Isolation of an Intermediate in Clavulanic Acid Biosynthesis

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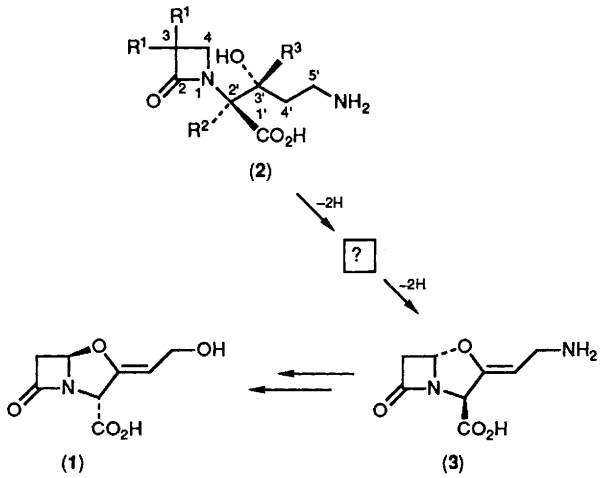
The operation of a primary isotope effect was utilised to enable a hitherto unknown intermediate in clavulanic acid biosynthesis to be isolated and characterised.

The key cyclisation step in clavulanic acid (**1**) biosynthesis has recently been shown to be the oxidative cyclisation of proclavaminic acid (**2a**) to clavaminic acid (**3**) (Scheme 1).[1,2] The enzyme, clavaminic acid synthase, has been purified to homogeneity and shown to have a requirement for Fe^{2+}, dioxygen, and 2-oxoglutarate.[1,2] Thus, this enzyme shows similar requirements to other oxygenase enzymes of β-lactam biosynthesis, such as deacetoxycephalosporin C synthase.[3] It has recently been shown[4] that in the enzymatic conversion of (**2a**) to (**3**), the oxygen of the hydroxy moiety of proclavaminic acid was retained and that there was no loss of deuterium label from positions C-2′ and C-3 of proclavaminic acids (**2b**) and (**2c**).

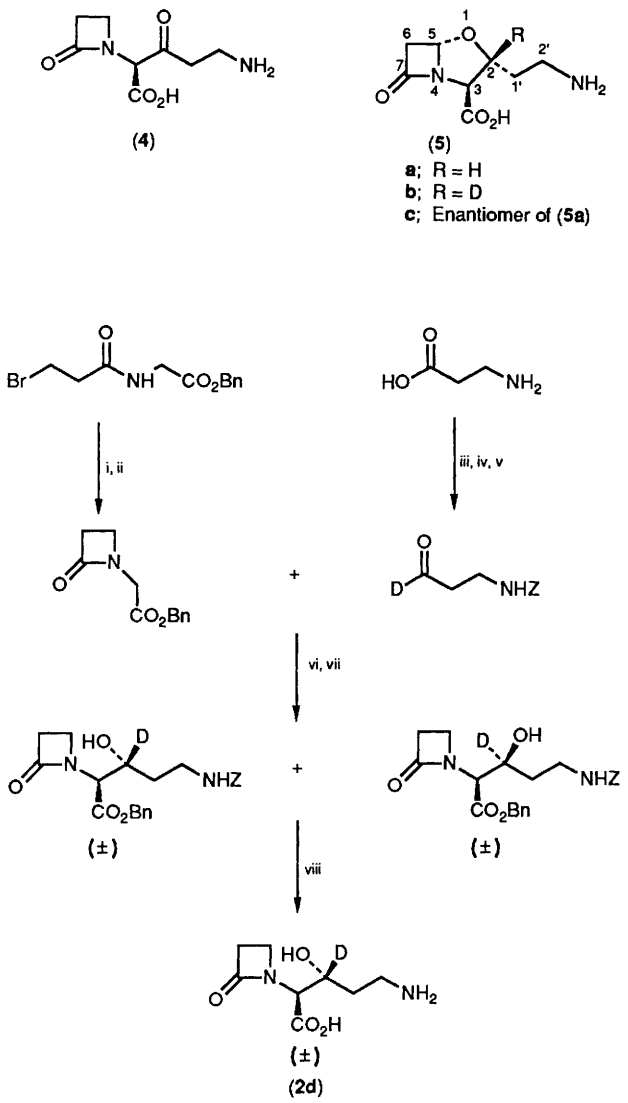
Although (**2a**) is apparently converted to (**3**) by a single enzyme, two distinct chemical events must occur, namely closure of the oxazolidine ring and desaturation to form the exocyclic double bond. Each stage requires the abstraction of two hydrogen atoms, and, as no intermediates have been detected, the order of these events remains unknown. Two possible intermediate structures are the 3′-keto compound (**4**) or the bicyclic clavam (**5a**). The experiments described below support (**5a**) as the intermediate, rather than (**4**).

Initially we examined the product composition of an incubation of fully protiated proclavaminic acid (**2a**) with



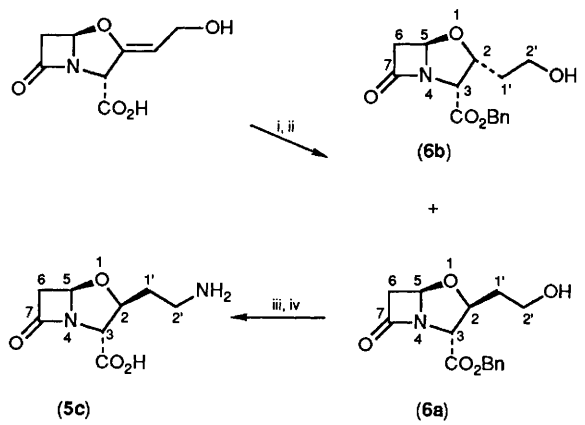
For (**2**): **a**; $R^1 = R^2 = R^3 = H$
b; $R^1 = R^3 = H$, $R^2 = D$
c; $R^2 = R^3 = H$, $R^1 = D$
d; $R^1 = R^2 = H$, $R^3 = D$

Scheme 1



Scheme 2. *Reagents and conditions:* i, NaI, acetone; ii, NaH, DMF, −10 °C; iii, $PhCH_2OCOCl$, THF/H_2O, 0 °C; iv, EtOCOCl, Et_3N, THF, −10 °C, then $NaBD_4$; v, $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −50 °C; vi, $(Me_3Si)_2NLi$, THF, −78 °C, then H_3O^+; vii, DBN, CH_2Cl_2, then separate diastereoisomers by flash chromatography; viii, H_2, Pd/C (10%), $EtOH/H_2O$.

Z = $PhCH_2OCO$; Bn = $PhCH_2$; DMF = dimethylformamide; THF = tetrahydrofuran; DMSO = dimethyl sulphoxide; DBN = 1,5-diazabicyclo[4.3.0]non-5-ene.



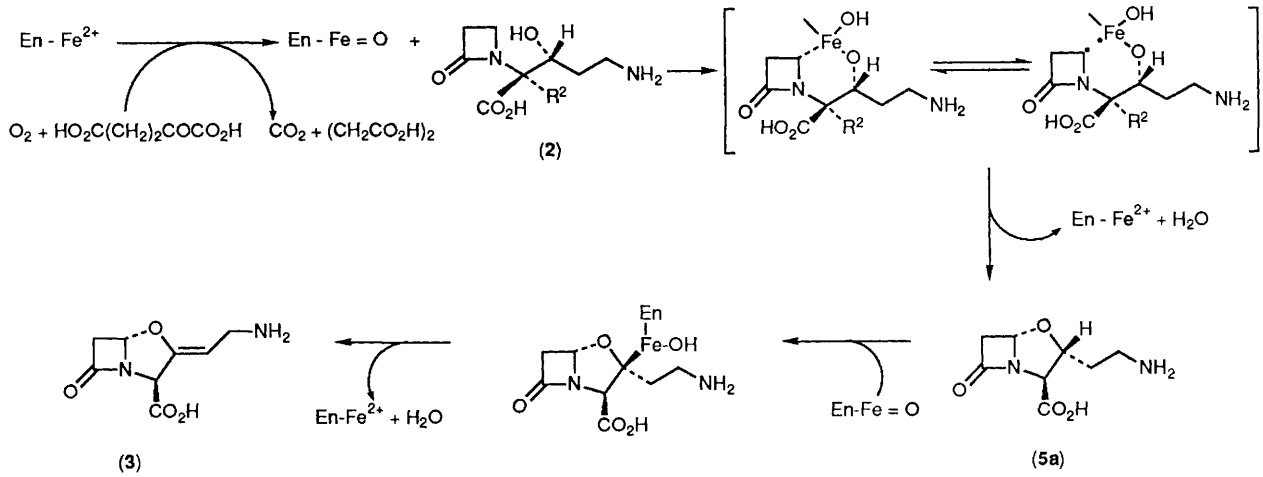
Scheme 3. *Reagents and conditions:* i, H_2, Pd/C (10%); ii, $PhCH_2Br$, DMF, ratio (**6a**): (**6b**) 1:4; iii, Ph_3P, diethyl azodicarboxylate, diphenylphosphoryl azide, THF; iv, H_2, Pd/C (5%), $EtOH/H_2O$, 1:1.

partially purified clavaminic acid synthase from *Streptomyces clavuligerus*[5] by 500 MHz ^1H NMR spectroscopy and saw, in addition to signals corresponding to clavaminic acid (**3**), a minor resonance at δ *ca.* 5.4, equivalent to approximately 5—10% of the intensity of the clavaminic acid resonances. This resonance occurred as a doublet with an apparent coupling constant of 2.5 Hz, and was thus reminiscent of the C-5 proton of a 1',2-dihydroclavulanate system.[6] Reasoning that this might be an intermediate between proclavaminic acid (**2a**) and clavaminic acid (**3**), we investigated the incubation of a deuteriated proclavaminic substrate (**2d**), in the hope that an isotope effect would slow the formation of the exocyclic double bond and enable the putative bicyclic saturated intermediate to be isolated. Previously, we have successfully used this strategy with enzymic reactions in penicillin and cephalosporin biosynthetic studies.[3]

Racemic proclavaminic acid labelled with deuterium at the C-3' position (**2d**) was synthesised (Scheme 2), and when incubated with partially purified clavaminic acid synthase gave the same two products, but now in an approximately 1:1 ratio. The new material was purified and isolated by HPLC [reverse phase octadecylsilane column] and characterised by ^1H NMR spectroscopy (500 MHz; D_2O; ref. to sodium 3-trimethylsilyl-tetradeuteriopropionate); δ_H 2.09—2.14 (1H, m, 1'-H), 2.28—2.31 (1H m, 1'-H), 2.96 (1H, d, *J* 16.5 Hz, 6-H), 3.21 (2H, *ca.* t, *J* 7 Hz, 2'-H), 3.44 (1H, dd, *J* 16.5, 2.5 Hz, 6-H), 4.14 (1H s, 3-H), 5.40 (1H, d, *J* 2.5 Hz, 5-H) (2-H was deuteriated). This spectrum was consistent with the structure (**5b**) and our assignment was subsequently confirmed by doping a solution of (**5b**) with an authentic sample of the enantiomer of dihydroclavaminic acid (**5c**) [prepared from calvulanic acid (Scheme 3)], followed by ^1H NMR and HPLC analysis. The relative stereochemistry of (**5c**) was determined by nuclear Overhauser enhancement (NOE) experiments.† Assuming that the *S*-configuration at C-2' of proclavaminic acid remains unchanged during the conversion then the absolute stereochemistry of the natural dihydroclavaminate is (2*R*,3*S*,5*S*) as shown in (**5a**). On this basis the configuration at C-3' of proclavaminic acid is retained during formation of the oxazolidine ring.

We conclude from the above evidence that clavaminic acid synthase converts proclavaminic acid to clavaminic acid *via* a dihydroclavaminate intermediate. The possibility that (**5a**) is a shunt metabolite of the enzyme, rather than an intermediate, cannot be entirely ruled out by our experiments. However, the large increase in the ratio of (**5b**):(**3**) caused by the use of deuteriated proclavaminic acid, in the absence of any other detectable compound, suggests that this latter theory is unlikely. A possible mechanism for the formation of the oxazolidine ring, involving an enzyme-bound iron oxene moiety, is indicated in Scheme 4.

† For (**5c**) irradiation of 2-H gave an NOE (8%) to 5-H, whereas irradiation of 1'-H gave no NOE to 5-H. For (**6a**) irradiation of 2-H gave an NOE (5%) to 5-H, whereas irradiation of 1'H have no NOE to 5-H. For (**6b**) irradiation of 2-H gave no NOE to 5-H, whereas irradiation of 1'-H gave an NOE (3%) to 5-H.



Scheme 4

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‡ *Note added in proof*: pure (**5b**) has been converted to (**3**), by highly purified clavaminic acid synthase, demonstrating the intermediacy of (**5**) in the conversion of (**2**) to (**3**).

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5 Details of enzyme purification and incubation conditions will be published elsewhere.
6 For a discussion see: P. C. Cherry and C. E. Newall, in 'Chemistry and Biology of β-Lactam Antibiotics,' eds. R. B. Morin and M. Gorman, Academic Press, London, 1982, vol. 2, p. 372.