Pergamon

Tetrahedron Letters, Vol. 35, No. 17, pp. 2783-2786, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $6.00+0.00

0040-4039(94)E0379-C

Enzymes of Valclavam Biosynthesis

Jack E. Baldwin, Yoshiyuki Fujishima, Kee-Chuan Goh and Christopher J. Schofield

The Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, UK

Abstract: Two enzymes of the clavulanic acid pathway, clavaminic acid synthase (CAS) and proclavaminic acid amidino hydrolase (PAH), were detected in mycelia of *Streptomyces antibioticus* ssp *antibioticus* Tü 1718. These enzymes are presumably involved in the biosynthesis of valclavam, indicating a shared biosynthetic pathway between clavulanic acid and valclavam.

Clavulanic acid (**1**), clavam-2-carboxylate (**2**) [both produced by *Streptomyces clavuligerus*] and valclavam (**3**) [produced by *Streptomyces antibioticus*] are the only members of the clavam series of β-lactam antibiotics for which biosynthetic studies have been performed. Our investigations,[1,2] together with those of Townsend *et al*,[3,4] have suggested an initial overlap in the biosynthetic pathways leading to these clavams, at least up to and including the monocyclic β-lactam intermediate, proclavaminic acid (**4**) (Scheme 1).

Two enzymes have been identified in the biosynthesis of clavulanic acid (**1**) in *S. clavuligerus*: the non-haem iron and α-ketoglutarate-dependent dioxygenase clavaminic acid synthase (CAS)[5] and proclavaminic acid amidino hydrolase (PAH).[6] CAS exists as two isozymes in *S. clavuligerus* and has been sequenced,[7] cloned and over-expressed in *E. coli*.[8] CAS and PAH catalyse a remarkable sequence of reactions in the biosynthesis of clavulanic acid (**1**). Firstly, 5-guanidino-2-(2-oxoazetidin-1-yl)pentanoic acid (**5**) is hydroxylated by CAS to give 3-hydroxy-5-guanidino-2-(2-oxoazetidin-1-yl)pentanoic acid (**6**).[9] PAH then hydrolyses the guanidino side chain of (**6**) to give proclavaminic acid (**4**), which then undergoes CAS-mediated oxidative cyclisation to give dihydroclavaminic acid (**7**). Desaturation of (**7**), again catalysed by CAS, gives clavaminic acid (**8**).[10] We report in this Letter the detection of both CAS and PAH activities in *S. antibioticus* ssp *antibioticus* Tü 1718.

Fermentation of *S. antibioticus* cells was carried out over a 6-day period in a glycerol-based medium as described.[1] Valclavam (**3**) production commenced about three days after inoculation. Cell-free extracts were monitored for activities of CAS (using N_{α}-acetyl-*L*-arginine as a substrate)[11,12] and PAH [using (**6**) as a substrate][6]. Significant CAS activity was detected in the crude cell lysates from the 2nd, 3rd and 4th-day harvests. PAH activity was also detected after the 2nd day and apparently activity did not decrease after the 3rd day. Since CAS activity has been reported in clavulanic acid-producing organisms other than *S. clavuligerus*, *e.g. S. jumonjinensis*[13] and *S. katsurahamanus*,[14] we monitored for clavulanic acid (**1**) production during the fermentations but were unable to detect any. This suggests the involvement of PAH and CAS in valclavam (**3**) biosynthesis. Details of an initial purification of CAS using the crude cell lysates from the third day harvest are shown in Table 1.

The major protein from the most active fraction in the last purification step (Mono Q chromatography) was blotted from the SDS-PAGE gel and subjected to N-terminal analysis. The 33-residue N-terminal sequence obtained, when aligned with N-terminal sequences[7] of the CAS isozymes from *S. clavuligerus*, convincingly

identified this protein as a CAS isozyme from *S. antibioticus* (Figure 1). At this stage, it is not possible to rule out the presence of multiple CAS isozymes in *S. antibioticus* ssp *antibioticus* Tü 1718.

Step	Total protein (mg)	Total activity		Specific activity (IU/mg)	Purification (fold)
		IU	Recovery		
Crude sonicate	120	3.27	100	0.028	1
Q-Sepharose FF pool	7.5	1.13	34	0.151	5.4
Superdex 75 pool	0.71	0.087	2.7	0.123	4.4
Mono Q pool	0.14	0.088	2.7	0.629	22.5

Table 1 Preliminary purification of CAS from *S. antibioticus* ssp *antibioticus* Tü 1718

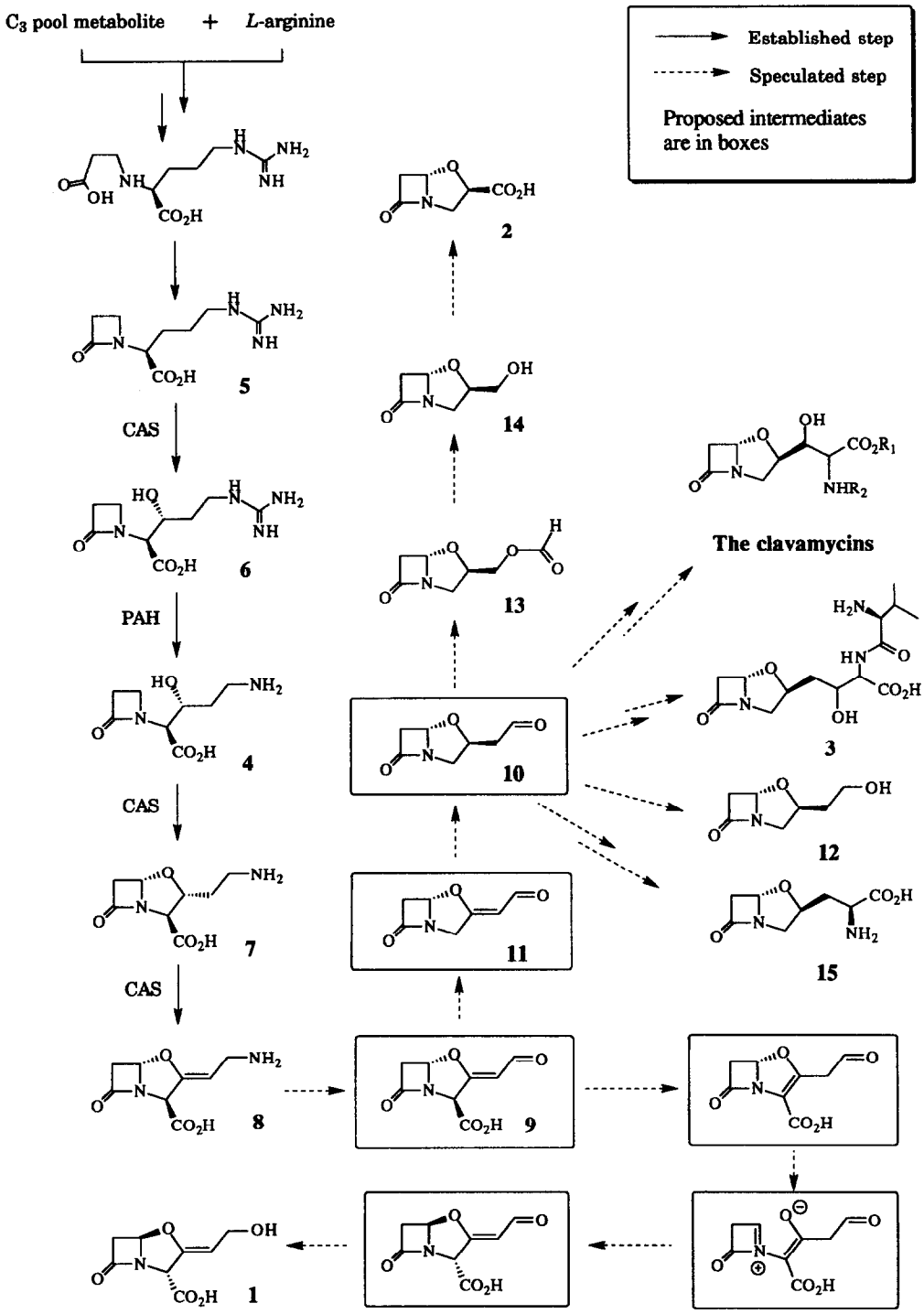
sc_1CAS	TSVDCTAYGPELRALAARLPRTPRADLYAFLDA	(Published)[7]
saCAS	TVVDCSEYSADLLALASRLPRIPRQDLYGFLDA	(Our data)
sc_2CAS	PIVDCTPYRDELLALASELPEVPRADLHGFLDE	(Published)[7]

(Legend : Single underline = conservative change, Double underline = identity
sa = *S. antibioticus*, sc_1 = *S. clavuligerus* isozyme 1, sc_2 = *S. clavuligerus* isozyme 2)

Figure 1 Comparison of 33-residue N-terminal sequences from different CAS enzymes

The results presented herein and described elsewhere[1-4] imply that clavams share a large part of their biosynthetic pathways, as shown in Scheme 1. Aldehydes **9** and **10** are proposed as the branch-points determining which type of clavam is biosynthesised. Aldehyde (**9**), has been proposed to be an intermediate in the 'double inversion' (at C-3 and C-5) of clavaminic acid (**8**) to clavulanic acid (**1**).[15] It may arise from clavaminic acid (**8**) through oxidative deamination, possibly mediated by a 2-oxo acid dependent dioxygenase related to CAS. Aldehyde (**9**) is also anticipated to undergo ready decarboxylation to yield **11** (or its endocyclic double bond isomer). Reduction of the double bond of **11** would give saturated aldehyde (**10**) which is proposed as the branch-point to all the other clavams produced by *Streptomyces* bacteria. Thus, reduction of the aldehyde (**10**) would produce 2-(2-hydroxyethyl)clavam (**12**).[16] Baeyer-Villiger oxidation of (**10**), a reaction encountered in biological systems,[17] would yield 2-formyloxymethylclavam (**13**), which may be hydrolysed and oxidised to give (**2**) *via* (**14**). Alternatively (**10**) may be diverted down other pathways to give valclavam (**3**), clavalanine (**15**)[18] or the clavamycins from *S. hygroscopicus*.[19] Validation of the biosynthetic proposals indicated in Scheme 1 will require demonstration of the intermediacy of the key aldehydes **9**, **10** and **11** and will be the subject of future investigations.

We thank Prof Dr Hans Zähner (University of Tübingen) for the generous gift of a slope of *S. antibioticus* ssp *antibioticus* Tü 1718, Mr John W. Keeping for carrying out the fermentations, Mr Anthony C. Willis for N-terminal sequence analysis, Mr Stephen W. Elson, Dr Keith H. Baggaley, and Dr Neville H. Nicholson for helpful discussions and the Singapore Economic Development Board for a scholarship to K.C.G.



Scheme 1 Proposed biosynthetic pathways to known clavams

REFERENCES

1. Baldwin, J. E.; Goh, K.-C.; Schofield, C. J., preceding paper.
2. Baldwin, J. E.; Claridge, T. D. W.; Goh, K.-C.; Keeping, J. W.; Schofield, C. J. *Tetrahedron Lett.* **1993**, *34*, 5645-5648.
3. Iwata-Reuyl, D.; Townsend, C. A. *J. Am. Chem. Soc.* **1992**, *114*, 2762-2763.
4. Janc, J. W.; Egan, L. A.; Townsend, C. A. *Bioorg. & Med. Chem. Lett.* **1993**, *3*, 2313-2316.
5. Elson, S. W.; Baggaley, K. H.; Gillett, J.; Holland, S.; Nicholson, N.; Sime, J. T.; Woroniecki, S. R. *J. Chem. Soc., Chem. Commun.* **1987**, 1736-1738.
6. Elson, S. W.; Baggaley, K. H.; Davison, M.; Fulston, M.; Nicholson, N. H.; Risbridger, G. D.; Tyler, J. W. *J. Chem. Soc., Chem. Commun.* **1993**, 1212-1214.
7. Marsh, E. N.; Chang, M. D.-T.; Townsend, C. A. *Biochemistry* **1992**, *31*, 12648-12657.
8. Lawlor, E.; Lloyd, M. D.; Elson, S. W.; Holland, S.; Schofield, C. J.; Cassels, R.; Baldwin, J. E.; Hodgson, J., manuscript in preparation.
9. Baldwin, J. E.; Lloyd, M. D.; Son, B.-W.; Schofield, C. J.; Sewell, T.; Elson, S. W.; Baggaley, K. H.; Nicholson, N. H. *J. Chem. Soc., Chem. Commun.* **1993**, 500-502.
10. Baldwin, J. E.; Adlington, R. M.; Bryans, J. S.; Bringhen, A. O.; Coates, J. B.; Crouch, N. P.; Lloyd, M. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H.; Cassels, R.; Nicholson, N. *Tetrahedron* **1991**, *47*, 4089-4100.
11. Baldwin, J. E.; Lee, V.; Lloyd, M. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H. *J. Chem. Soc., Chem. Commun.* **1993**, 1694-1696.
12. Lloyd, M. D., *D.Phil* Thesis, University of Oxford, **1992**.
13. Vidal, C. M., Spanish Patent ES 550549, **1987**.
14. Kitano, K.; Kintaka, K.; Katamoto, K., Japanese Patent JP 53-104769, **1978**.
15. Townsend, C. A.; Krol, W. J. *J. Chem. Soc., Chem. Commun.* **1988**, 1234-1236.
16. Wanning, M.; Zähner, H.; Krone, B.; Zeeck, A. *Tetrahedron Lett.* **1981**, *22*, 2539-2540.
17. For example: (i) Petit, F.; Furstoss, R. *Tetrahedron: Asymmetry* **1993**, *4*, 1341-1352. (ii) Taschner, M. J.; Black, D. J.; Chen, Q.-Z. *Tetrahedron: Asymmetry* **1993**, *4*, 1387-1390.
18. Pruess, D. L.; Kellett, M. *J. Antibiot.* **1983**, *36*, 208-212.
19. King, H. D.; Langhärig, J.; Sanglier, J. J. *J. Antibiot.* **1986**, *39*, 510-515.

(*Received in UK* 27 *January* 1994; *accepted* 18 *February* 1994)