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

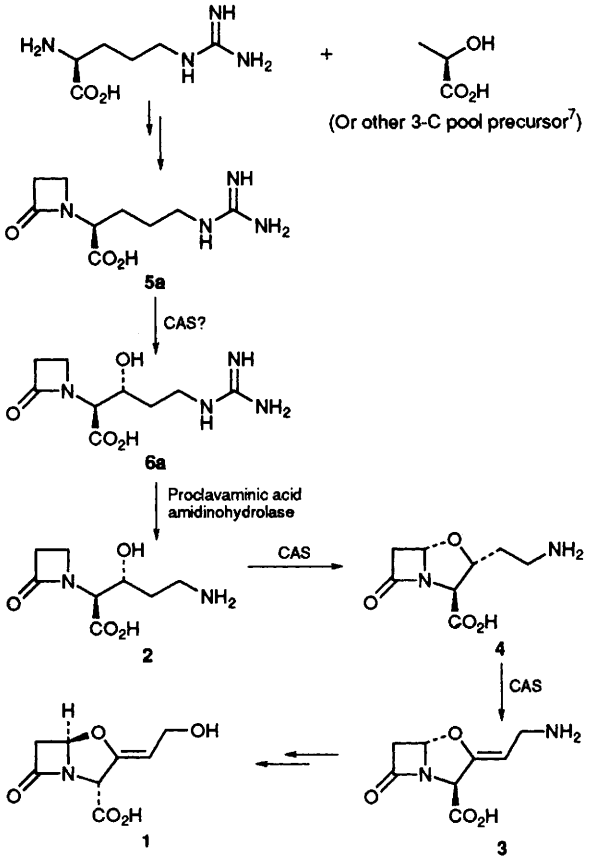
Jack E. Baldwin,[a] Kirsten D. Merritt,[a] Christopher J. Schofield,[a] Stephen W. Elson[b]† and Keith H. Baggaley[b]

[a] *The Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, South Parks Road, Oxford, UK OX1 3QY*
[b] *SmithKline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, UK RH3 7AJ*

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.



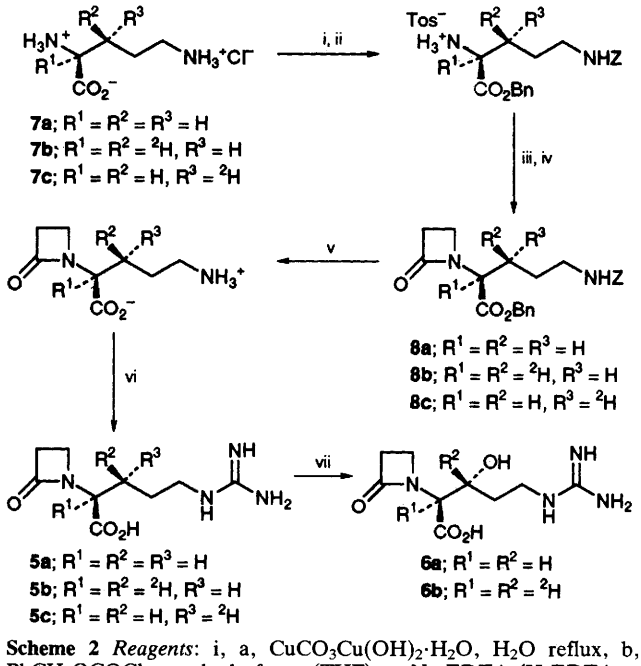
Scheme 1 Biosynthesis of clavulanic acid

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 = $OCOCH_2Ph$.

† *Present address*: Smithkline Beecham Pharmaceuticals, Centro de Investigacion Basica, Parque Technologico de Madrid, 28760 Tres Cantos Madrid, Spain.

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]

We warmly thank Mrs H. Edwards and Dr Y. Fujishima for providing the recombinant enzyme preparation, the SERC for support, Drs C. Robinson and R. T. Aplin for mass spectrometry, and Dr M. D. Lloyd for assistance.

Received, 26th April 1993; Com. 3/02397D

References

1 A. G. Brown, D. Butterworth, M. Cole, G. Hanscomb, J. D. Hood, C. Reading and G. N. Rolinson, *J. Antibiot.*, 1976, **29**, 668.

2 (*a*) S. W. Elson, K. H. Baggaley, J. Gillett, S. Holland, N. H. Nicholson, J. T. Sime and S. R. Woroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1736; (*b*) K. H. Baggaley, J. T. Sime, N. H. Nicholson, S. W. Elson, J. Gillett, S. Holland and S. R. Woroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1738; (*c*) S. W. Elson, K. H. Baggaley, J. Gillett, S. Holland, N. H. Nicholson, J. T. Sime and S. R. Woroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1739; (*d*) K. H. Baggaley, S. W. Elson, N. H. Nicholson and J. T. Sime, *J. Chem. Soc., Perkin Trans. 1*, 1990, 1521.

3 (*a*) S. W. Elson, S. R. Woroniecki and K. H. Baggaley, Eur. Pat. 213 914 (11.3.87); (*b*) S. P. Salowe, E. N. Marsh and C. A. Townsend, *Biochemistry*, 1990, **29**, 6499.

4 (*a*) J. E. Baldwin, R. M. Adlington, J. S. Bryans, A. O. Bringham, J. B. Coates, N. P. Crouch, M. D. Lloyd, C. J. Schofield, S. W. Elson, K. H. Baggaley, R. Cassels and N. H. Nicholson, *J. Chem. Soc., Chem. Commun.*, 1990, 617; (*b*) J. E. Baldwin, R. M. Adlington, J. S. Bryans, A. O. Bringham, J. B. Coates, N. P. Crouch, M. D. Lloyd, C. J. Schofield, S. W. Elson, K. H. Baggaley, R. Cassels and N. H. Nicholson, *Tetrahedron*, 1991, **47**, 4089.

5 J. E. Baldwin, M. D. Lloyd, B. Wha-Son, C. J. Schofield, T. J. Sewell, S. W. Elson, K. H. Baggaley and N. H. Nicholson, *J. Chem. Soc., Chem. Commun.*, 1993, 500.

6 B. P. Valentine, C. R. Bailey, A. Doherty, J. Morris, S. W. Elson, K. H. Baggaley and N. H. Nicholson, *J. Chem. Soc., Chem. Commun.*, in the press.

7 S. W. Elson, K. H. Baggaley, M. Davison, M. Fulston, N. H. Nicholson, G. D. Risbridger and J. W. Tyler, *J. Chem. Soc., Chem. Commun.*, in the press.

8 J. E. Baldwin, K. D. Merritt and C. J. Schofield, *Tetrahedron Lett.*, 1993, **34**, 3919.

9 M. S. Bernatowicz, Y. Wu and G. R. Matsueda, *J. Org. Chem.*, 1992, **57**, 2497.

10 The recombinant CAS used in these experiments was produced *via* cloning of the CAS gene which corresponds to the CS 2 gene of the clavulanic acid biosynthesis gene cluster as reported by Townsend *et al.*: see E. N. Marsh, M. D.-T. Chang and C. A. Townsend, *Biochemistry*, 1992, **31**, 12648.

11 Incubations utilised 0.2–0.4 IU of CAS. CAS activities were based on the use of *N*-acetylarginine as a substrate for CAS; J. E. Baldwin, M. D. Lloyd, V. Lee and C. J. Schofield, S. W. Elson and K. H. Baggaley, manuscript in preparation.

12 (*a*) C. A. Townsend and E. B. Barrabee, *J. Chem. Soc., Chem. Commun.*, 1984, 1586; (*b*) C. A. Townsend, *J. Nat. Prod.*, 1985, **48**, 708; (*c*) S. Englard, J. S. Blanchard and C. F. Midelfort, *Biochemistry*, 1985, **24**, 1110; (*d*) J. Stubbe, *J. Biol. Chem.*, 1985, **260**, 9972.

13 A. Basak, S. P. Salowe and C. A. Townsend, *J. Am. Chem. Soc.*, 1990, **112**, 1654.