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Product-Substrate Engineering by Bacteria: Studies on Clavaminate Synthase, a Trifunctional Dioxygenase[1]

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Abstract:

Evidence is presented that clavaminate synthase (CS) catalyses three oxidative reactions in the clavulanic acid biosynthetic pathway. The first CS catalysed step (hydroxylation) is separated from the latter two (oxidative cyclisation and desaturation) by the action of a hydrolytic enzyme, proclavaminate amidinohydrolase, which modifies (or 'mutates') the sidechain of the product of the first reaction thereby converting it into a substrate for the second CS catalysed reaction.

Keywords: Amidines; Enzyme reactions; Hydroxylations.

Introduction:

Clavulanic acid (**1**) is a medicinally important inhibitor of class A 'serine' β-lactamases which play an important role in bacterial resistance to penicillins.[2] Biosynthetic studies on clavulanic acid (**1**) have shown that the three carbon atoms of its β-lactam ring are derived from pyruvate[3] whilst the remaining five carbon atoms are derived from *L*-arginine[4a] (**Scheme 1**). In the early stages of the pathway *L*-arginine and pyruvate are joined to give *L*-N^2-carboxyethylarginine (**2**)[4b,4c] which has been recently shown to be converted into the monocyclic β-lactam (**3**) in a MgATP-dependent reaction catalysed by a β-lactam synthase (BLS).[5] Mutant strains of *Streptomyces clavuligerus* deficient in BLS accumulate (**2**), consistent with its role in the *in vivo* biosynthetic pathway.[5b]

β-Lactam (**3**) is hydroxylated in a reaction catalysed by an iron(II) and 2-oxoglutarate (2-OG)-dependent dioxygenase, clavaminate synthase (CS).[1a] Hydrolysis of the amidino group of (**4**), catalysed by proclavaminate amidinohydrolase (PAH)[4c,6], gives proclavaminate (**5**). Both the oxidative cyclisation of (**5**) to dihydroclavaminate (**6**)[7] and the desaturation of the latter to clavaminate (**7**)[8] are also catalysed by CS. The desaturation of (**6**) has been shown to (at least predominately) proceed with *syn*-elimination of the requisite hydrogens.[9] In *S. clavuligerus* there are CS two isoenzymes (CS1 and CS2),[10] whilst only one isoenzyme has been isolated from the valclavam (**9**) producer *S. antibioticus*.[11] Both isoenzymes of CS from *S. clavuligerus* have been cloned and over-produced in *Escherichia coli*.[10b,10c,12]

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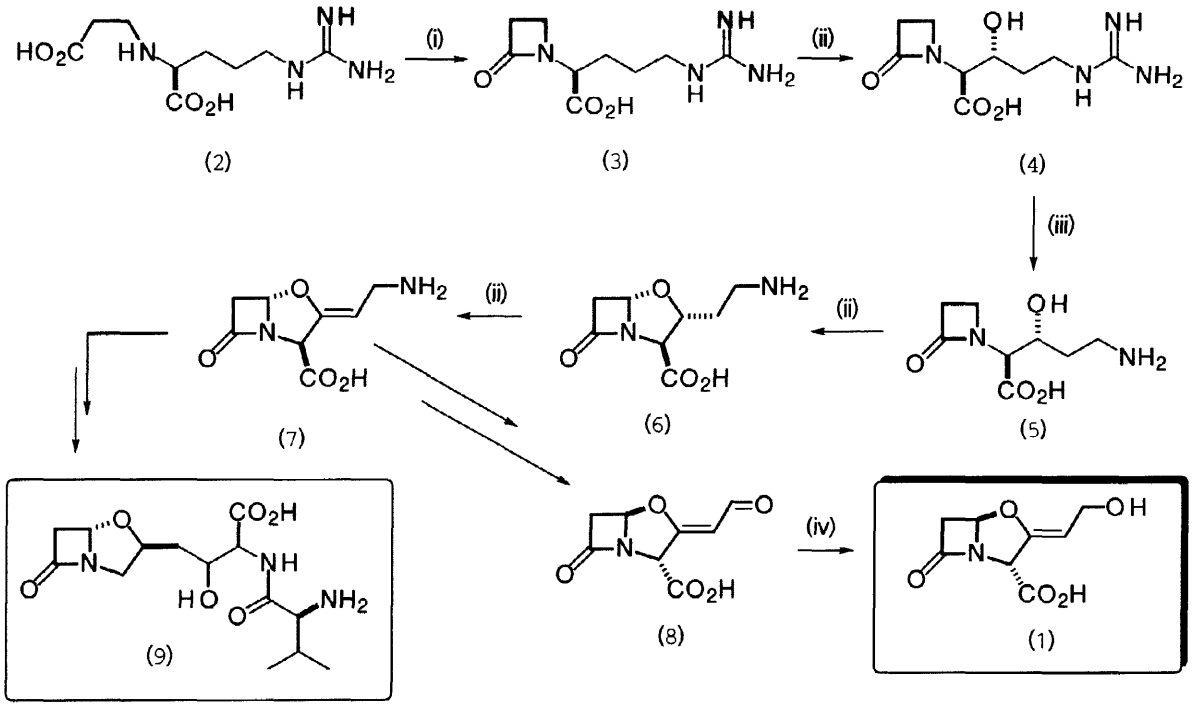


Scheme 1: The biosynthesis of clavulanate (**1**). Enzymes and cofactors/cosubstrates: (i) β-lactam synthetase. MgATP; (ii) clavaminate synthase (CS), Fe(II), 2-oxoglutarate, O_2; (iii) proclavaminate amidinohydrolase (PAH). Mn(II); (iv) clavulanate dehydrogenase (CD), NADPH.

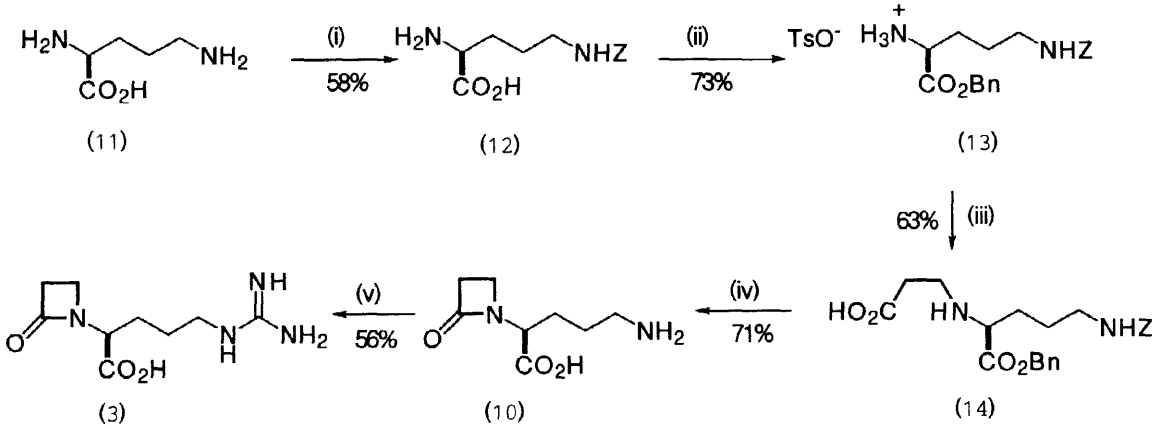
Aldehyde (**8**) is the immediate precursor of clavulanic acid (**1**) and is reduced in an NADPH-dependent process catalysed by clavulanate dehydrogenase (CD) to give (**1**).[13] Intermediates in the remarkable process which results in the inversion of the (2*S*) and (5*S*) stereochemistry of (**7**) to the (2*R*) and (5*R*) stereochemistry of (**1**) are unclear. Synthetic ester derivatives of aldehyde (**8**) undergo facile racemisation,[14] but it seems likely that several steps are involved in the conversion of (**7**) to (**8**). Recently, Egan *et al.* have provided evidence that clavaminate (**7**), rather than an earlier intermediate or aldehyde (**8**), is the branch point between the biosynthesis of clavulanic acid (**1**) and all other clavams.[15] It is noteworthy that clavulanic acid (**1**) and aldehyde (**8**) are atypical clavams in that they have the (5*R*)-stereochemistry, whilst the majority [*e.g.* valclavam (**9**)] have the (5*S*)-stereochemistry. All available evidence suggests that the steps of the pathway before (**7**) are common to the biosynthesis of all clavams. Thus, CS plays an essential role not just in the biosynthesis of clavams with the (5*R*) stereochemistry such as (**1**), but also in those with the (5*S*)-stereochemistry such as valclavam (**9**).

Attempts to elucidate the pathway prior to proclavaminate (**5**) followed two approaches at Oxford and SmithKline Beecham. The isolation of possible precursors from mutant strains of *S. clavuligerus* deficient in clavulanic acid (**1**) biosynthesis coupled with the use of *in vivo* ^{13}C-labelling studies,[4b,4c] and the *in vitro* incubation of potential substrates with purified enzymes. Herein, we report experimental details of the *in vitro* hydroxylation of the proposed precursor (**3**) to give (**4**)[1a], the proposed role of this reaction in the biosynthesis of clavams, which demonstrating the trifunctional role of CS in the biosynthesis of clavulanate (**1**). These studies also demonstrate that it is possible to alter the type of reaction (*e.g.* hydroxylation or desaturation) catalysed by an oxygenase enzyme by modifications to the substrate at a site which is 'remote' from the oxidising centre, suggesting that it may be possible to engineer the active site of CS to catalyse other desired reactions.

Early *in vivo* studies also showed that the oxygen in the heterocyclic ring of (1) was (at least partially) derived from dioxygen.[16] Further *in vitro* studies showed that this oxygen was retained during the conversion of proclavaminate (5) to clavaminate (7) using purified CS[17], suggesting that the hydroxyl group of proclavaminate (5) is derived from dioxygen *via* the action of an oxygenase. CS is a member of the family of iron(II), 2-OG-dependent oxygenases, many of which catalyse the hydroxylation of unactivated carbon-hydrogen bonds. Known substrates for hydroxylation reactions catalysed by 2-OG dependent oxygenases include both free amino acids (*e.g.* proline[18]) and amino acid residues in proteins (*e.g.* prolyl[19] and asparty[20] residues). Subsequent to the discovery that CS catalysed the conversion of (5) to (6) and (6) to (7), it was proposed that CS or a closely related enzyme catalysed the introduction of the hydroxyl group of proclavaminate (5) and we initially speculated that ornithine derivative (10) (**Scheme 2**) might be the direct precursor of proclavaminate (5).

Synthesis of substrates:

The proposed substrate (10) was synthesised from ornithine (11), *via* (12), following methodology developed for the synthesis of proclavaminate (5).[7b] Thus, Michael reaction of acrylic acid with the diprotected ornithine derivative (13) gave β-amino acid (14) in 63% yield (**Scheme 2**). Ring closure in 71% yield to the desired β-lactam *via* a mixed anhydride, generated from methanesulphonyl chloride and base followed by hydrogenolytic deprotection gave (10) in quantitative yield. An alternative potential CS substrate (3) with a guanidino sidechain (see below) was synthesised from (11). Initially the method of Bodanszky *et al.*[21a] was used, but it was subsequently found that the procedure of Bernatowicz *et al.*,[21b] using 1-amidino-4-azapyrazole rather than 1*H*-pyrazole-1-carboxamidine hydrochloride, gave (3) in higher yield (56%).

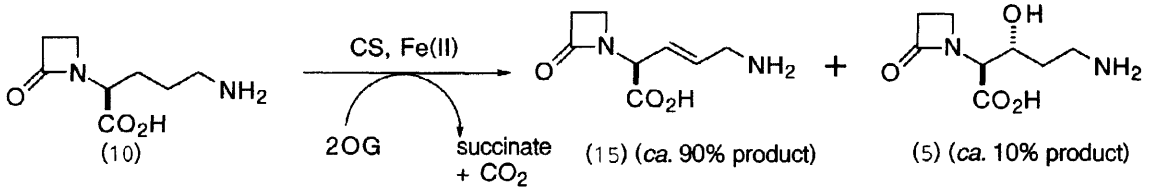


Scheme 2: (i) (a) $CuCO_3.Cu(OH)_2.H_2O$ (b) MgO, $C_6H_5CH_2OCOCl$ (c) EDTA; (ii) BnOH, benzene, TsOH, reflux; (iii) acrylic acid, MeCN; (iv) (a) MsCl, $NaHCO_3$, MeCN (b) Pd/C/H_2, EtOH, H_2O; (v) a) 1-amidino-3,5-dimethylpyrazole-HNO_3, $HCONMe_2$, H_2O pH 8.0-9.0[21a]; or b) 1-amidino-4-azapyrazole, 1M Na_2CO_3.[21b]

Incubation of potential substrates with CS:

Incubation of the proposed substrate (10) with CS derived from *S. clavuligerus* or recombinant *E. coli* cells (CS2) gave qualitatively identical results. The presence of two resonances at 5.8-5.9 and 5.9-6.0 p.p.m. in the ^1H NMR spectrum (500 MHz) analyses of the crude incubation mixture suggested the unexpected production of an alkene product. A relatively small amount of proclavaminate (5) at about 10% of the level of the unidentified material was also observed by ^1H NMR spectroscopy. Purification of the crude incubation mixture resulted in

the isolation of *E*-(2*S*)-5-amino-2(2'-oxoazetidin-1-yl)-pent-3,4-enoate (**15**) as the major product (*ca.* 90% of the total identified products) (**Scheme 3**).

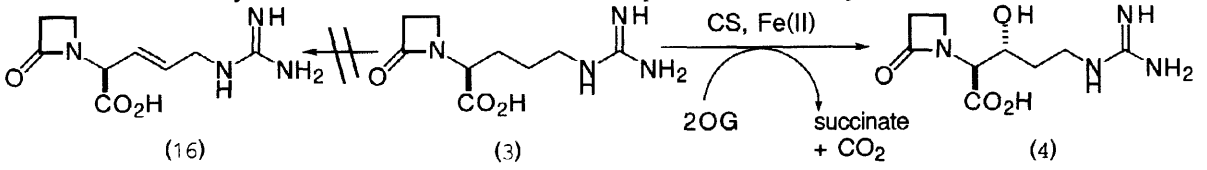


Scheme 3: Incubation of β-lactam (**10**) with CS.

The stereochemistry of the double bond was assigned on the basis of the 14 Hz H-3 to H-4 coupling constant, derived from a homonuclear decoupling experiment. The proclavaminate (**5**) which was also isolated from the crude incubation mixture, had an identical HPLC retention volume (3.1 mL) to synthetic *threo*-proclavaminate (**5**) and was well separated from *erythro*-proclavaminate (retention volume 3.4 mL). ^{1}H NMR doping experiments and mass spectrometric analyses confirmed the identity of the alcohol product as (**5**).

The observation that the incubation of (**10**) with CS led to the production of proclavaminate (**5**) as only a minor product, suggests that (**10**) is not the *in vivo* substrate for the biosynthesis of proclavaminate (**5**). Although it was considered possible that the ratio of products is biased *in vivo* towards hydroxylation, perhaps as a result of post-translational modification, this scenario was considered unlikely since both native and recombinant CS enzymes gave the same result. We speculated either that an alternative enzyme related to CS may be responsible for the *in vivo* biosynthesis of (**5**) from (**10**), or that an alternative, related intermediate was the true substrate for a hydroxylation reaction catalysed CS. Cognisant with the observation that both ornithine and arginine had been efficiently incorporated into (**1**) in whole cell labelling studies,[4a,4c] we investigated the latter possibility by the synthesis and incubation of the arginine derivative (**3**).

The guanidino substrate (**3**) was incubated with recombinant and wild type CS as before, and the products were analysed by ^{1}H NMR (500 MHz) before purification by reverse phase HPLC. Incubation of (**3**) with CS appeared to give quantitative conversion to the hydroxylated product (**4**), with no apparent production of the guanidino alkene (**16**) by ^{1}H NMR (500 MHz) or reversed phase HPLC analyses (**Scheme 4**).

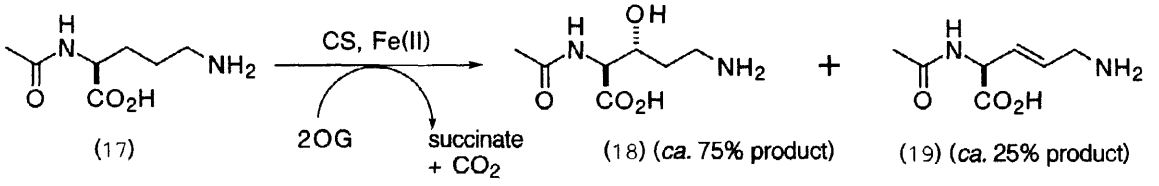


Scheme 4: Incubation of β-lactam (**3**) with CS.

Preliminary kinetic analysis on this reaction gave the following apparent kinetic parameters: For a mixture of the two isoenzymes derived from *S. clavuligerus*, K_m = 180 ± 20 μM, V_{max} = 2.3 ± 0.05 nmol/min/mg protein; for the single CS 2 isoenzyme derived from recombinant *E. coli* cells, K_m = 250 ± 25 μM, K_{cat} = 14.7 ± 0.15 S^{-1}. Although this type of preliminary kinetic data on 2-OG dependent oxygenases should be treated with caution,[22] taken together with the observations from the NMR studies it seems that guanidine (**3**) is the most efficient substrate for CS so far discovered.[1a] Subsequent studies with specifically labelled substrates showed that the hydroxylation reaction proceeded with retention of configuration.[23] The alcohol product (**4**) of the CS catalysed hydroxylation reaction was examined as a substrate for the CS catalysed cyclisation and desaturation reactions. Analysis of the derived incubation products by ^{1}H NMR (500 MHz) failed to show the production of

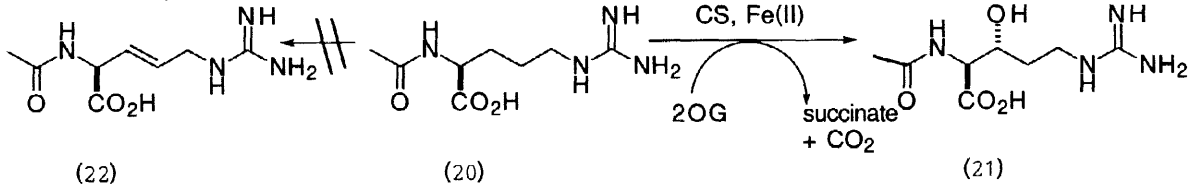
any bicyclic clavams or alkenes. Imidazole derivatisation followed by HPLC analysis[24] suggested the production of very low [less than 5% of those observed with proclavaminate (5)] amounts of bicyclic clavams. It seems possible that their production was due to slow removal the amidino group of (4) to form proclavaminate (5). It has been reported that incubation of (3) with CS and PAH (expressed as a fusion protein) *in vitro* resulted in its conversion *via* four enzyme catalysed steps to clavaminate (7).[6] The results imply clearly that CS is involved in three oxidative steps in the biosynthesis of clavulanate (1) from pyruvate and arginine.

In order to explore the substrate selectivity of CS, and to develop an efficient and convenient alternative assay, the incubation of commercially available N-α-acetyl amino acids with CS was investigated.[1b] Incubation of *L*-N-α-acetylornithine (17) gave both alcohol (18) and alkene (19) products as observed for the incubation of the analogous β-lactam substrate (**Scheme 5**).



Scheme 5: Incubation of *L*-N-α-acetylornithine (17) with CS.

However, the ratio of alcohol: alkene produced was reversed when comparing the N-α-acetyl and β-lactam derivatives. The ratio of (18):(19) was *ca.* 3:1 upon incubation of (17), whilst in the case of the β-lactam substrate (10) the ratio of (5):(15) was *ca.* 1:10. Incubation of *L*-N-α-acetylarginine (20) gave the expected alcohol product (21), with no evidence for the formation of the alkene product (22) (**Scheme 6**).[1b] Incubation of *D*-N-α-acetylarginine with CS resulted in a very low level of conversion (<2%) to a product, which had an identical retention time to (21) by reversed phase HPLC analysis. It is unclear if this low level of conversion was due to traces of contaminating *L*-N-α-acetylarginine (20). Kinetic parameters for the conversion of (20) to (21) for the single CS 2 isoenzyme derived from recombinant *E. coli* cells, $K_m = 230 \pm 20$ μM, $K_{cat} = 6.4 \pm 0.8$ S^{-1}.



Scheme 6: Incubation of *L*-N-α-acetylarginine (20) with CS.

Oxygen incorporation experiments

In order to demonstrate that the CS catalysed hydroxylation reaction was responsible for the introduction of a dioxygen derived oxygen into (4), CS derived from recombinant *E. coli* cells was incubated with (3) under an atmosphere of $^{18}O_2$. Analysis of the reaction products by electrospray ionisation mass spectrometry (ESI MS) revealed that the majority of product (4) contained ^{18}O label. However, a substantial proportion (*ca.* 30%) of (4) was not labelled with ^{18}O (**Table 1**). Incubation of (3) in the presence of ^{18}O-labelled water (*ca.* 47.5 atom %) showed that some ^{18}O was incorporated into (4), thus demonstrating a water-mediated exchange process. However, the level of labelling was insufficient to fully account for the results of the ^{18}O dioxygen labelling experiment. Since the labelling experiments were carried out only once, the most likely explanation for the discrepancy in the stoichiometry of incorporation from the $^{18}O_2$ and $H_2{}^{18}O$ labelling experiments is that

there was incomplete degassing of the enzyme solution. However, in the absence of multiple determinations and careful kinetic studies (to eliminate possible isotope effects / the operation of more than one kinetic mechanism) it cannot be ruled out that a cofactor/cosubstrate mediated exchange process is occurring.

Table 1: Incorporation of ^{18}O into (**4**) Derived from (**3**)

	m/z (Da)							
incubation	242	243	244	245	246	247	248	249
under air	0	0	0	100	12	1.5	0.1	0
under $^{18}O_2$	0	1	0	30	5	100	12	2
$H_2{}^{16}O/H_2{}^{18}O$ (52.5:47.5)	0	0	0	100	13	6	2	1

The hydroxylation of *L*-N-α-acetylarginine (**20**) was also investigated using ^{18}O labelling studies (**Table 2**). Incubations performed in the presence of $^{18}O_2$ revealed a high but incomplete level of isotopic incorporation. However, unlike with the β-lactam substrate (**10**), the level of incorporation of ^{18}O from labelled water accounted for the less than stoichiometric incorporation from labelled dioxygen. This result also lends support to the proposal that the discrepancy in the levels of incorporation of label from dioxygen and water in the case of (3) results from a 'technical imperfection' rather than reflecting an intrinsic property of the enzyme.

Table 2: Incorporation of ^{18}O into (**21**) Derived from (**20**)

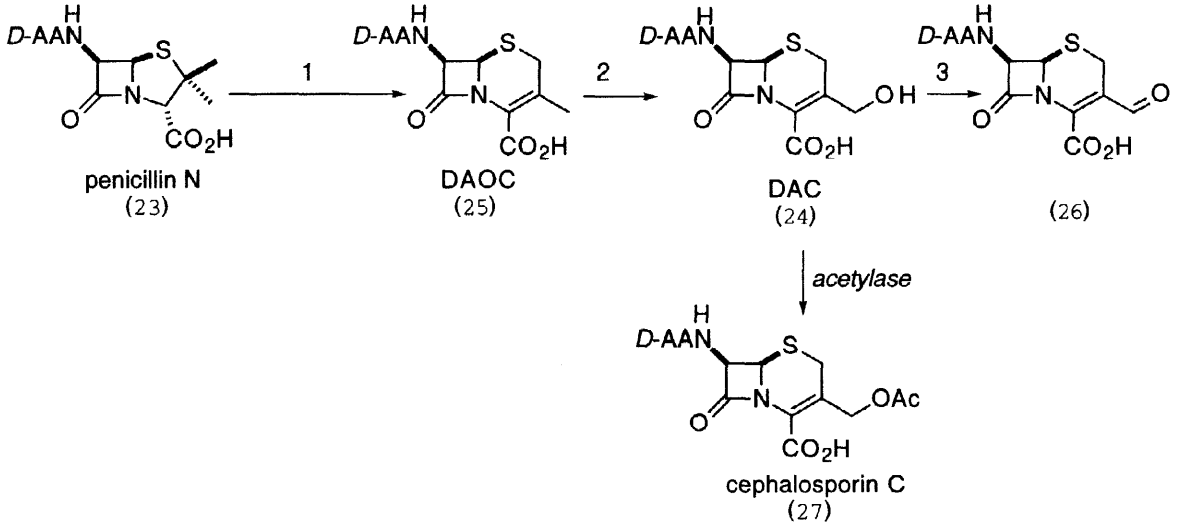
	m/z (Da)							
incubation	231	232	233	234	235	236	237	238
under air	0	0	100	11	1	0	0	0
under $^{18}O_2$	0.5	0.4	17	5	100	11	4	0.6
$H_2{}^{16}O/H_2{}^{18}O$ (52.5:47.5)	2	2	100	12	10	2	2	1

The less than stoichiometric incorporation of ^{18}O label from dioxygen into the products of 2-oxo acid dependent oxygenases has been well documented[25] and has been studied using substrate analogues in the case of deacetoxy/deacetylcephalosporin C synthase (DAOC/DAC synthase).[26] Presently it is unclear at which stage of the mechanism the oxygen exchange process occurs and whether or not the same exchange mechanism operates for all the 2-oxo acid dependent oxygenases/oxidases and for all types of substrate.

Discussion:

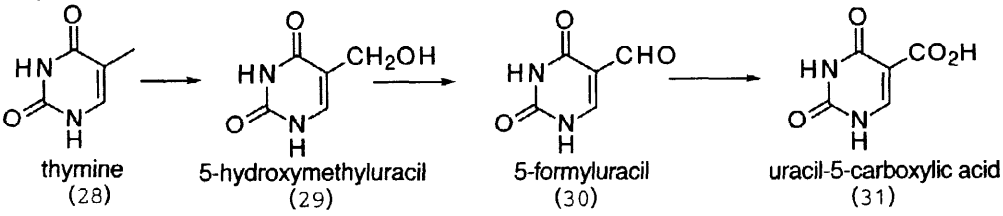
Both the substrate and product selectivities of the CS catalysed reactions are interesting, both from the perspective of the evolution of the pathway and from that of understanding fundamental properties of oxidative enzymes. The results presented herein are consistent with a biosynthetic pathway leading to clavulanic acid (**1**) from *L*-arginine and pyruvate (**Scheme 1**), in which CS plays (at least) an unprecedented trifunctional role. Other examples of non-haem, iron(II) and 2-OG-dependent dioxygenases which catalyse multiple steps in a metabolic pathway have been reported (**Schemes 7, 8, 9**). These include enzymes involved in cephalosporin[27a] uracil[27b] and gibberellin biosynthesis.[27c] In eukaryotic organisms producing cephalosporins a single enzyme, DAOC/DAC synthase, catalyses the oxidative ring expansion and hydroxylation of penicillin N (**23**) to give deacetylcephalosporin C (DAC **24**) *via* deacetoxycephalosporin C (DAOC **25**). In prokaryotes these steps are

catalysed by separate enzymes with a high (but imperfect) degree of selectivity for either penicillin N (**23**) or DAOC (**25**) as a substrate. *In vitro* DAOC/DACS also catalyses the conversion of DAC (**24**) to the unstable aldehyde (**26**), which rapidly undergoes hydrolytic ring opening to give an inactive cephalosporinate (**Scheme 7**). *In vivo* the 'undesirable' third oxidation is prevented by acetylation of DAC (**24**) to give cephalosporin C (**27**).[27b]



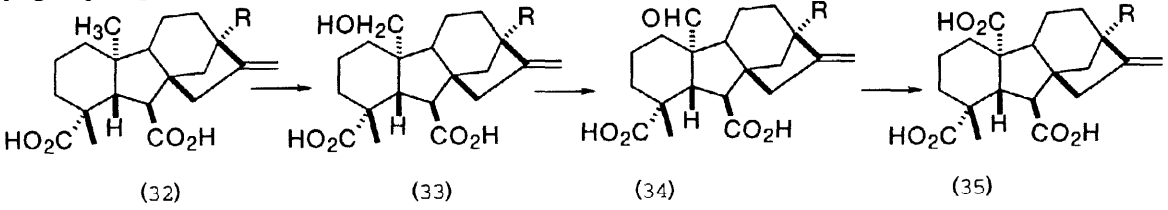
Scheme 7: Reactions of deactoxy/deacetylcephalosporin C synthase (1, 2, and 3)

Thymine hydroxylase catalyses three sequential oxidations of thymine (**28**) to give uracil-5-carboxylate (**31**) *via* 5-hydroxymethyluracil (**29**) and 5-formyluracil (**30**) (**Scheme 8**).[27c]



Scheme 8: The reactions of thymine hydroxylase.

The plant 2-OG oxygenase, gibberellin C-20 oxidase[27d], catalyses a related sequence of oxidations of the C-20 methyl group of gibberellin (**32**) to the C-20 carboxylate of (**35**) (**Scheme 9**).

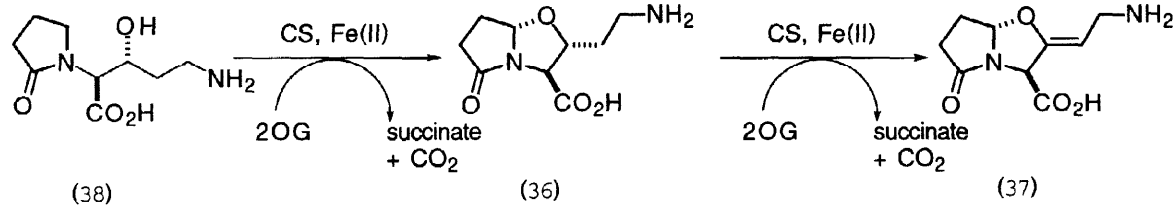


Scheme 9: The reactions of gibberellin C-20 oxidase; R = H or OH.

CS is exceptional amongst the family of 2-OG oxygenases, both in terms of the diversity of the three types of oxidative reactions it catalyses (hydroxylation, oxidative ring formation and desaturation)[1,7,8] and in that one of the reactions it catalyses (hydroxylation) is separated from the other two by the action of a separate hydrolytic enzyme (PAH).[4,6] To the extent of our knowledge the diversity of oxidative reaction catalysed by CS is

unprecedented by that of any other known enzyme. More generally it seems that the 2-OG and related oxygenases/oxidases are able to carry out a wider range of chemistry than their haem-dependent counterparts. It is possible that this difference reflects the utilisation of the haem cofactor, which of necessity fills four of the possible metal co-ordination sites rendering substrate co-ordination to the metal at the same time as a reactive form of dioxygen (superoxide or ferryl) impossible. The use of a rigid haem cofactor may also limit the degree to which generation of the reactive species can be co-ordinated with productive binding of the prime substrate *via* conformational changes within the active site.[28]

The results obtained from the incubation of CS with guanidino and (**3**) and amino (**10**) substrates demonstrate that relatively small changes in the enzyme-substrate complex can bring about significant changes in the type of oxidation reaction catalysed, *i.e.* hydroxylation or desaturation. In the case of (**10**) the dominating reaction is desaturation whilst in the case of (**3**) the presence of an additional amidino group completely biases the oxidative reaction catalysed from desaturation to hydroxylation. The presence of the same amidino group (probably completely) excludes the product (**4**) from being a substrate for the CS catalysed cyclisation and desaturation reactions for which its amino analogue (**5**) is a substrate.



(38) (36) (37)

Scheme 10: Cyclisation of γ-Lactam (**38**) to (**36**) and (**37**).

Both in comparison with each other and with their β-lactam analogues, the incubation results obtained with *L*-N-α-acetylornithine (**17**) and *L*-N-α-acetyl-arginine (**20**) demonstrate how subtle modifications in substrate analogues can modify the product selectivity of an oxidative enzyme. It also notable that substitution of the β-lactam of (**5**) for a γ-lactam ring also resulted in a decrease in the rate of desaturation of (**36**) to (**37**) relative to the rate of cyclisation of (**38**) to (**36**) (*vide supra*, **Scheme 10**).[29] Although this is a much less dramatic change than the bias in product selectivity from hydroxylation to desaturation, it provides yet another example of the sensitive product selectivity of CS.

The concept of evolution of new enzyme functions and thus biosynthetic pathways *via* gene-duplications followed by mutations resulting in altered selectivities is well established. The section of the clavulanate (**1**) biosynthetic pathway encompassing the PAH and the three CS catalysed reactions suggests an alternative scenario, *i.e.* that steps in a pathway may evolve *via* a modification of a product leading to the 'extraction' of another catalytic activity from an enzyme already active in the pathway. In effect the sidechain of the product of one enzyme catalysed reaction [*i.e.* (**4**)] may be mutated by the action of another enzyme (or by a non-enzymatic reaction) to give another substrate [*i.e.* (**5**)] for the first enzyme. Thus, in one sense, the PAH catalysed conversion of (**4**), which is not a CS substrate, to (**5**), which is a CS substrate, may be viewed as a product-substrate 'mutation' process.

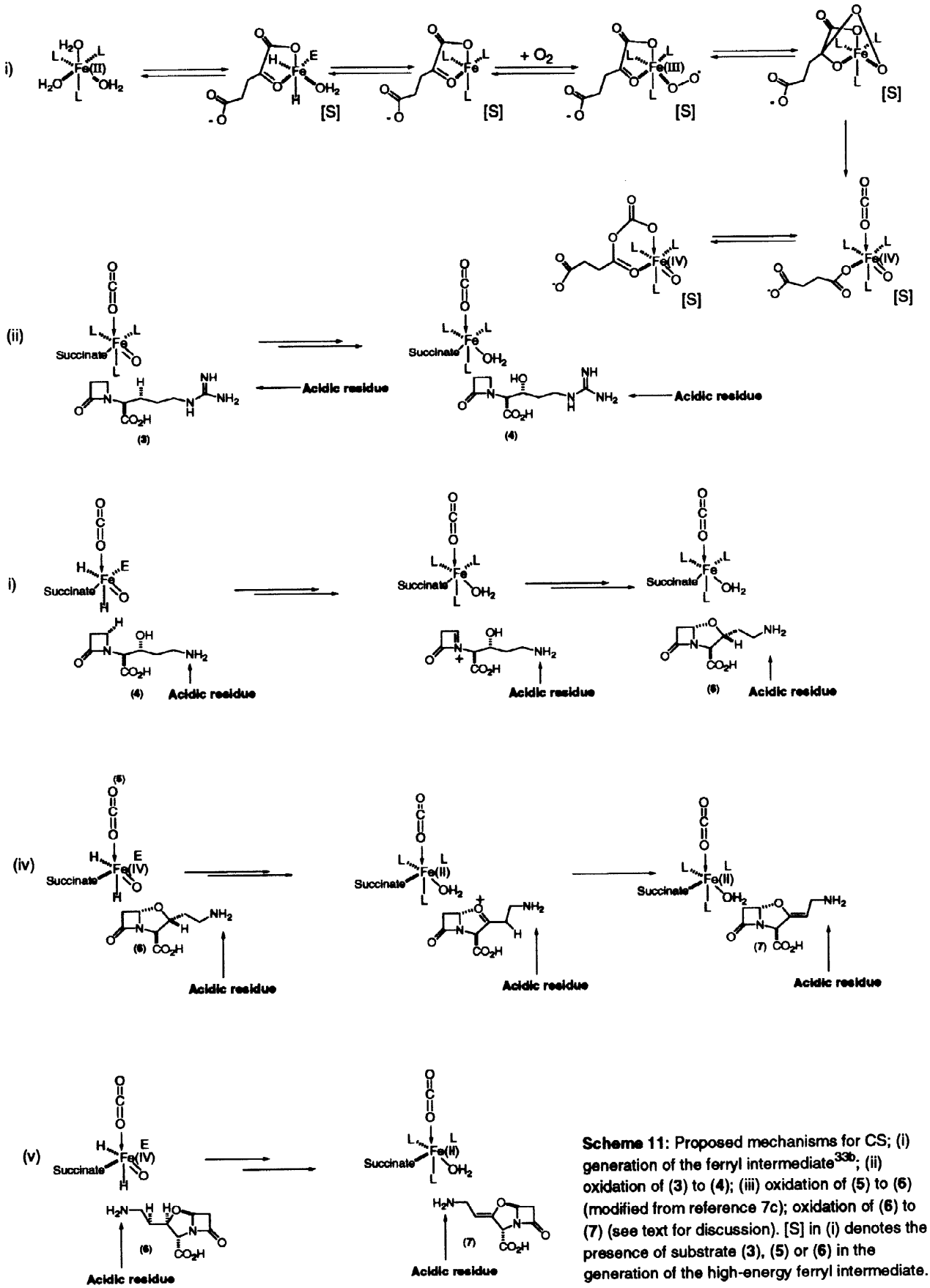
Site-directed mutagenesis has been used for probing enzyme-substrate interactions and for altering the selectivity of enzymes. The alterations of the (dominant) type of oxidative reaction catalysed by CS resulting from changes in the substrate at sites 'remote' from those oxidised suggests that protein-engineering of CS to alter its product selectivity may be a productive exercise. It is of interest that a recent study on plant di-iron lipid

oxygenases[30] showed that recombinant plants containing oxygenase with as few as four mutated residues produced lipids with a radically different composition. These results demonstrate that relatively few modifications to an oxygenase enzymes may dramatically alter its product selectivity, and suggest that mutation studies aimed at altering the product selectivity of CS may be profitable.

The presently proposed mechanism for iron(II) and 2-OG dependent oxygenases involves sequential (and productive) initial binding of 2-OG and 'prime' substrate followed by that of dioxygen.[31] The reaction may then proceed to form a reactive ferryl species, which is responsible for the oxidation of the prime substrate, together with anhydride or succinate/CO_2 ligands [**Scheme 11**, (i)]. The relationship between the ferryl species and the prime substrate is likely to be the key factor in determining the product selectivity of the enzyme. Recently, Zhou *et al.*[32] reported that the six-membered octahedral complex produced on binding of 2-oxoglutarate to the iron(II)-CS complex was relatively unreactive towards dioxygen. Binding of (3) to this complex changed the co-ordination state of the iron from six to five by loss of water, thus rendering the complex reactive to dioxygen. Note that in the absence of prime substrate or in the case of appropriate substrate analogues 'uncoupled' turnover of 2-OG to succinate and CO_2 can still occur[10a,33], albeit normally at reduced levels relative to the prime substrate coupled reaction. The uncoupled turnover of 2-OG may represent an editing mechanism by which unwanted or incorrectly bound substrates are rejected and may be (partly) responsible for the product selectivity of CS.[32] Such a mechanism is, however, unlikely to represent the only method which determines the substrate selectivity of CS in its natural environment and binding interactions before generation of reactive oxidising species will be important.

Any detailed understanding of the origins of the remarkable selectivity of CS must await knowledge of its three dimensional structure and those of its enzyme-substrate/intermediate complexes. Indeed CS is seemingly an excellent case study of how substrate and product selectivity are achieved by metal dependent oxidising enzymes in general. To date only one structure of a 2-OG dependent oxygenase has been reported, that of DAOCS from *Streptomyces clavuligerus* complexed to iron and 2-OG.[34] DAOCS catalyses the oxidative ring expansion of penicillin N (**23**) to DAOC (**25**). This reaction is related to the CS catalysed oxidative ring closure of (**5**) to (**6**) and to the isopenicillin N synthase (IPNS) catalysed four electron oxidation of a tripeptide to give the penicillin nucleus (a 2-OG cosubstrate is not utilised by this enzyme). DAOCS and IPNS, for which crystal structures have also been reported,[35] are clearly related by sequence to each other and to many other 2-OG dependent oxygenases which have probably evolved from a common ancestor. However, the degree of sequence similarity between CS and the DAOCS/IPNS subfamily is limited with even highly conserved motifs within the sub-family being apparently absent in the CS sequences. These include an HisXAsp (HXD) motif, present in the DAOCS/IPNS sub-family, which is responsible for providing two of three conserved His, His, carboxylate, triad of iron ligands in the wider family of non-haem iron oxygenases/oxidases.[28]

Although the HXD motif of CS may be replaced by an HTE sequence (residues 172-174 in CS1 and 173-175 in CS2)[12a] and CS does show similarity to some other 2-OG dependent oxygenases[36], the lack of overall sequence similarity between CS and DAOCS makes proposals regarding the CS active site based on that of DAOCS speculative. The side chains of two arginines of an RXR motif (160-162) in the DAOCS active site have been proposed to bind to the carboxylate of the penicillin N (**23**) substrate (**Figure 1**). Docking of CS substrates (**3**) and (**5**) similarly into the DAOCS active site suggests that similar binding of their carboxylates to the side chains of the two arginines will orientate the oxidised C-H bonds towards the ferryl intermediate.



Scheme 11: Proposed mechanisms for CS; (i) generation of the ferryl intermediate[33b]; (ii) oxidation of (**3**) to (**4**); (iii) oxidation of (**5**) to (**6**) (modified from reference 7c); oxidation of (**6**) to (**7**) (see text for discussion). [S] in (i) denotes the presence of substrate (**3**), (**5**) or (**6**) in the generation of the high-energy ferryl intermediate.

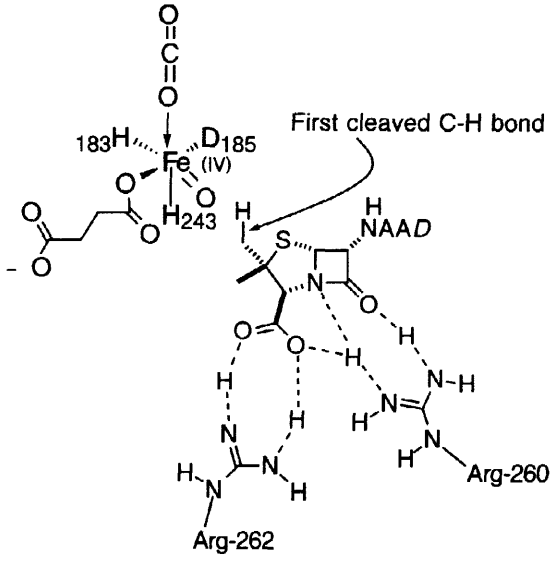


Figure 1: Possible binding mode of (**23**) in the active site of DAOCS; *D*AA = *D*-δ-(α-aminoadipoyl)-.[34b]

By analogy with mechanism proposed for IPNS it has also been suggested that the ring closure reaction [(**5**) to (**6**)] catalysed by CS involves co-ordination of the hydroxyl group of (**5**) to the metal centre.[7c] The high-energy ferryl intermediate [generated as in **Scheme 11**, (i)] is believed to perform the substrate oxidation. This may react in an insertion type mechanism with substrates (**3**) and (**5**) [as shown in **Scheme 11**, (ii) and (iii)]. The observed stereochemistries in the conversions of (**3**) to (**4**)[23a] and (**5**) to (**6**)[9] are consistent with the crude model based on the DAOCS structure.

Ligation of the alcohol of (**5**), during the conversion of (**5**) to (**6**), to the iron in CS would almost certainly require displacement of one of the other ligands. It would seem most likely that succinate or CO_2/bicarbonate are displaced, but the possibility of displacement of one of the protein-derived ligands from the iron can not be excluded. Alternatively, the hydroxyl group of (**5**) may not be ligated to the metal and general base catalysis may be involved in the cyclisation reaction.

Hydrolysis of the guanidino sidechain of (**3**) or (**4**) to give (**10**) or (**5**) clearly influences the selection of which bonds in the substrate are oxidised by CS. A possible explanation is that there is an acidic residue(s) at the active site of CS, which bind the basic substrate sidechain and orientates it relative to the ferryl such that a particular bond is cleaved. Thus loss of the amidine from (**3**) may result in the substrate being pulled towards the putative acidic residue resulting in oxidation of the β-lactam 4-H', rather than the 3-H of the sidechain, as in the conversion of (**3**) to (**4**). However, such a simple explanation does not account for the difference in alkene:alcohol product ratio from (**3**) and (**10**), nor for the conversion of dihydroclavaminate (**6**) to clavaminate (**7**).

Docking of dihydroclavaminate (**6**) into the model in a similar manner projects the two hydrogens which must be removed in the desaturation process[9] away from the metal centre [*vide supra*, **Scheme 11** (iv)]. Salowe *et al.*[7c] have previously proposed that the conversion of (**6**) to (**7**) may occur *via* oxidation of the oxygen followed by loss of a proton [**Scheme 11**, (iv)]. An alternative proposal to account for the apparent change in the stereochemical relationship between the ferryl and the bonds oxidised was that the ferryl intermediate may 'flip'[37] from one position on the iron to another dependent on whether (**3**)/(**5**) or (**6**) is the CS substrate. However, based on the DAOCS model, such a flip of the ferryl from opposite His 183 (the proposed dioxygen binding site for

DAOCS) for the oxidation of (3) and (5) by CS to opposite His-243 (DAOCS) (displacing CO_2) or Asp-185 (displacing succinate) would not project the ferryl closer to the substrate without other conformational changes. It may thus be that different residues are involved in binding of the basic side chain of the substrates (and possibly the carboxylate) in the case of dihydroclavaminate (6) compared to (3) and/or (5) resulting in rotation of (6) in the active site relative to (3) and (5) [**Scheme 11**, (v)].

Possible implications of the trifunctional role of CS for the biosynthesis of other metabolites:

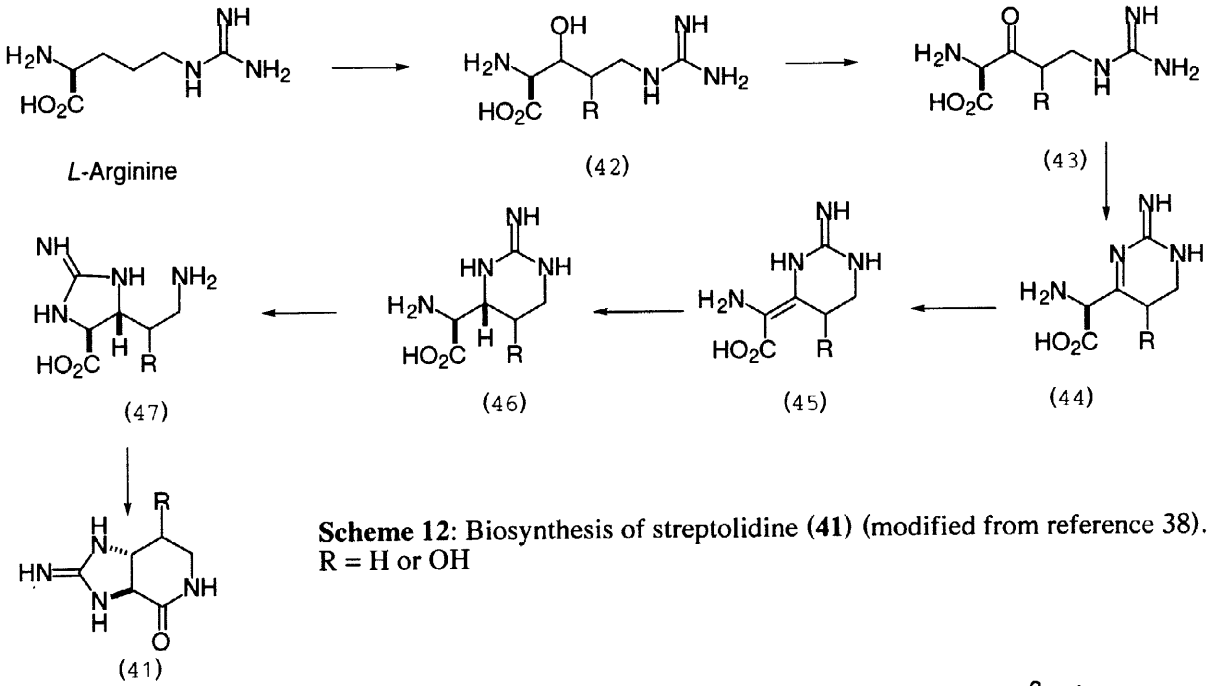
The results presented herein demonstrating the versatile nature of the oxidative transformations catalysed by CS may have implications for the biosynthesis of other metabolites derived by oxidation of arginine or its derivatives. Metabolites apparently derived by oxidation of arginine include the streptothricin group of antibiotics[38], the hexapeptide antibiotics K-582A and K-582B [which contain (2*S*, 3*R*)-hydroxyarginine][39], Anatoxin-a(s) (**39**)[40] and carnosidine (**40**).[41]

Martinkus *et al.* propose that the biosynthesis of streptolidine (**41**) from 3-hydroxyarginine (**42**) as outlined (**Scheme 12**).[38] Clear parallels exist with the clavam biosynthesis pathway, including the oxidation of arginine derivatives at both the 3- and 4-positions. Labelling studies suggest that alcohol (**42**) was oxidised to the ketone (**43**) (or a derivative) and cyclised to give imine (**44**). Rearrangement to (**45**) followed by reduction to (**46**) and ring switching leads to (**47**) which is cyclised (*c.f.* the BLS catalysed reaction in clavam biosynthesis) to give the bicyclic lactam core of streptolidine (**41**). A second hydroxyl group is introduced at C-4 of arginine during this pathway. The trifunctional role of CS in the clavam biosynthesis pathway suggests that a similar multifunctional oxygenase might be involved in streptolidine (**42**) biosynthesis.

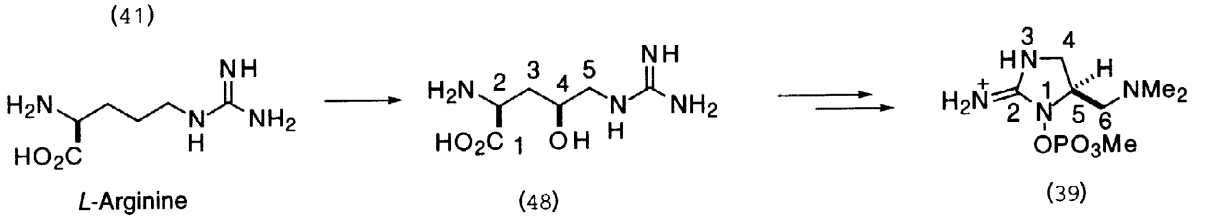
Anatoxin-a(s) (**39**) is also derived from arginine *via* (2*S*,4*S*)-hydroxyarginine (**48**) (**Scheme 13**). Labelling studies have demonstrated that both C-3 hydrogens are lost during anatoxin-a(s) (**39**) biosynthesis, suggesting oxidation and cyclisation *via* a ketone,[40] analogous to the streptolidine and clavam pathways.

Another 2-OG dependent oxygenase from *Pseudomonus syringae* pv. *phaseolicola* PK2 has been shown to directly hydroxylate arginine to 5-hydroxyarginine (**49**),[42] which decomposes to guanidine (**50**) and (*L*)-Δ1-pyrroline-5-carboxylate (**51**) (**Scheme 14**). In addition to this 'typical' 2-OG dependent oxygenase reaction in which the other products are succinate and carbon dioxide, in the presence of arginine the enzyme can also catalyse the production of ethylene from 2-OG. This interesting reaction presumably also results in the formation of three carbon dioxide molecules or a carbon monoxide and 2 carbon dioxide molecules, dependent on whether the 2-OG undergoes a four or two electron oxidation. Unlike in clavulanate (**1**), streptolidine (**41**), anatoxin-a(s), and carnosidine (**40**) biosynthesis, it appears that 2-oxoglutarate is the substrate and arginine is the cosubstrate in this reaction.

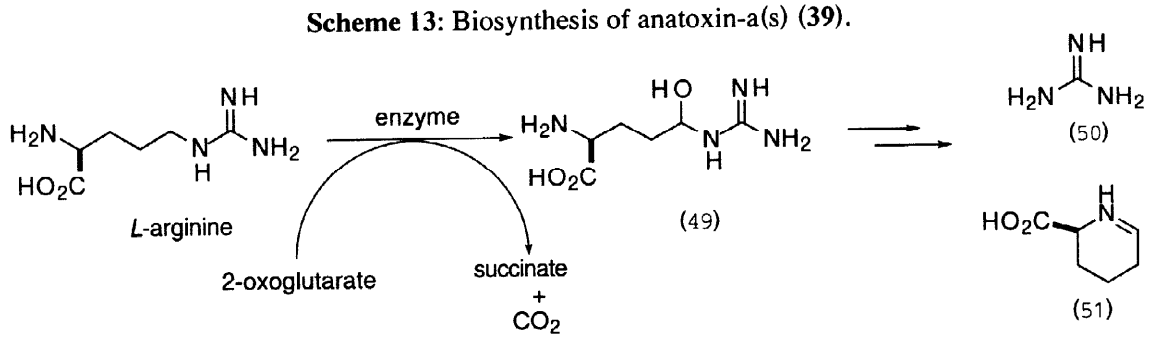
The biosynthesis of carnosadine[41] (**40**), a cyclopropyl derivative of arginine, can be most efficiently envisaged occuring from arginine in a single step *via* a two electron oxidation. There is no precedent for oxygenase catalysed formation of a three membered ring. However, isopenicillin N synthase[35], which is related by sequence and structure to the 2-OG dependent oxygenases, catalyses the formation of the strained bicyclic fused β-lactam-thiazolidine nucleus of the penicillins from a simple peptide precursor. Thus, it seems reasonable to propose that the conversion of arginine to carnosadine (**40**) may be mediated in a single step *via* an oxygenase related to CS. Alternatively a hydroxyarginine derivative (**52**) may be an intermediate in this cyclisation (**Scheme 15**).



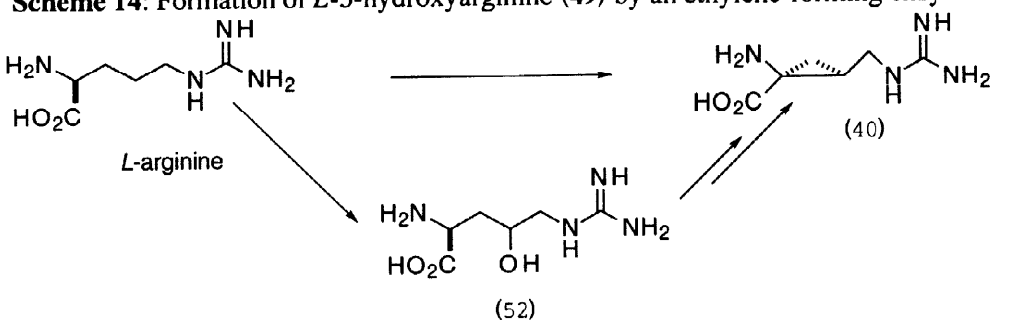
Scheme 12: Biosynthesis of streptolidine (**41**) (modified from reference 38). R = H or OH



Scheme 13: Biosynthesis of anatoxin-a(s) (**39**).



Scheme 14: Formation of *L*-5-hydroxyarginine (**49**) by an ethylene-forming enzyme.



Scheme 15: Proposed biosynthetic pathways to carnosidine (**40**).

Experimental:

All chemicals were obtained from the Sigma-Aldrich Chemical Co. and were used without further

purification unless otherwise stated. Infrared spectra were recorded on a Perkin-Elmer 1750 Fourier Transform spectrometer with only selected absorbences being recorded. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on either a Varian Gemini 200 operating at 200 MHz or a Brüker AM500 operating at 500 MHz. The spectra of synthetic compounds were referenced to residual protonated solvent residues as an internal standard. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on either a Varian Gemini 200 operating at 50.3 MHz or a Brüker AM500 operating at 125.8 MHz. The spectra were referenced to solvent carbon residues or 1,4-dioxan, δ_C = 67.3 p.p.m, for samples in D_2O. DEPT editing was used unless no indication of the number of protons attached to the carbon atom is given. All chemical shifts (δ_H, δ_C) are in parts per million (p.p.m.) down field from tetramethylsilane (TMS), in the solvent indicated Mass spectra were recorded on either a V. G. 20-250 or a BIO-Q instrument, with the modes of ionisation being indicated as Desorption Chemical Ionisation (DCI), Probe Chemical Ionisation (CI) or Direct Electron Impact (DEI). Only major peaks were recorded, as the mass-to-charge (m/z) ratio. Accurate masses were recorded by the EPSRC mass spectrometry service centre (University of Wales, Swansea). Optical rotations were determined using a Perkin-Elmer 241 polarimeter with concentrations given in g/100 mL. Melting points (m.p.) were determined using a Büchi 510 capillary melting point apparatus and are uncorrected. Elemental analyses were performed within the Dyson Perrins laboratory.

Thin layer chromatography was performed on Merck DC-Alufólien 60F254 0.2 mm precoated plates. Spots were detected by quenching of ultraviolet fluorescence (λmax 254 nm) or 5% (w/v) dodecamolybdophosphoric acid in ethanol followed by heating. Amino acids were located on t.l.c. by 3% (w/v) ninhydrin in ethanol. Flash chromatography was carried out on Baker silica gel (30-60 mm). Light petroleum refers to the fraction of petroleum ether which boils between 40-60°C. This was distilled before use, as were ethyl acetate, diethyl ether, benzene, dichloromethane. Tetrahydrofuran was distilled from sodium benzophenone ketyl under an atmosphere of nitrogen. Acetonitrile was dried by distillation from calcium hydride and stored over activated 4 Å molecular sieves under argon. Water was produced by distillation or by a Milli-Q$_{PLUS}$™ purification system (Millipore). HPLC grade methanol was obtained from Rathburn Chemical Ltd (Walkerburn, Scotland).

Synthesis of (2S)-N^5-benzyloxycarbonylornithine (12)

A solution of *L*-ornithine.HCl (300 mg, 1.76 mmol) and basic copper carbonate ($CuCO_3.Cu(OH)_2.H_2O$) (530 mg, 1.96 mmol) in water (2 mL) was heated under reflux for 30 minutes, filtered while hot, and washed with hot water (3 x 3 mL). The resulting solution was cooled to room temperature and basified with magnesium oxide (100 mg, 2.48 mmol). After further cooling to <5 °C, a solution of benzylchloroformate (0.5 mL, 3.33 mmol) in tetrahydrofuran (10 mL) was added dropwise over 30 minutes. The solution was warmed slowly to room temperature and stirred for 22 hours. The resulting blue precipitate was collected by vacuum filtration, washed with water (3 x 10 mL), ethanol (3 x 10 mL) and diethyl ether (2 x20 mL), and dried *in vacuo* to yield the crude copper complex (407 mg, 77%): m.p. 206-208 °C (dec.); ν_{max}(KBr)/cm^{-1} 3333s (N-H), 2927m, 2123w, 1697s (urethane C=O), 1582s, 1549s, 1412m, 1277m, 1143m and 781m.

EDTA (2.53 g, 8.67 mmol) was added to a stirred solution of sodium hydrogen carbonate (1.42 g, 16.9 mmol) in water (20 mL). The powdered crude copper complex (400 mg, 0.67 mmol) was added and heated under reflux for 2 hours. The solution was cooled, filtered, and the white powder washed with water (3 x 20 mL) and dried *in vacuo* to yield (**12**) (273 mg, 58% overall): m.p. 212-214 °C (dec.) [lit.[43] 254 °C (dec.)]; $[\alpha]_D^{25}$ +18.4 (c 1.0 in 6 M HCl); ν_{max}(KBr)/cm^{-1} 3333s (N-H), 3080m (N-H), 2930m, 2128w, 1687s (urethane C=O).

1582s (CO_2^-), 1412m and 1295m; ^1H NMR (200 MHz; 2 M DCl) δ_H 0.73-1.06 (2H, m, H-4), 1.06-1.39 (2H, m, H-3), 2.43 (2H, t, J=6.5 Hz, H-5), 3.39 (1H, t, J=6 Hz, H-2), 4.32 (2H, s, CH_2Ph) and 6.66 (5H, s, aromatic H); ^{13}C NMR (50.3 MHz; 2 M DCl) δ_C 24.0 (C-4), 26.3 (C-3), 39.1 (C-5), 51.9 (C-2), 66.3 (CH_2Ph), 127.2, 127.9, 128.4, and 136.0 (aromatic C), 158.0 ($NHCO_2$) and 171.3 (C-1); *m/z* (DCI-NH_3) 267 (MH^+, 20%), 204 (12), 115 (100), 108(30), 106 (22), 99 (20), 91 (36) and 70 (46).

Synthesis of (2S)-N^5-benzyloxycarbonylornithine benzyl ester, p-toluenesulphonate salt (13)

Monoprotected *(2S)*-ornithine (**12**) (150 mg, 0.56 mmol) and benzyl alcohol (0.60 mL, 5.80 mmol) were stirred together with benzene (35 mL) and *p*-toluenesulphonic acid monohydrate (117 mg, 0.62 mmol). The solution was heated under reflux in a modified Dean-Stark apparatus (using 4 Å molecular sieves) for 23 hours, cooled to room temperature, the benzene evaporated *in vacuo*, and the flask flushed with toluene (3 x 5 mL) to yield a colourless oil. Trituration with petroleum ether (200 mL) yielded (**13**) as a white solid (215 mg, 73%): m.p. 84-86 °C (dec.); $[\alpha]_D^{24}$ -2.4 (c 1.0 in $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3440br s (N-H), 2930m, 1754m (ester C=O), 1693m (urethane C=O), 1453m, 1216s and 1125s; ^1H NMR (200 MHz; $CDCl_3$) δ_H 1.37-1.58 (2H, br m, H-4), 1.78-1.97 (2H, br m, H-3), 2.26 (3H, s, $C_6H_4CH_3$), 2.92-3.04 (2H, br m, H-5), 3.96-4.08 (1H, br m, H-2), 4.91-5.10 (4H, br m, 2 x CH_2Ph), 7.01 (2H, br s, 2 x NH), 7.10-7.38 (10H, m, 2 x C_6H_5) and 7.70 and 8.20 (2 x 2H, 2 x br s, $CH_3C_6H_4SO_3^-$), other 2 x NH not observed; ^{13}C NMR (50.3 MHz; $CDCl_3$) δ_C 21.2 ($C_6H_4CH_3$), 24.5 (C-4), 27.2 (C-3), 40.0 (C-5), 52.8 (C-2), 66.6 and 68.0 (2 x CH_2Ph), 126.4, 127.3, 127.7, 128.1, 128.8, 129.3, 134.8, 136.9 and 141.3 (aromatic C), 156.1 ($NHCO_2CH_2Ph$) and 169.7 (C-1); *m/z* (DCI-NH_3) 357 (MH^+, 87%), 249 (15), 221 (25), 204 (18), 190 (22), 115 (100), 108 (42), 91 (80) and 70 (27).

Synthesis of (2S)-N^5-benzyloxycarbonyl-N^2-(2-carboxyethyl)ornithine benzyl ester (14)

The tosylate salt (**13**) (205 mg, 0.39 mmol) was partitioned between ethyl acetate (20 mL) and saturated sodium hydrogen carbonate (15 mL), and the aqueous layer extracted with further ethyl acetate (15 mL). The organic layer was washed with water (20 mL), dried (sodium sulphate) and evaporated *in vacuo* to yield the free amine as a colourless oil. The free amine was used to prepare (**14**) by the method of Baggaley *et al.* Spectroscopic properties were consistent with those previously reported.[44]

Synthesis of (2S)-benzyl 5-benzyloxycarbonylamino-2-(2'-oxoazetidin-1'-yl)pentanoate

To methanesulphonyl chloride (27 μL, 0.35 mmol) in acetonitrile (1.8 mL) containing suspended sodium hydrogen carbonate (146 mg, 1.74 mmol) at 60 °C, was added a solution of the acid (**14**) (124 mg, 0.29 mmol) in acetonitrile (10 mL) dropwise over 45 minutes. The solution was stirred for a further 3 hours, cooled to 0 °C, filtered, and the solvent evaporated *in vacuo* to yield a yellow oil. Purification by flash chromatography, eluting with ethyl acetate-petroleum ether (11:9), yielded the diprotected analogue of (**10**) as a colourless oil (84 mg, 71%): R_f 0.3 (EtOAc:PE 3:2); $[\alpha]_D^{25}$ -6.0 (c 1.0 in $CHCl_3$) [lit.[44] $[\alpha]_D^{20}$ -10.95 (c 2.0 in EtOH)]; ν_{max}(thin film)/cm^{-1} 3340br w (N-H), 2930w, 1750sh s, 1734s and 1718s (3 x C=O), 1541m and 1250m; ^1H NMR (200 MHz; $CDCl_3$) δ_H 1.48-1.70 (2H, m, H-4), 1.70-2.04 (2H, m, H-3), 2.94 (2H, t, J=4 Hz, H-3'), 3.15-3.33 and 3.37-3.48 (3H + 1H, 2 x m, H-5 and H-4'), 4.41 (1H, dd, J=9.5 and 5 Hz, H-2), 4.90 (1H, br s, NH), 5.10 and 5.17 (2 x 2H, 2 x s, 2 x CH_2Ph) and 7.36 (10H, s, aromatic H); ^{13}C NMR (50.3 MHz; $CDCl_3$) δ_C 26.7 and 26.8 (C-3 and C-4), 36.4, 37.8 and 40.2 (C-3', C-4' and C-5), 53.9 (C-2), 66.7 and 67.2 (2 x CH_2Ph), 128.3, 128.5, 128.7, 128.9, 135.4 and 136.8 (aromatic C), 156.7 ($NHCO_2CH_2Ph$) and 168.4 and 170.5 (2 x C=O, ester

and β-lactam); *m/z* (CI-NH_3) 411 (MH^+, 74%), 320 (MH^+ - $PhCH_2$, 27), 303 (100), 259 (30), 204 (24), 169 (30), 167 (41), 160 (28), 125 (34), 108 (30) and 91 (80).

Synthesis of (2S)-5-amino-2-(2'-oxazetidin-1'-yl)pentanoic acid (10)

(**10**) was prepared by the method of Baggaley *et al.*[44] A small amount was purified by reverse phase HPLC [ODS (250 x 7 mm), 12.5 mM NH_4HCO_3, 1.5 mL/min] with a retention volume of 9.0 mL for use in biological experiments. Spectroscopic properties were consistent with those previously reported.[44]

Synthesis of (2S)-5-guanidino-2-(2'-oxazetidin-1'-yl)pentanoic acid (3)

A solution of (**10**) (22 mg, 0.12 mmol) and 1-amidino-4-azapyrazole (17 mg, 0.12 mmol) in 1 M aqueous sodium carbonate (117 μL, 0.12 mmol) was stirred at room temperature for 24 hours and then freeze-dried to yield a white solid. Purification by reverse phase HPLC [ODS (250 x 7 mm), H_2O, 1.5 mL/min] yielded (**3**) as a white solid (15 mg, 56%) with a retention volume of 31.0 mL: ν_{max}(KBr)/cm^{-1} 3436s (N-H), 2938m, 1735s (β-lactam C=O), 1654s (CO_2^-), 1559m and 1398m; ^1H NMR (500 MHz; D_2O) δ_H 1.59-1.70 (2H, m, H-4), 1.75-1.84 and 1.84-1.96 (1H + 1H, 2 x m, H-3), 2.93-3.02 (2H, m, H-3'), 3.23 (2H, *ca.* t, J=7 Hz, H-5), 3.38-3.44 and 3.44-3.51 (1H + 1H, 2 x m, H-4') and 4.11 (1H, dd, J=11 and 5 Hz, H-2); *m/z* (ESI MS) 229 (MH^+, 100).

Synthesis of (2R)-2-acetamido-5-guanidino-pentanoate (D-N-α-acetylarginine dihydrate)

D-Arginine hydrochloride (0.50g, 2.38 mmol) in water (3 mL) was cooled in ice-water and sodium hydrogen carbonate (0.20g, 2.38 mmol) was added. Acetic anhydride (0.29 mL, 3.07 mmol) and sodium hydrogen carbonate (0.20g, 2.38 mmol) were added alternatively in small portions to this stirred mixture. Addition of further sodium carbonate (0.16g, 1.90 mmol) gave a thick white precipitate which was stirred for 30 minutes before storing at 6 °C. After three hours the white precipitate was filtered off and washed with ice cold water (3 mL) to give the desired product (0.36 g, 1.41 mmol, 59%): m.p. 139-141 ° C; $[\alpha]^{23}_D$-10.16 (0.85 in H_2O); ^1H NMR (200 MHz, D_2O) δ_H 1.20-1.20 (m, 4 H, H-3 and H-4), 1.77 (s, 3H, CH_3), 2.94 (t, 2H, J=6.5 Hz, H-5), 3.89 (m, 1H, H-2); ^{13}C NMR (50.3 MHz, $CDCl_3$) δ_C 21.50 ($\underline{C}H_3$), 24.21 (C-3 or 4), 28.36 (C-3 or 4), 40.34 (C-5), 54.55 (C-2), 156.8 ($CH_3\underline{C}O$), 173.7 (C-1), 179.0 (C=NH); m/z (APCI) 239 (M+ Na^+, 7%), 217 (MH^+, 80%), 199 (12%), 175 (10%), 157 (85%), 139 (12%), 115 (100%).

Incubation experiments

Incubations with CS from *S. clavuligerus* SC2 or recombinant *E. coli* cells were carried out by the reported protocols.[7b] The amount of CS is reported in IU (μmols/min) for the conversion of (**5**) to (**7**) under the stated conditions.[10b] HPLC separations used ODS Hypersil columns with a Waters Associates 600E pump, a 600E system controller, a 490E programmable wavelength detector, a SE 120 chart recorder and a Rainin injector fitted with a 200 μL injection loop. Solvents were sparged with helium at 100 mL/min for at least 10 minutes before commencing the experiment and at 20 mL/min thereafter. Crude incubation mixtures were filtered through a 0.22 μm filter (Anachem). Products were characterised by ^1H NMR (500 MHz) referenced to TSP with 0.5 Hz line broadening and by positive ion ESI MS. Assays using (**3**) and (**20**) as substrates were carried out in a total volume of 100 μL at ambient temperature in a 5 mL tube[10b], and quenched by flash heating in a microwave for 10 seconds before HPLC analysis [ODS (250 x 4.6 mm), H_2O, 1 mL/min, λ = 218 nm at 0.1 AUFS]. Standard curves of (**4**) or (**20**) were used to quantify products. Kinetic analyses were at 30 °C in a total volume of 200 μL and the HPLC eluate was monitored at 0.05 AUFS and parameters were determined using reported methods.[10b]

Incubation of (2S)-5-amino-2-(2-oxoazetidin-1-yl)pentanoate (10)

Incubation of (**10**) with native CS (1.2 mg, 0.34 IU/mg) gave a *ca.* 7% conversion to (**15**). HPLC purification [ODS column (250 x 7 mm), 25 mM NH_4HCO_3 at 1.5 mL/min] gave (**15**) with a retention volume of 9.15 mL (96 μg): ^{1}H NMR (500 MHz, D_2O) δ_H 2.95-3.0 (2H, m, H-6), 3.4-3.5 (2H, m, H-7), 3.5-3.6 (2H, m, H-5), 5.8-5.9 (1H, m, H-4), 5.9-6.0 (1H, m, H-3). A 2D COSY correlation spectrum was consistent with the proposed structure of (**15**) and indicated the presence of a signal at *ca.* 4.6 p.p.m. under the residual HOD peak; m/z (ESI MS) = 185 (MH^+, 100%). Homonuclear decoupling experiments showed $J_{3H,4H}$ = 14.0 Hz, indicating *E* stereochemistry. Incubation of (**10**) with recombinant CS (6 mg, 0.034 IU/mg) gave a *ca.* 30% conversion to (**15**) and <5% conversion to (**5**). Purification as before gave (**15**) (*ca.* 200 μg) with identical properties to that from incubations with native CS, and (**5**) (<<5 μg) with a retention volume of 6.9 mL. It was not possible to completely assign the ^{1}H NMR spectrum (500 MHz) of (**5**), but the resonances were consistent with the proposed structure. m/z (ESI MS) = 203 (MH^+, 100%).

Incubation of (2S)-5-guanidino-2-(2-oxoazetidin-1-yl)pentanoate (3)

Incubation of (**3**) with native CS (2 mg, 0.3 IU/mg) and recombinant CS (0.18 mg, 0.37 IU/mg) gave a >85% conversion to (**4**). HPLC purification [ODS (250 x 4.6 mm), H_2O, 1 mL/min] led to the isolation of (**4**) (340 μg and 350 μg) with a retention volume of 7.5 mL: ^{1}H NMR (500 MHz, D_2O) δ_H 1.7-1.8 (1H, m, H-4), 1.8-1.9 (1H, m, H-4), 3.0 (2H, t, J=4 Hz, H-6), 3.36 (2H, t, J=7 Hz, H-5), 3.5-3.55 (1H, m, H-7), 3.59-3.61 (1H, m, H-7), 4.1 (1H, d, J=5.5 Hz, H-2), 4.15-4.2 (1H, m, H-3). A 2D COSY correlation spectrum was consistent with the proposed structure of (**4**). m/z (ESI MS) = 245 (MH^+, 100%).

Incubation of (2S)-2-acetamido-5-aminopentanoate (L-N-α-acetylornithine) (17)

Incubation of (**17**) with recombinant CAS (6 mg, 0.035 IU/mg) gave a *ca.* 17% conversion to (2*S*)-2-acetamido-5-amino-3-hydroxypentanoic acid (**18**) and a *ca.* 5% conversion to *E*-(2*S*)-5-amino-2-acetamidopent-3,4-enoic acid (**19**). HPLC purification [Bondapak amine (250 x 7 mm), ODS guard column, 0.015 M HCO_2H, 2 mL/min] gave (**19**) (30 μg) with a retention volume of 9.4-10.0 mL: ^{1}H NMR (500 MHz) δ_H 2.0 (3H, s, CH_3-), 2.33 (2H, d, J=5 Hz, H-5), 3.55 (1H, m, H-2), 5.65-5.75 (1H, m, H-4), 5.9-6.0 (1H, m, H-3). A 2D COSY correlation spectrum was consistent with the connectivities of (**19**). Homonuclear decoupling experiments showed $J_{3H,4H}$ = 15.2 Hz, indicating *E* stereochemistry; m/z (ESI MS) = 173 (MH^+, 100%). Further HPLC purification of the fraction between 8-9 mL [ODS (250 x 4.6 mm), 0.05% (v/v) aqueous HCO_2H, 1 mL/min] gave (**18**) (99 μg) with a retention volume of 5.5-9.0 mL: ^{1}H NMR (500 MHz, D_2O) δ_H 1.75-1.85 (2H, m, H-4), 2.1 (3H, s, CH_3-), 3.15 (2H, t, J=5 Hz, H-5), 4.2-4.25 (1H, m, H-3), 4.30 (1H, d, J=3.5 Hz, H-2). A 2D COSY correlation spectrum was consistent with the connectivities of (**18**); m/z (ESI MS) = 191 (MH^+, 100%)

Incubation of (2S)-2-acetamido-5-guanidino-pentanoate (L-N-α-acetylarginine) (20)

Incubation of (**20**) with recombinant CS (6 mg, 0.035 IU/mg) gave >85% conversion to (**21**). HPLC purification [ODS (250 x 4.6 mm), H_2O, 1 mL/min] led to the isolation of (**21**) (320 μg) with a retention volume of 4.5 mL: ^{1}H NMR (500 MHz, D_2O) δ_H 1.8-2.0 (2H, m, H-4), 2.1 (3H, s, CH_3-), 3.15 (2H, m, H-5), 4.20-4.22 (1H, m, H-3), 4.25 (1H, d, J=3.5 Hz, H-2); A 2D COSY correlation spectrum was consistent with the connectivities of (**21**); m/z (ESI MS) = 236 (0%), 235 (1%), 234 (11%), 233 (MH^+, 100%), 232 (0%), 231 (0%). Incubation of (2*R*)-2-acetamido-5-guanidino-pentanoate with recombinant CS (26 mg, 0.084 IU/mg) gave

<< 5% conversion to (**21**). HPLC analysis [ODS (250 x 4.6 mm), H_2O, 1 mL/min] confirmed trace conversion to (**21**). Incubation of (**20**) under identical conditions gave a *ca.* 65% conversion to (**21**).

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Abbreviations:
AUFS, absorbance units full scale; BnOH, benzyl alcohol; CD, clavulanate dehydrogenase; CS, clavaminate synthase; *D*AA, *D*-δ-(α-aminoadipoyl)-; DAC, deacetylcephalosporin C; DACS, deacetylcephalosporin C synthase; DAOC, deacetoxycephalosporin C; DAOCS, deacetoxycephalosporin C synthase; DAOC/DACS, deacetoxy/deacetylcephalosporin C synthase; EDTA, ethylenediaminetetraacetic acid; ESI MS, electrospray ionisation mass spectrometry; I.U., international unit; PAH proclavaminate amidinohydrolase; MsCl methylsulphonyl chloride; TMS, tetramethylsilane; TsOH, *p*-tolunesulphonic acid; TSP, [2,2',3,3'-$^2H_4$]-trimethylsilylpropanoate, sodium salt.

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