A Substrate Analogue Study on Clavaminic Acid Synthase: Possible Clues to the Biosynthetic Origin of Proclavamic Acid

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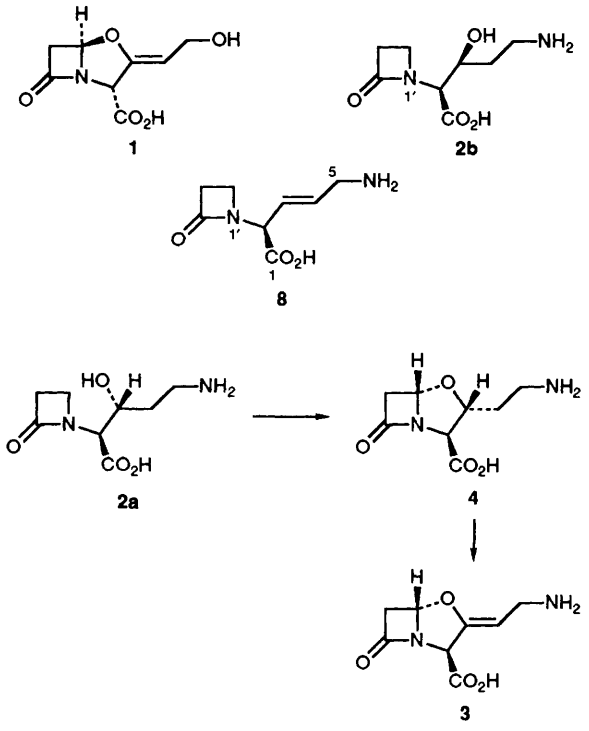
Incubation of (2*S*)-5-amino-(2′-oxoazetidin-1′-yl)pentanoic acid with clavaminic acid synthase gave (2*S*)-5-amino-2-(2′-oxoazetidin-1′-yl)pent-3,4-enoic acid as the major product, together with a small amount of proclavaminic acid; modification of the amino group to the guanyl group biased the mode of reactivity from desaturation to hydroxylation.

The use of cell-free and labelling experiments has established that the biosynthesis of the β-lactamase inhibitor, clavulanic acid **1**, in *Streptomyces clavuligerus* ATCC 27064, proceeds *via* the intermediates proclavaminic acid **2a** and clavaminic acid **3**[1] (Scheme 1). The enzyme responsible for the cyclisation and desaturation of **2a**, clavaminic acid synthase (CAS), has been isolated and partially characterised.[2] Recently, we demonstrated that *in vitro* conversion of **2a** to **3** proceeds *via* the saturated clavam **4**, establishing that cyclisation precedes desaturation.[3,4] It would seem probable that the *in vivo*



Scheme 1

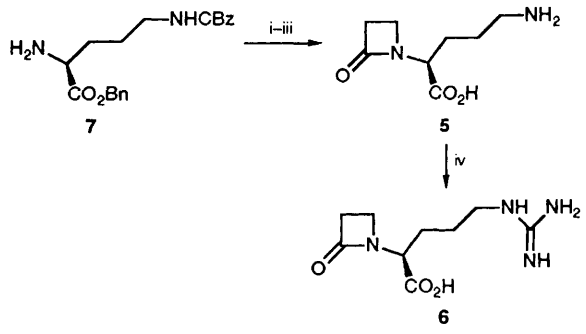
conversion of **2a** to **4** and the subsequent conversion to **3** is carried out either by a bifunctional single enzyme, or by two closely related enzymes.

The biosynthetic pathway to **2a** remains obscure. Labelling studies *in vivo* have demonstrated that the C-3 skeleton of **1** can be derived from glycerol or glycerate,[5] and that the C-5 skeleton is derived from the amino acids of the urea cycle.[6] Townsend and Krol have demonstrated[7] that the ring oxygen of **1** is derived from dioxygen, and that the hydroxy oxygen of **2a** is not exchanged in the conversion to **3**.[8] We speculated that the hydroxy group of **2a** was introduced by an α-ketoglutarate-dependent dioxygenase, such as CAS or a closely related enzyme. We report herein results obtained from the incubation of (2*S*)-5-amino-(2′-oxoazetidin-1′-yl)pentanoic acid **5**, and a related substrate **6**, with CAS. The enzymes used in these studies were obtained from *S. clavuligerus*, and also from a recombinant source.[9]†

The desired substrate **5**[1d] was synthesised in three steps from diprotected ornithine (Scheme 2). Thus Michael addition of **7** to acrylic acid was followed by ring closure to the β-lactam using methanesulfonyl chloride and base[10] and subsequent deprotection to give **5**.

Incubation of **5** with either native or recombinant CAS preparations and the appropriate cofactors gave similar results. A low level of conversion (<10%) to proclavaminic acid **2a** and products **3** and **4**, formed subsequently, was observed and confirmed by ^{1}H NMR (500 MHz) and mass spectrometry. Doping experiments by HPLC and NMR indicated the products were identical to authentic materials. However, the major product **8** [observed **2a**:**8**, <1:9] was assigned as the (*E*)-olefin **8** ($J_{3\text{-}4H}$ 14 Hz), which was purified by reverse-phase HPLC (25 mmol dm^{-3} NH_4HCO_3, octadecylsilane column): δ_H {500 MHz, D_2O, referenced to sodium 3-trimethylsilyl [2,2,3,4,-$^2H_4$]propionate (TSP)]}

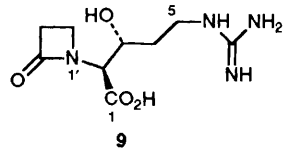
† The wildtype material was partially purified and contained two isozymes which catalysed the conversion of **2** → **4** → **3**. See also ref. 2(*b*). The recombinant material contained a single isozyme (>90% pure by SDS-PAGE, M_r, *ca.* 35600) that catalysed the same conversions. Full details will be published elsewhere.[9]



Scheme 2 *Reagents and conditions*: i, CH_2=$CHCO_2H$, MeCN, 60 °C, 60%; ii, $MeSO_2Cl$, $NaHCO_3$(aq.), MeCN, 60 °C; iii, 10% Pd/C/H_2, EtOH:H_2O (2:1); iv, 1-amidino-3,5-dimethylpyrazole-HNO_3, dimethylformamide-H_2O, pH 8–9, 45% plus 40% recovered **5**

Table 1 Electrospray mass spectrometry results (% observed) for **9** produced by incubation of **6** with a recombinant CAS isozyme

	m/z							
Incubation	242	243	244	245.0	246.0	247.0	248	249
Under air	—	—	—	100	12	1.5	0.1	—
Under $^{18}O_2$ atmosphere	0	1	0	30	5	100	12	2
In $H_2{}^{16}O$-$H_2{}^{18}O$ (52.5:47)	—	—	—	100	13	6	2	1

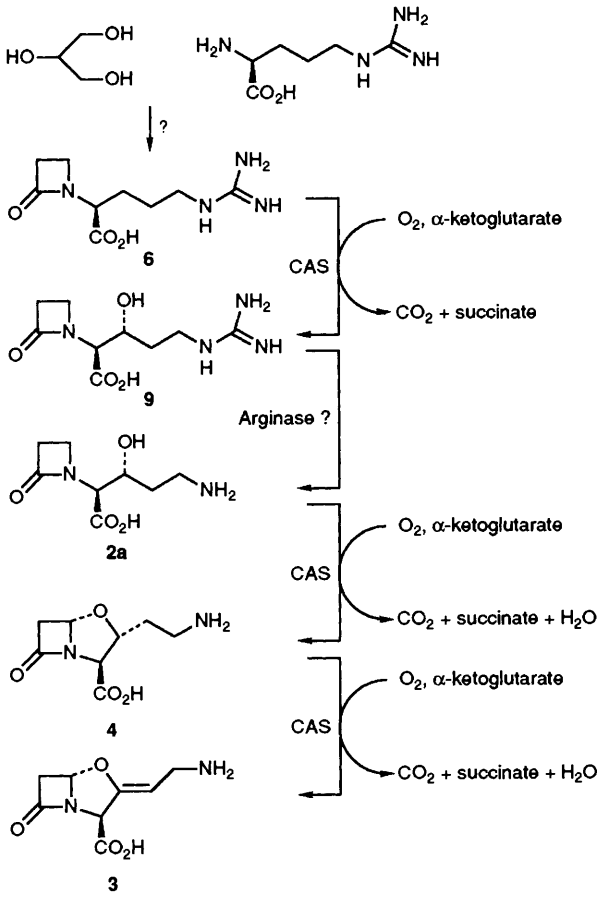


9

2.80–2.85 and 3.26–3.33 (2 × 2H, m, 2 × 3′-H and 2 × 4′-H), 3.36–3.47 (2H, m, 2 × 5-H), 5.64–5.72 (1H, m, 4-H), 5.77–5.81 (1H, m, 3-H). 2-H was obscured by the HOD suppression. A 2D COSY experiment was consistent with the connectivity as indicated; *m/z* (electrospray) 185 (MH^+). We were unable to find any evidence for the formation of the *erythro* diastereoisomer **2b** of proclavaminic acid.

These results indicate that the hydroxylation of **5** by CAS is unlikely to be a major pathway for the biosynthetic production of **2a** (although *in vivo* it is possible the ratio may be biased to the production of **2a**). However, it seemed possible that an enzyme structurally related to CAS, with slightly altered substrate specificity, or a related substrate may result in the bias of the oxygenase activity from desaturation to hydroxylation.

We investigated the latter possibility by the synthesis [Scheme 2, (iv)[11]] and incubation of **6**, in which the amine group of **5** was replaced by a guanidino group. We chose this modification since in addition to ornithine, arginine is efficiently incorporated into the C-5 skeleton of **1**,[6] and evidence has been accumulated for retention of the guanyl group in the early stages of clavulanic acid biosynthesis.[12] Furthermore it has been shown that extracts of *S. clavuligerus* contain arginase activity.[13] It was found that **6** was processed efficiently by CAS to the guanidino alcohol **9** (>85%), which was isolated by reversed-phase HPLC (H_2O, octadecylsilane column): δ_H (500 MHz, D_2O, TSP reference) 1.70–1.80, 1.80–1.90 (2H, 2 × m, 2 × 4-H), 3.02–3.04 (2H, *ca.* t, *J* 7 Hz, 2 × 3′-H), 3.36 (2H, *ca.* t, *J* 7 Hz, 5-H), 3.50–3.55 (1H, m, 4′-H), 3.60–3.65 (1H, m, 4′-H), 4.09 (1H, d, *J* 5.5 Hz, 2-H), 4.17–4.20 (1H, m, 3-H); *m/z* (electrospray) 245 (MH^+). The ^{1}H NMR mass spectrometry and CD data for compound **9**



Scheme 3 Proposed biosynthesis of clavaminic acid

were consistent with those for synthetic material prepared from **2a**.[11]

There was no evidence for the formation of unsaturated products during the incubation of **6**, indicating almost complete bias of CAS activity towards hydroxylation. The presence of small amounts of a bicyclic material, was indicated by the sensitive imidazole assay[14] (≪5% of that produced from **2a** under identical conditions) which *may* arise from the slow conversion of **9** to **2a**. Incubation of **6** with CAS under an atmosphere of $^{18}O_2$ showed *ca.* 77% (unoptimised) incorporation of a single ^{18}O atom into **9** (Table 1). Incubation of **6** in the presence of water enriched to 47 atom% with ^{18}O resulted in a *ca.* 10% incorporation of ^{18}O into **9** (Table 1).

Preliminary steady-state kinetic studies showed that at 30 °C for partially purified CAS activities from the *S. clavuligerus* ATCC 27064, the K_m for **6** was 180 ± 20 μmol dm^{-3}, and V_{max} was 2.3 ± 0.05 μmol min^{-1} mg^{-1} of protein. Preliminary kinetic analysis for the recombinant CAS isozyme purified from recombinant *Escherichia coli* cells under the same conditions gave a K_m of 250 ± 25 μmol dm^{-3} and a k_{cat} of 882 ± 9 min^{-1}. These results show that k_{cat} for the hydroxylation reaction by recombinant CAS is *ca.* 60 times that of the cyclisation and desaturation of (2*S*,3*R*)-proclavaminic acid **2a**, which for the recombinant CAS was 14.6 ± 0.6 min^{-1} under similar conditions.[9]

These studies are consistent with a sequence of events in clavulanic acid **1** biosynthesis in which the use of the CAS activity is maximised, as in the hypothesised pathway shown (Scheme 3). The pathway proposed implies the intimate association of an arginase activity with the biosynthesis of **1**.

The related iron(II), α-ketoglutarate dependent dioxygenase, DAOC/DAC (deacetoxycephalosporin C/deacetylcephalosporin C) synthase from *Cephalosporium acremonium*, has been shown to catalyse three sequential oxidations *in vitro*,[14] so there is some precedent in the literature for this type of reaction pathway. The CAS isozymes appear to be remarkable in the range of chemistry performed, hydroxylation, oxidative cyclisation and desaturation. In addition if our speculation is correct, one of these steps (hydroxylation) is 'separated' from the other two sequential reactions (cyclisation and desaturation) by the action of a mechanistically unrelated enzyme (an arginase). Alternatively, it is possible that more specific oxygenases (as yet undiscovered) may catalyse the different oxidative steps.

This study also demonstrates clearly how a relatively small modification to a substrate, at a site removed from that functionalised, can change dramatically the mode of catalytic reactivity of the enzyme, in this case the balance between hydroxylation and desaturation.‡

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‡ It is also of interest that β-hydroxyarginine has been proposed as an intermediate in the biosynthesis of streptothricin F[16].