Investigation of the Stereospecificity of Clavaminic Acid Synthase in the Desaturation of Dihydroclavaminic Acid to Clavaminic Acid

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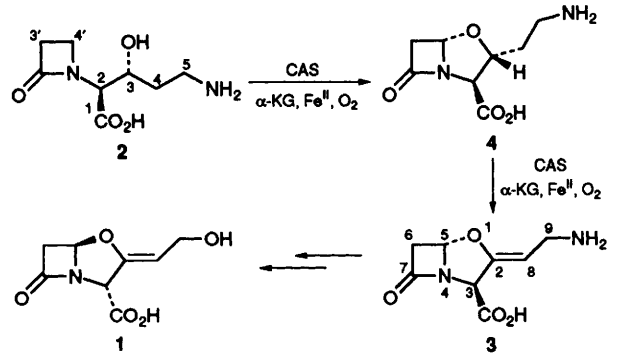
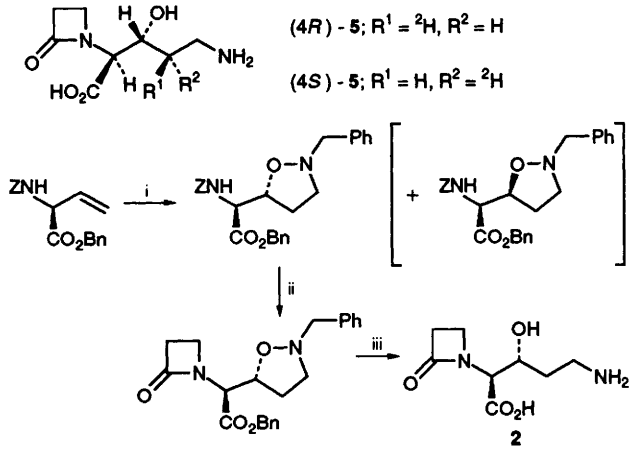
Incubations of (4*R*)- and (4*S*)-[4-$^2H_1$]-proclavaminic acid with clavaminic acid synthase resulted in the stereospecific removal of the deuterium and hydrogen respectively from C-4, in their conversions to clavaminic acid, suggesting an enzyme catalysed *syn*-elimination for the desaturation of dihydroclavaminic acid to clavaminic acid.

Clavaminic acid synthase (CAS),[1] isolated from *Streptomyces clavuligerus* ATCC 27064,[2] is one of the enzymes involved in the biosynthesis of the β-lactamase inhibitor clavulanic acid **1**.[2,3] The key function of CAS is conversion of the monocyclic β-lactam, proclavaminic acid **2**[4,5] to the bicyclic clavaminic acid **3**,[4] *via* the dihydroclavaminic acid intermediate **4**[6] (Scheme 1). This process requires ferrous ion as co-factor and α-ketoglutarate (α-KG) and molecular oxygen as co-substrates and involves two distinct chemical events, namely closure of the oxazolidine ring to give **4** and subsequent desaturation to form the exocyclic double bond of **3**.[6]

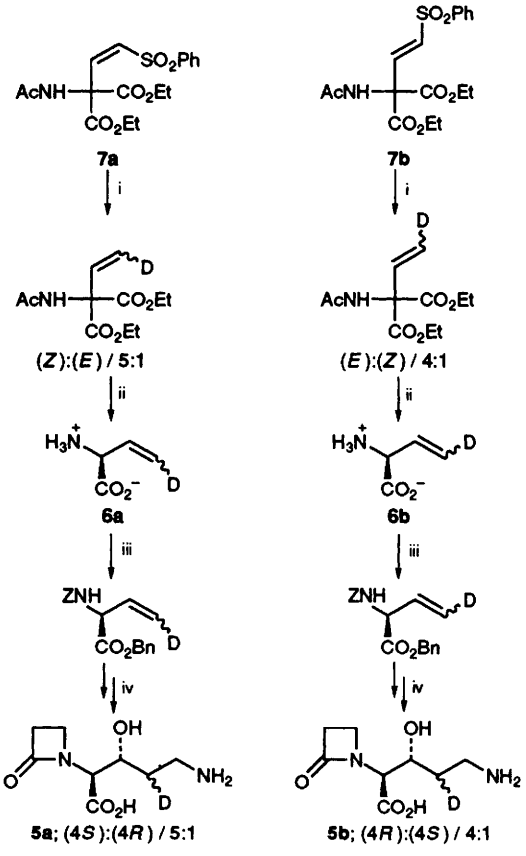
Labelling experiments have shown that the cyclisation of **2** to **3** (*via* **4**) occurs stereospecifically,[7] with the 4′-pro-(*S*) hydrogen being replaced by oxygen from the substrate. The aim of this work was to investigate the stereospecificity of the desaturation of **4** to **3** by incubation of (4*R*)- and (4*S*)-[4-$^2H_1$]-proclavaminic acids **5** with CAS.

The synthesis of hydroxyornithine, based on a 1,3-dipolar cycloaddition of *N*-benzyl nitrone to vinyl glycine, has been described by Wityak and Gould.[8] Elaboration of a β-lactam moiety onto the cycloadduct and hydrogenation provides Townsend's route to proclavaminic acid (Scheme 2).[9] In order to incorporate the deuterium label in **5**, (*E*)- and (*Z*)-[4-$^2H_1$]-vinyl glycines **6** were required, the stereospecific olefinic synthesis of these amino acids in racemic form had been described by Sawada and Hill from the (*E*)- and (*Z*)-vinyl sulfones **7**, respectively, by reduction with aluminium amalgam.[10] Despite repeated efforts the stereospecificity of the sulfone reduction could not be reproduced in greater than a 5:1 ratio of retention to inversion. This was considered adequate to proceed as the (*E*):(*Z*) ratio of deuterium





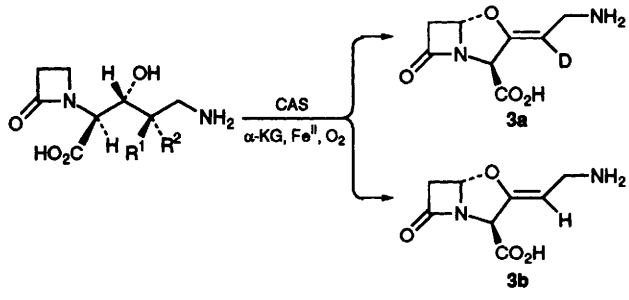
Scheme 2 *Reagents and conditions*: i, (*a*) *N*-benzyl nitrone, benzene, reflux, (*b*) separation of diastereoisomers by chromatography; ii, (*a*) HBr in AcOH, (*b*) *tert*-butyl acrylate, (*c*) TFA, (*d*) PPh_3, dipyridyl disulfide, MeCN, reflux; iii, $Pd(OH)_2/C$, H_2 (3 atm.), EtOH:H_2O (1:1 v/v)



Scheme 3 *Reagents and conditions*: i, Al/Hg (D_2O), dioxan, 10 °C, 48 h; ii, (*a*) 6 mol dm^{-3} HCl, reflux, 4 h (*b*) $ClCH_2COCl$, 1 mol dm^{-3} NaOH, $Et_2O:H_2O$ (1:1 v/v); (*c*) enzymatic resolution using Acylase I from porcine kidney; iii, (*a*) *N*-benzyloxycarbonyloxysuccinimide, CH_2Cl_2, NEt_3, then H_3O^+, extract; (*b*) $PhCHN_2$; iv, reagents as shown in Scheme 2

incorporation in the vinyl compounds was clearly discernible by 500 MHz NMR and, since the protons at C-4 in **2** are diastereotopic, the ratio of deuterium incorporation in **5** could also be clearly established. The (2*S*)-[4-$^2H_1$]-vinyl glycines **6a** and **6b** were obtained by enzymatic resolution[11] of the racemic olefins; a necessary procedure for the synthesis of the optically pure proclavaminic acids. The route to (4*R*)- and (4*S*)-**5** is shown in Scheme 3. Electrospray ionisation mass spectrometry revealed the deuterium incorporation in **5** to be greater than 98%; 500 MHz NMR showed **5a** to have 5 : 1/(*S*) : (*R*) stereochemistry at C-4 and **5b** to have 4 : 1/(*R*) : (*S*) stereochemistry at C-4.

Incubation of **5a** with CAS† resulted in the formation of **3a** as the major enzymatic product, whereas incubation of **5b** with CAS resulted in the formation of **3b** as the major enzymatic product (Scheme 4). The ratio of **3a** : **3b** in each case was consistent with the ratio of deuterium incorporation at C-4 in the substrates. The products from the incubation mixture were isolated by reverse phase HPLC and characterised by ^1H NMR spectroscopy and mass spectrometry [^1H NMR data as previously reported[6] with the absence of the triplet at δ 4.86 in the case of **3a** (*m*/*z*, Table 1)]. Recovered **5** from each incubation mixture showed the same (*S*) : (*R*) ratio for deuterium incorporation at C-4 as in the fed substrate, implying that a kinetic isotope effect was not observable for



Scheme 4

Table 1 Electrospray ionisation mass spectrometry results, *m*/*z* for M + H, for clavaminic acid **3a/b** produced by incubation of **2**, **5a** and **5b** with CAS; (*a*) incubation of **2**, (b_1, b_2) incubation of **5a**, (c_1, c_2) incubation of **5b**.

m/*z* (%)	198	199	200	201	202	Ratio of **3a** : **3b**
(*a*)	0.0	100	10.5	10.0	0.0	—
(b_1)	2.0	19.5	100	13.0	6.0	5.0 : 1
(b_2)	0.0	18.0	100	11.0	6.0	5.4 : 1
(c_1)	0.5	100	37.5	6.0	2.5	1 : 3.7
(c_2)	3.0	100	33.5	6.0	4.0	1 : 4.3

hydrogen/deuterium abstraction at this centre. The experiment was repeated with identical results.

These results, combined with the previously reported structure of dihydroclavaminic acid **4**[6] and the known double bond geometry in clavaminic acid **3**, suggest that the desaturation step occurs *via* a CAS catalysed *syn*-elimination. This stereochemical result is the same as was observed for the desaturation of stearic acid to oleic acid in bacteria[12] and algae.[13]

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Footnote

† Incubations were carried out under standard conditions[6] utilising 1.1–1.2 IU of CAS with 0.5 mg of the deuterated substrate.

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