Studies on the Stereospecificity of the Clavaminic Acid Synthase Catalysed Hydroxylation Reaction

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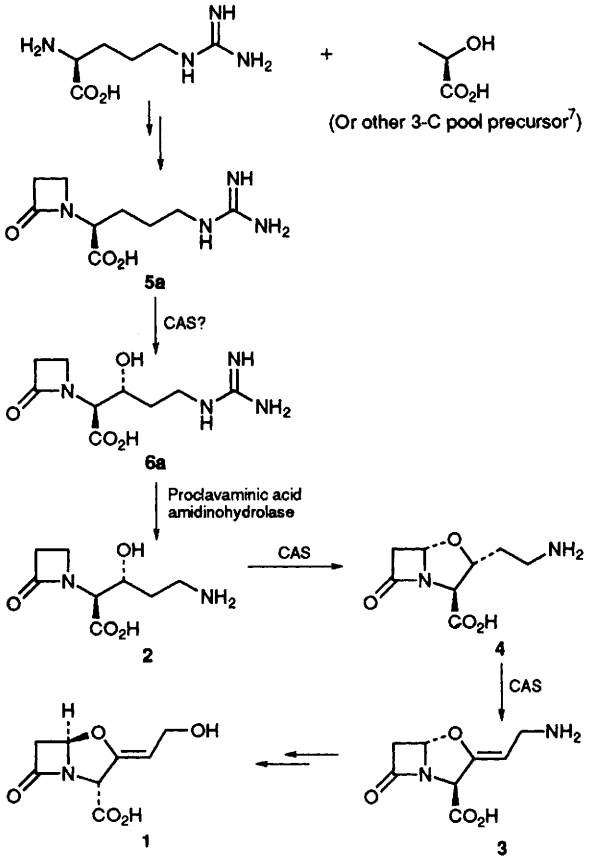
Incubation of (2*S*,3*S*)-5-guanidino[2,3-$^2H_2$]-2-(2′-oxoazetidin-1′-yl)pentanoic acid and (2*S*,3*R*)-5-guanidino-[3-$^2H_1$]-2-(2′-oxoazetidin-1′-yl)pentanoic acid with clavaminic acid synthase resulted in highly stereospecific hydroxylation at C-3, with removal of the *pro-R* hydrogen or deuterium, respectively.

The β-lactamase inhibitor clavulanic acid **1** is produced by *Streptomyces clavuligerus* ATCC 27064.[1] The biosynthesis of **1** proceeds *via* the intermediates proclavaminic acid **2** and clavaminic acid **3** (Scheme 1).[2] Proclavaminic acid **2** is converted to clavaminic acid **3** by the ferrous ion, α-ketoglutarate dependent oxygenase clavaminic acid synthase (CAS),[2a] which has been isolated and characterised.[3] In the conversion of **2** to **3** it has been shown that CAS catalyses two distinct reactions, initially converting **2** to dihydroclavaminic acid **4**, and subsequently desaturating **4** to form **3**.[4] Recent work[5] has shown that CAS also has the ability to catalyse the hydroxylation of (2*S*)-5-guanidino-2-(2′-oxoazetidin-1′-yl)pentanoic acid **5a** to **6a**. The biosynthesis of **2** has been shown to proceed from arginine[6] *via* **5a** and **6a**,[7] with the latter then being converted to **2** by proclavaminic acid amidino hydrolase.[7] These observations have led to speculation that CAS *may* have a trifunctional role in the clavulanic acid **1** biosynthetic pathway.[5]

In order to investigate the stereospecificity of the hydroxylation reaction we report herein the syntheses of **5** stereospecifically labelled at the C-3 position (**5b** and **5c**) and the results of the incubation of these compounds with CAS.



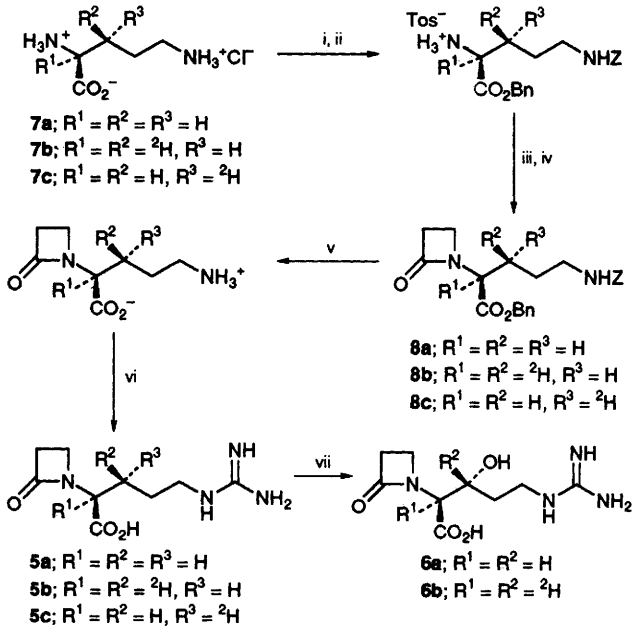
Scheme 1 Biosynthesis of clavulanic acid

The stereospecifically deuteriated ornithines **7b** and **7c** (>90% enantiomeric excess, e.e.) were synthesised as described elsewhere[8] and then converted to the desired substrates **5b** and **5c** using minor modifications of previously reported work[5,9] (Scheme 2). The ratio of L to D diastereoisomers in **8b** and **8c** was checked by ^1H NMR spectroscopy in the presence of a chiral solvating reagent, (*R*)-2,2,2-trifluoro-1-(9-anthryl)-ethanol.[2d] This revealed that a low level (<10%) of epimerisation had occurred during functionalisation of **7**. The labelled compounds **5b** and **5c** were considered adequate for incubation since the enantiomer of **5a** is not a substrate for CAS.[2d,3b]

Incubation of the guanidino compound **5b** with partially purified recombinant CAS[10,11] gave the hydroxylated product

Table 1 Electrospray ionisation mass spectrometry results for **6** produced by incubation of **5** with a recombinant CAS isozyme; (*a*) incubation of **5a**, (*b*) incubation of **5b**, (*c*) incubation of **5c**

	m/z							
	242	243	244	245	246	247	248	249
% Observed								
(*a*)	—	—	—	100	13	1.5	0.5	—
(*b*)	1.5	0.5	0.5	2.5	—	100	13	1.5
(*c*)	2.5	12	3.5	100	14.5	4	1.5	1.5



Scheme 2 *Reagents*: i, a, $CuCO_3Cu(OH)_2 \cdot H_2O$, H_2O reflux, b, $PhCH_2OCOCl$, tetrahydrofuran (THF), c, Na_2EDTA (H_4EDTA = ethylenediaminetetraacetic acid), H_2O, reflux; ii, *p*-$MeC_6H_4SO_3H$, $PhCH_2OH$, benzene, reflux; iii, acrylic acid, MeCN; iv, $MeSO_2Cl$, $NaHCO_3$, MeCN, 60 °C; v, H_2, 10% Pd–C, EtOH–H_2O (2:1); vi, $C_2N_3C(NH)NH_2 \cdot HCl$, 1 mol l^{-1} Na_2CO_3;[9] vii, Incubation with CAS and appropriate cofactors. Bn = CH_2Ph, Z = $OCOCH_2Ph$.

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6b in >85% conversion, which was isolated by reverse phase HPLC (H_2O, octadecylsilane column) and characterised by ^1H NMR spectroscopy and mass spectrometry [*m/z*, Table 1, entry (*b*)], in which the majority of both deuterium labels were retained; δ_H (500 MHz; D_2O, ref. to 1,4-dioxane) 1.70–1.78 and 1.78–1.86 (2 × 1H, 2 × m, 2 × 4-H), 3.01, and 3.48–3.54 and 3.57–3.62 (2H and 2 × 1H, t and 2 × m, *J* 4 Hz, 2 × 3′-H and 2 × 4′-H) and 3.35 (2H, *ca.* t, *J* 6 Hz, 2 × 5-H). Incubation of **5c** under standard conditions[11] gave predominantly product **6a** in >80% conversion [*m/z*, Table 1, entry (*c*)]; ^1H NMR data as previously reported.[5]

These results indicate that the hydroxylation of **5** as catalysed by CAS proceeds with a high degree (>95%) of retention of configuration at C-3. This is precedented in other hydroxylations catalysed by ferrous dependent oxygenases[12] and also in the formation of the oxazolidine ring of **3** from **2** by insertion of the oxygen at C-4′.[13]

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