their 300 MHz ^{1}H NMR spectrum. After considerable experimentation, it was found that functionalization[11] of the C-3 hydroxy as its *p*-nitrobenzoate (PNB) **8**† rendered the ^{1}H NMR signals of these two hydrogens sufficiently well separated (δ 3.74 and 3.51) that their individual detection by deuterium NMR would become possible. In the event, ^{2}H NMR analysis at 76.7 MHz revealed weak deuterium enrichment at other predictable sites[12] in **8**, but substantial and equal incorporations of label at the two diastereotopic C-4′ loci were observed.

The findings of these experiments establish that no change in oxidation state of the *pro*-(*R*) hydroxymethylene occurs in the biosynthetic steps between glycerol **1** (or glycerate[12]) and proclavaminic acid **2**. In view of previous stereochemical information,[2,3] its utilization at C-4′ of the monocyclic β-lactam of **2**, therefore, must take place with overall retention of configuration during N–C-4′ bond formation. This stereochemical outcome is the opposite to that seen during β-lactam formation in nocardicin A **6**.[5,6] While the latter may be hypothesized as simply the result of a displacement process, the former must involve reactions occurring with an even number of inversions more consistent with, for example, elimination/addition processes in natural products biosynthesis. More broadly, these stereochemical observations further support the view of mechanistic diversity in the biochemically important task of β-lactam synthesis *in vivo* and of separately evolved biosynthetic solutions to the members of this antibiotic class.[13,14]

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