The enzymology of clavam and carbapenem biosynthesis

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The enzyme-catalysed reactions involved in formation of the bicyclic clavam and carbapenem nuclei, including β-amino acid and β-lactam formation, are discussed and compared with those involved in penicillin and cephalosporin biosynthesis. The common role of unusual oxidation reactions in the biosynthetic pathways and the lack of synthetic reagents available to effect them are highlighted.

Introduction

First used clinically over 50 years ago, the β-lactams remain amongst the most medicinally important antibiotics. However, soon after the clinical introduction of penicillin it was apparent that resistant strains of bacteria would threaten the widespread use of β-lactams.[1] The consequent need to combat resistance helped to motivate the search for new β-lactam antibiotics, which resulted in the discovery of the cephalosporins and carbapenems (Fig. 1). β-Lactamases, which function by hydrolysing the β-lactam ring, are significant mediators of resistance and some β-lactams, including clavulanic acid, were explicitly developed as β-lactamase antagonists.[2] These inhibitors react with β-lactamases to form acyl-enzyme complexes that are stable with respect to hydrolysis, a process closely related to the mode of action of β-lactam antibiotics which inhibit transpeptidases involved in cell wall biosynthesis (Fig. 2).[3–5]

Some medicinally used penicillins can be produced directly by fermentation and the intermediates 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporin(s) have served as starting materials for the production of 'semi-synthetic' β-lactams. Clavulanic acid is also produced by fermentation but it has not been possible to develop such methodology for the clinically used carbapenems. The development of synthetic methodology for carbapenem production is a significant achievement, but the requirement for total synthesis has probably hindered the development of new carbapenems.

Clavulanic acid is a potent inhibitor of Class A β-lactamases (penicillinases), but is much less potent against the Class C

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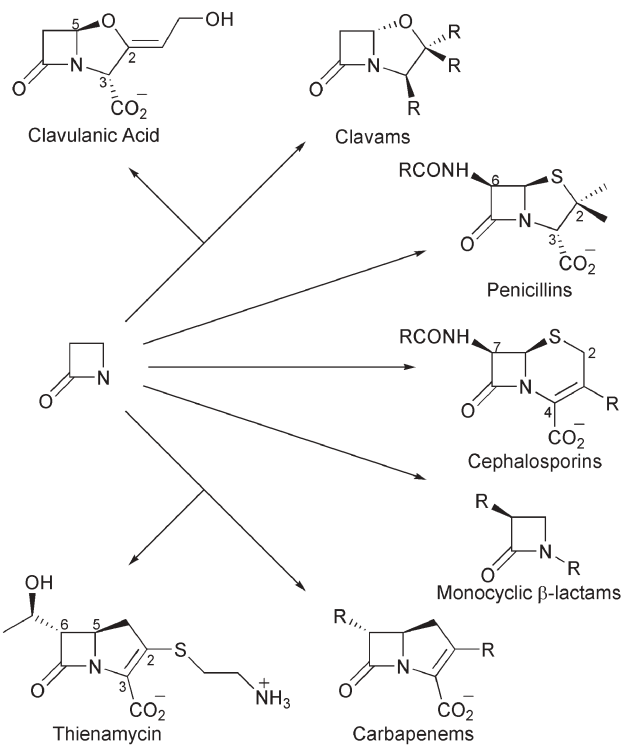

Fig. 1 The major families of clinically used β-lactams.

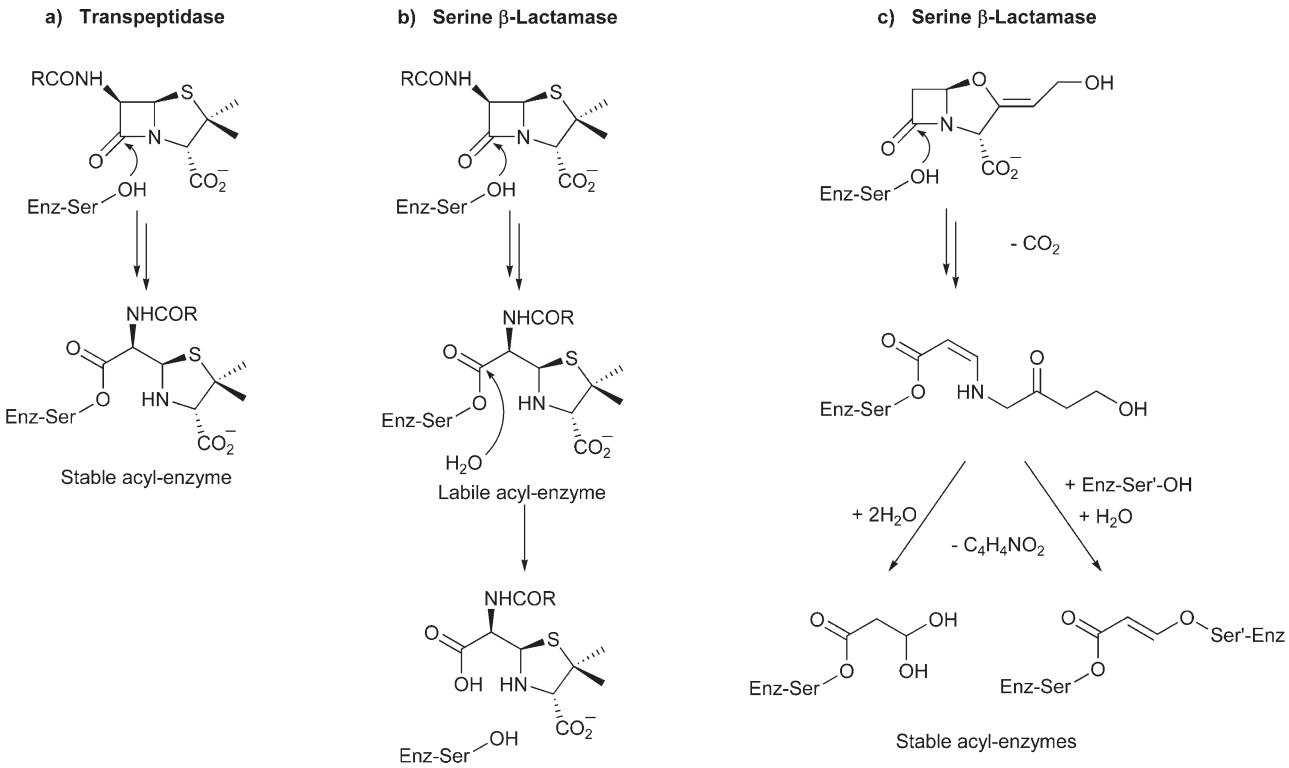

Fig. 2 (a) Inhibition of a cell-wall transpeptidase by a β-lactam antibiotic; (b) hydrolysis of a labile acyl-enzyme complex by a serine β-lactamase; (c) inhibition of a serine β-lactamase by clavulanic acid: formation of stable acyl-enzyme complexes.

β-lactamases (cephalosporinases). Although 6-substituted derivatives of clavulanic acid, and the structurally related oxapenems, (see *e.g.* reference 6) display activity against both the Class A and C β-lactamases, their development is hindered by lack of efficient production methodology. We have been studying enzymes involved in the biosynthesis of the bicyclic β-lactam nuclei of the clavam and carbapenem antibiotics with the aim of providing knowledge that might lead to new β-lactam antibiotics or enable the commercially viable production of otherwise inaccessible structures. In this article, comparisons are made between the enzymes involved in β-lactam production in the clavam and carbapenem pathways and those proposed for the other major structural families of β-lactam antibiotics, including the penicillins and cephalosporins. The article is not comprehensive in coverage of the pathways or individual enzymes, but rather seeks to introduce the reader to some of the remarkable reactions involved in β-lactam biosynthesis.

The biosynthetic pathways to β-lactams

From the perspective of the organic chemist, the pathway to the penicillin nucleus, which occurs in two enzyme-catalysed steps, is a paradigm of biosynthetic efficiency. Following ATP-dependent peptide synthetase mediated formation of an L,L,D-tripeptide (L-α-aminoadipoyl-L-cysteinyl-D-valine, ACV) from three L-amino acid precursors, isopenicillin N is formed in a single enzyme-catalysed step that utilises a molecule of dioxygen. This unique four-electron oxidative ring-closure, which provides the first-formed β-lactam intermediate in penicillin/cephalosporin biosynthesis, is catalysed by isopenicillin N synthase (IPNS), a non-haem, Fe(II)-dependent oxidase. The reaction proceeds *via* a monocyclic enzyme bound β-lactam intermediate, generated by N-4, C-5 bond formation, linked to a ferryl species (Fig. 3).[7–9] Two further steps catalysed by a third enzyme lead to medicinally useful antibiotics such as penicillin G (Fig. 4). The pathways leading

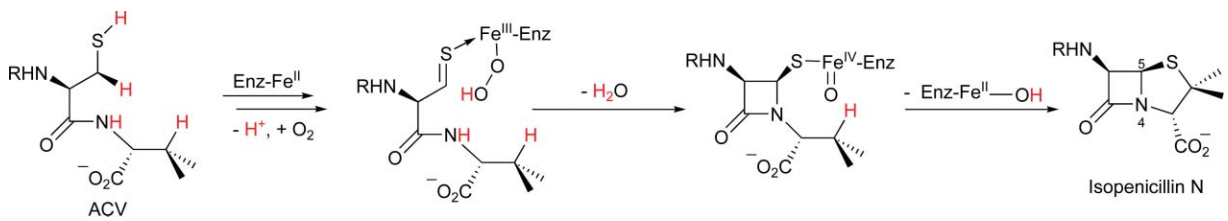

Fig. 3 Outline mechanism for isopenicillin N synthase showing the enzyme-bound monocyclic intermediate; R = L-δ-(α-aminoadipoyl).[7–9]

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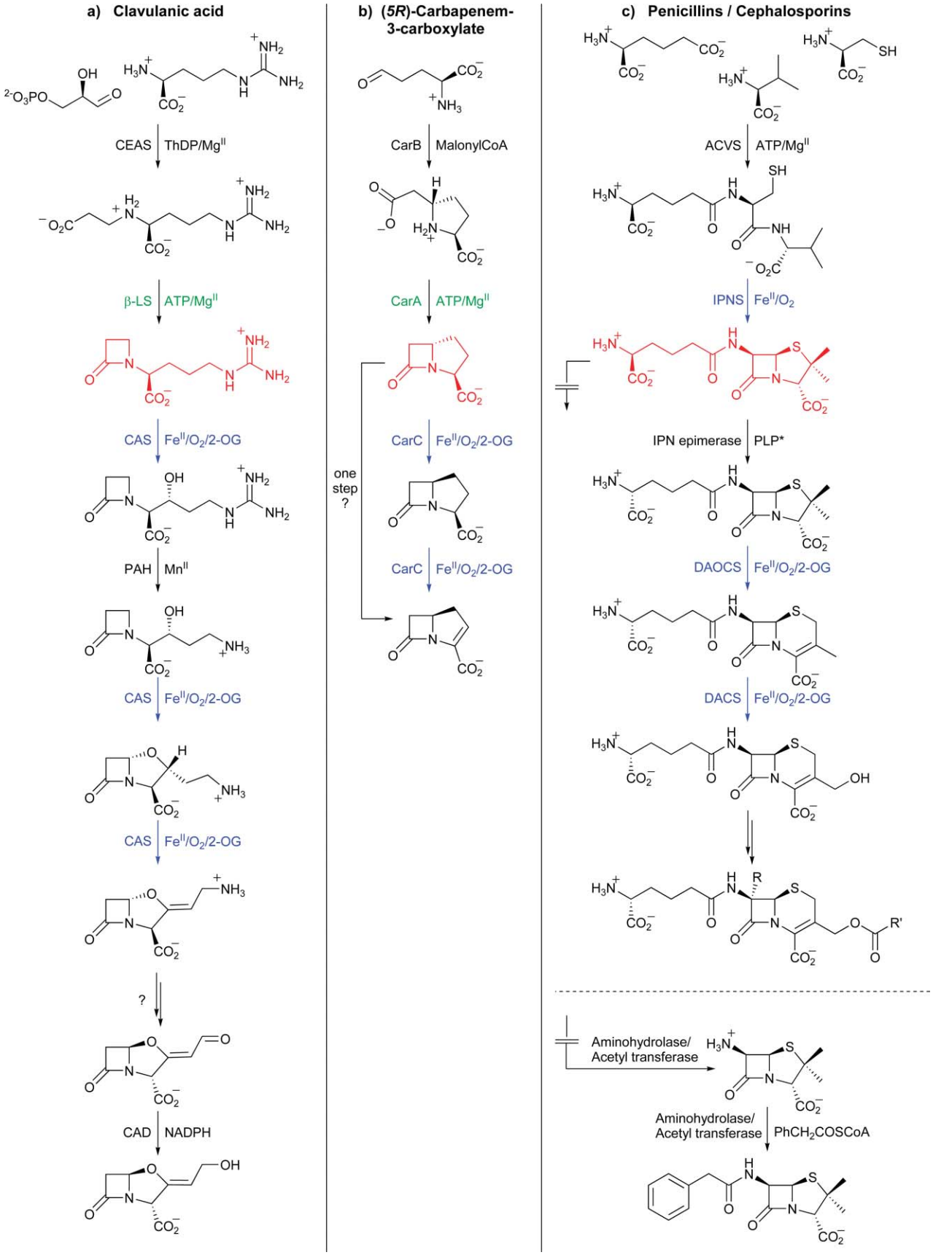

Fig. 4 Biosynthetic pathways to the bicyclic β-lactams: (a) clavulanic acid; (b) (5*R*)-carbapenem-3-carboxylate; (c) penicillins/cephalosporins, showing identified enzymes and their cofactors/co-substrates. *Pyridoxal phosphate (PLP)-dependent in bacteria; there is evidence that an alternative mechanism operates in fungi.[78,79]

to clavulanic acid, and some carbapenems, appear to be significantly longer and as a consequence present challenges in terms of investigation and manipulation, particularly in accessing potential intermediates. (See Fig. 4 for an overview of the pathways. For reviews on clavam and carbapenem biosynthesis see references 10–16.)

Formation of the β-amino acids

Unlike the mechanism of IPNS-mediated β-lactam formation in penicillin biosynthesis, which has little or no synthetic precedent, β-lactam formation in both the clavam and carbapenem pathways proceeds *via* cyclisation of a β-amino acid, one of the most commonly used reactions in the synthesis of β-lactams. The β-amino acid precursors of the clavams and carbapenems are produced by two very different types of enzyme, both distinct from the peptide synthetase of penicillin biosynthesis. Although the two enzymes belong to very different families, they may share a common mechanistic feature in carbon–nitrogen bond formation *via* a Michael reaction.

The first step in the biosynthesis of clavulanic acid, and possibly all clavams, is the condensation of D-glyceraldehyde-3-phosphate and L-arginine, catalysed by the unusual thiamine diphosphate (ThDP) dependent enzyme N^2-(2-carboxyethyl)-arginine synthase (CEAS).[17] Its three dimensional structure is similar to that of pyruvate decarboxylase, in possessing a tetrameric macromolecular arrangement (Fig. 5) and an active site at a dimer interface across which ThDP is bound in a characteristic 'V' conformation.[18]

CEAS is so far unique amongst the abundant ThDP-dependent enzymes in catalysing carbon–nitrogen bond formation (Fig. 6). In more typical reactions, addition of the C-2-thiazole ylid to a carbonyl group is followed by generation of a carbanion/enamine, *e.g.* by decarboxylation with pyruvate

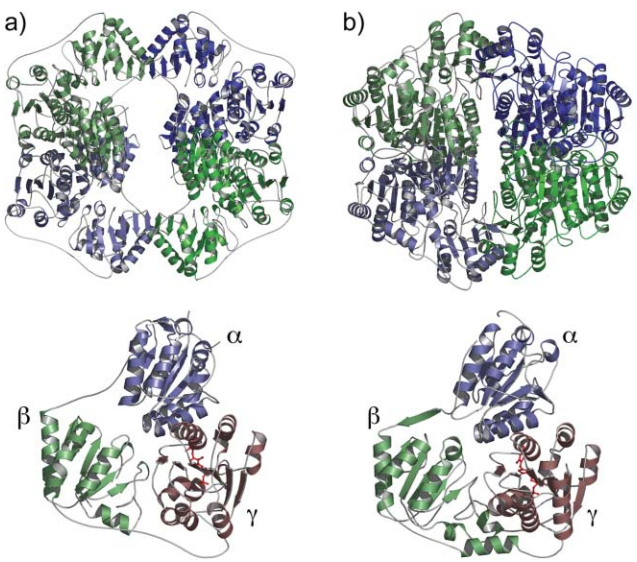

Fig. 5 Comparison between the quaternary and tertiary structures of: (a) pyruvate decarboxylase (PDB ID 1PVD);[86] (b) CEAS (PDB ID 1UPA).[18] The thiamine diphosphate cofactor is shown in red in the tertiary structures.

decarboxylase and acetohydroxyacid synthase.[19] Similarly for CEAS, the ThDP ylid is thought to react with the aldehyde of D-glyceraldehyde-3-phosphate, but beyond this point its mechanism must differ from those of other characterised ThDP-dependent enzymes. The CEAS mechanism is proposed to involve α-carbanion generation followed by elimination of β-hydroxyl and γ-phosphate groups, leading to a ThDP bound acryloyl group. Michael reaction of L-arginine followed by hydrolysis can then yield N^2-(2-carboxyethyl)arginine.[17] Crystallographic studies have suggested a central role for the 4′-amino group of the ThDP as a general acid/base at several stages of the reaction, including α-carbanion formation.[18]

The three-step biosynthetic pathway to the simplest carbapenem [(5*R*)-1-carbapen-2-em-3-carboxylate], in *Pectobacterium carotovora* proceeds *via* formation of (2*S*,5*S*)-carboxymethylproline, its cyclisation to give (3*S*,5*S*)-carbapenam-3-carboxylate and conversion of the latter to (5*R*)-carbapenem-3-carboxylate.[15,16] The first step is catalysed by (2*S*,5*S*)-carboxymethylproline synthase (CarB), a member of the crotonase family of enzymes, the mechanisms of which involve CoA ester derived enolates† (Fig. 7). CarB is unusual in that it produces a heterocycle (carboxymethylproline) *via* a C–C bond forming reaction with glutamate semi-aldehyde, catalyses a thioester hydrolysis and employs malonyl-CoA as a substrate.[20,21]

The observation that CarB catalyses the decarboxylation of malonyl-CoA in the absence of glutamate semi-aldehyde is consistent with a mechanism involving an initial decarboxylation to form an enzyme-bound enolate, as proposed for methylmalonyl-CoA decarboxylase.[22] However, in the CarB reaction instead of protonation, the enolate formed can react with glutamate semi-aldehyde/pyrroline-5-carboxylate to produce carboxymethylproline. The enolate may react with the imine form of pyrroline-5-carboxylate to give carboxymethylproline directly or *via* aldol reaction with glutamate semi-aldehyde to give, after loss of water, an alkene that can undergo intramolecular C–N bond-forming Michael reaction.[20,23]

Enzymes involved in β-lactam ring formation

In contrast to the biosynthesis of the β-amino acid precursors, two closely related enzymes catalyse β-lactam ring formation in the clavam and carbapenem pathways. Cyclisation of a β-amino acid precursor occurs in an ATP-dependent manner catalysed by β-lactam synthetase (β-LS/ORF3, clavam biosynthesis)[24–26] and (3*S*,5*S*)-carbapenam synthetase (CarA).[27] Both are related to asparagine synthetase type B (Asn-B)[28,29] which catalyses the synthesis of asparagine from glutamine and aspartate. Crystal structures have been reported for Asn-B,[28,29] β-LS[30,31] and CarA,[32] which together provide insights into the structure–function differences between the amide and lactam synthetases.

Asn-B comprises two domains: the N-terminal nucleophile (Ntn) hydrolase domain responsible for production of ammonia from glutamine and a C-terminal synthetase domain which catalyses production of β-aspartyl-AMP. Ammonia is proposed to travel along a tunnel linking the domains to react with the β-aspartyl-AMP to form asparagine (Fig. 8).[28,29]

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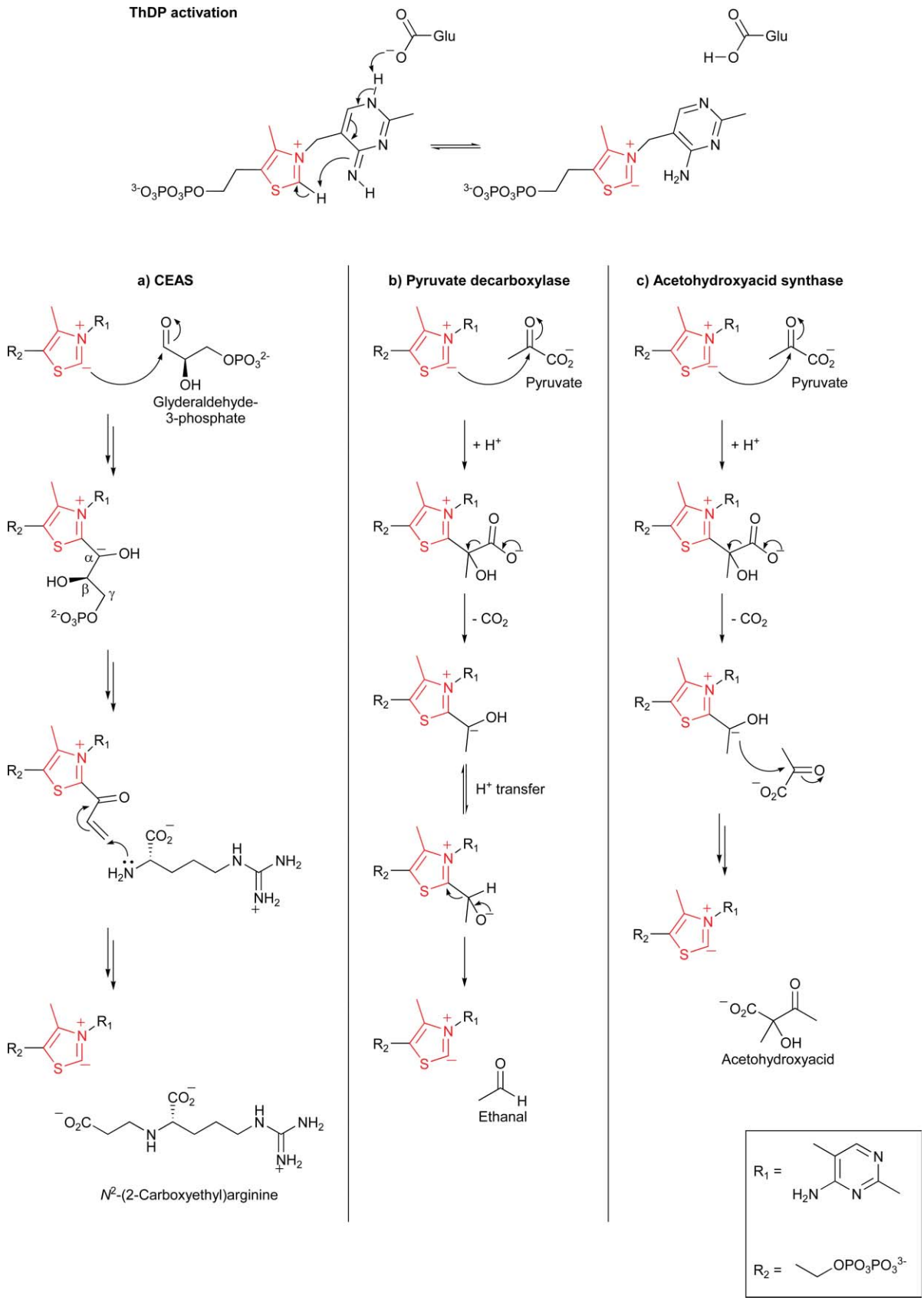

Fig. 6 Comparison between the proposed outline mechanisms for: (a) CEAS; (b) pyruvate decarboxylase; (c) acetohydroxyacid synthase. The mechanism of ThDP activation is also shown.

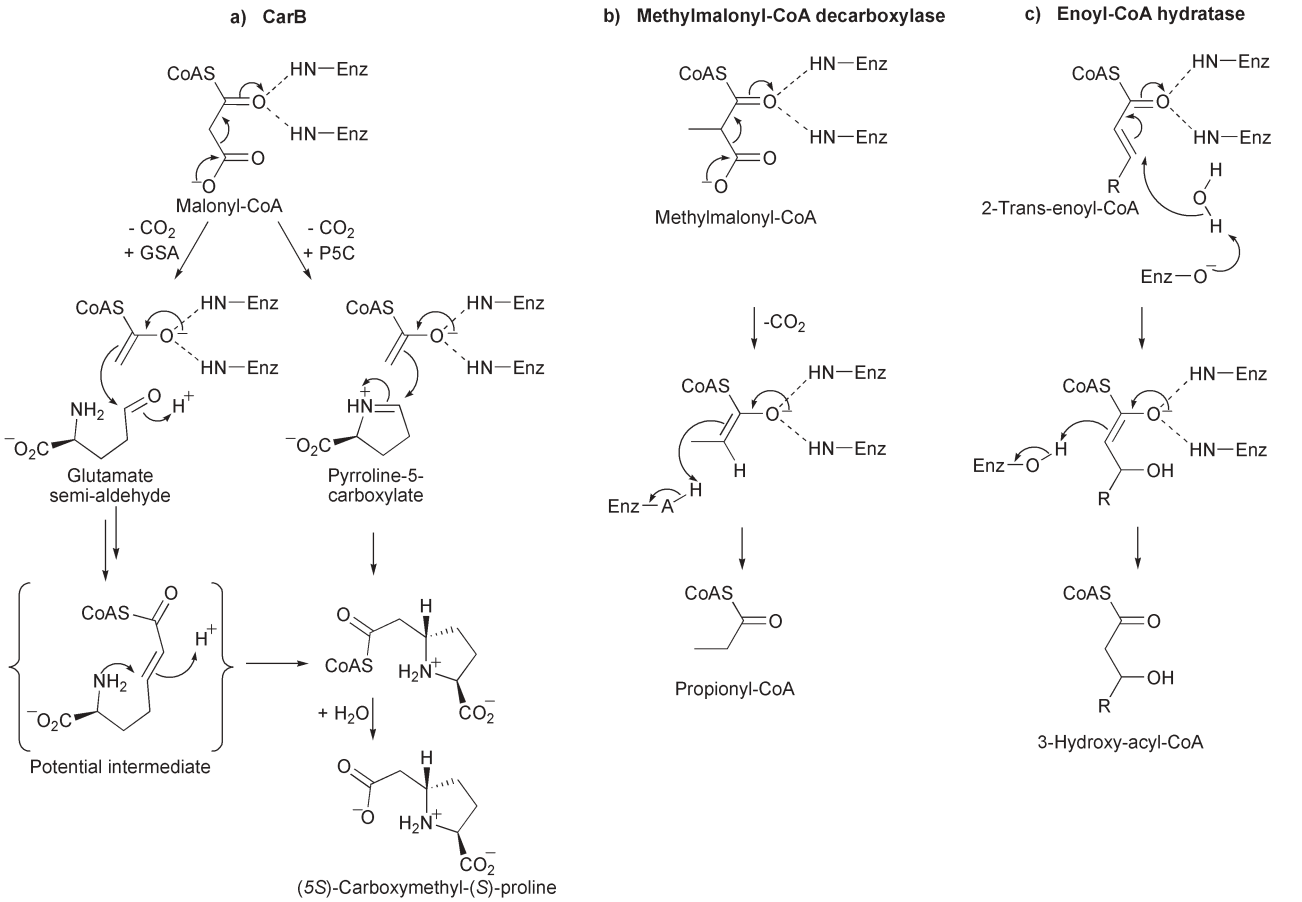

Fig. 7 Comparison between the proposed outline mechanisms for: (a) CarB; (b) methylmalonyl-CoA decarboxylase (AH = general acid); (c) enoyl-CoA hydratase.

Comparison of the β-lactam synthetases to Asn-B reveals that they have maintained the characteristic two domain fold.[30] Activation of a carboxyl as an acyl-adenylate followed by nucleophilic attack by nitrogen occurs in the C-terminal domain of all three enzymes (Fig. 9). However, as β-lactam formation is an intramolecular cyclisation it does not require the release of ammonia from glutamine, so the N-terminal domain of Asn-B may be expected to be mechanistically redundant in β-LS and CarA. Indeed, substitution of the N-terminal nucleophilic cysteinyl residue of Asn-B (which mediates hydrolysis of glutamine), with a glycine residue, together with its position in the operon, first alerted us to the possibility that *orf3* from the *S. clavuligerus* clavam biosynthesis cluster may encode a β-lactam synthetase.[26] The structure reveals that the position occupied by the N-terminal cysteine of Asn-B is replaced with a phenylalanine in β-LS and preceded by nine additional residues which occupy the corresponding glutamine binding pocket of Asn-B (Fig. 10).[30] In CarA, the cysteinyl residue is replaced with a potentially nucleophilic serinyl residue occupying a similar position, but other critical residues involved in glutamine binding are missing, which, along with some significant structural differences means that the glutamine binding pocket is effectively absent.[32] Hence the N-terminal domains in β-LS and CarA are not functional.

The precise mechanisms by which other families of natural β-lactams are produced are unclear. Labelling studies coupled with the recent sequencing of the biosynthetic gene cluster for Nocardicin A suggest that β-lactam formation is performed by an enzyme distinct from IPNS or the β-lactam synthetases. The biosynthesis of the nocardicins involves formation of a tripeptide precursor (Fig. 11), assembled by a non-ribosomal peptide synthetase.[33] It is proposed that β-lactam ring closure proceeds by an S_N2 type mechanism in which the amide nitrogen of the peptide precursor displaces an activated serinyl hydroxyl.[34] This could be carried out by the peptide synthetase itself or by other as-yet uncharacterised proteins.

The role of Fe(II)-dependent oxygenases and oxidases in β-lactam biosynthesis

In addition to IPNS, non-haem, Fe(II)-dependent oxygenases/oxidases belonging to the same structural family are involved in the pathways leading to the cephalosporins, clavams and carbapenems, where they catalyse synthetically challenging oxidative reactions including hydroxylation, desaturation, epimerisation and rearrangement. As well as Fe(II), these enzymes require 2-oxoglutarate (2-OG) as a cofactor, with the notable exception of IPNS[9] (and

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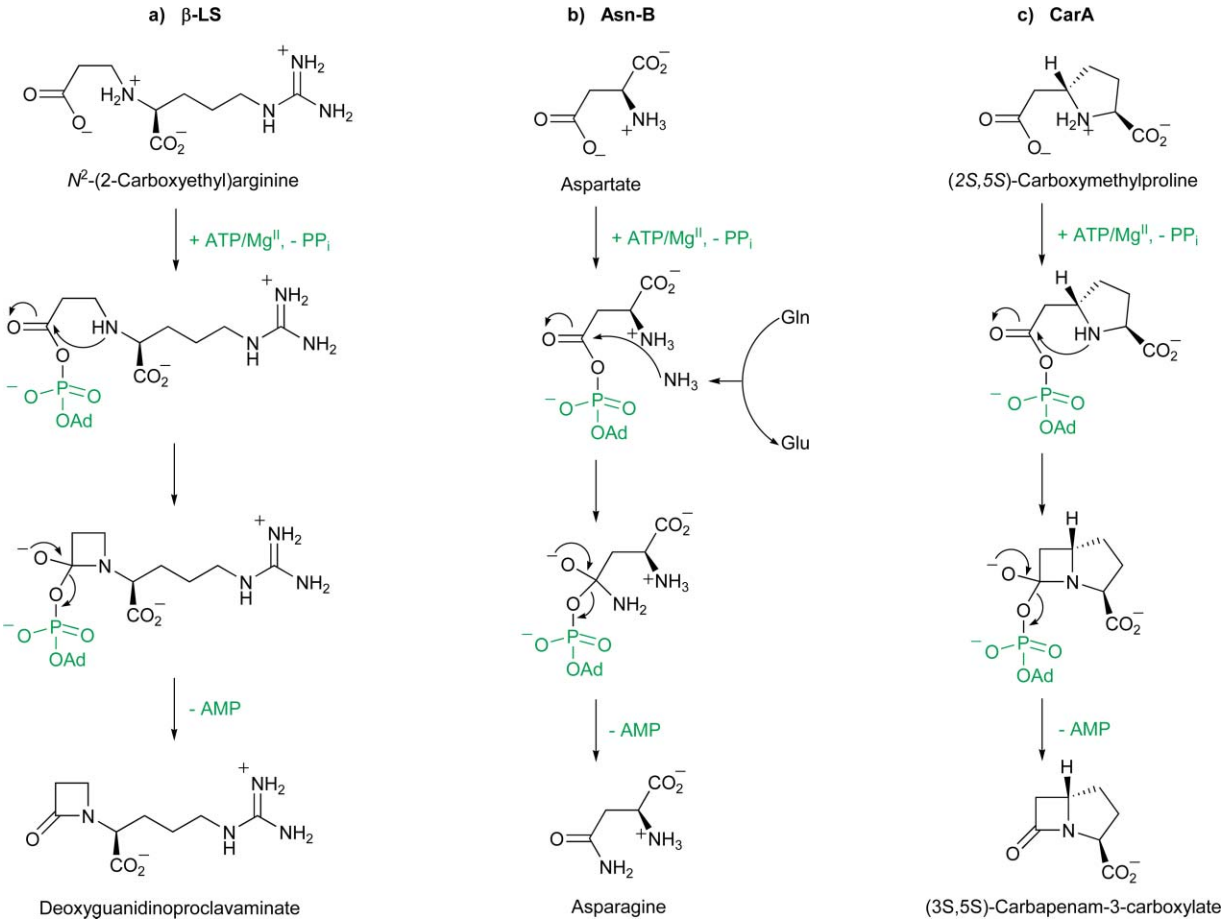

Fig. 8 Comparison between the proposed outline mechanisms for: (a) β-LS; (b) Asn-B; (c) CarA, showing the common mechanism of carboxylate activation.

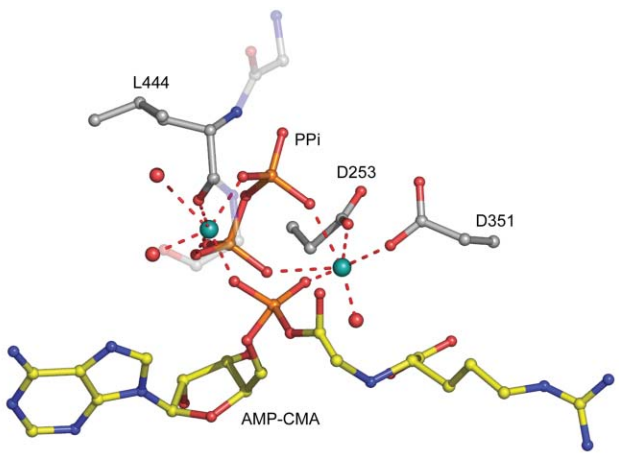

Fig. 9 View from the crystal structure of β-LS illustrating the AMP-N^2-(2-carboxymethyl)arginine (AMP-CMA) trapped product formed from the N^2-(2-carboxymethyl)arginine substrate analogue (PDB ID 1MBZ).[31] This acyl-adenylate is incapable of cyclising in a manner analogous to the true substrate (CEA) due to the shorter carbon chain which would require formation of a three-membered ring. Mg(II) is shown in green.

1-aminocyclopropane-1-carboxylic acid oxidase, the ethylene forming enzyme in plants[35,36]).

A prominent feature of the role of the 2-OG oxygenases in the β-lactam biosynthesis pathways is their ability to catalyse more than one reaction; notably within a pathway there is no duplication in the type of oxidation catalysed (Fig. 4a, b). In the clavam pathway CAS catalyses three different types of reaction;[37–41] a further feature of its role is that one of the reactions is separated from the other two by the action of another, mechanistically unrelated enzyme, proclavaminate amidino hydrolase (PAH) (Fig. 4a).[42–44] The first CAS-catalysed reaction is stereospecific hydroxylation of deoxyguanidinoproclavaminate to form a product (guanidinoproclavaminate) that is neither a substrate nor an (efficient) inhibitor of CAS.[45] The role of PAH is thus to modify ('mutate') the side chain of guanidinoproclavaminate from an amidino group to the amino group of proclavaminate in order to enable a further CAS-catalysed reaction. Secondly, CAS catalyses ring closure of proclavaminate to produce dihydroclavaminate (the first bicyclic intermediate),[46] and thirdly, desaturation of the latter to give clavaminic acid.

Carbapenem synthase (CarC) catalyses sequential reactions in the pathway from (3*S*,5*S*)-carbapenam-3-carboxylate to

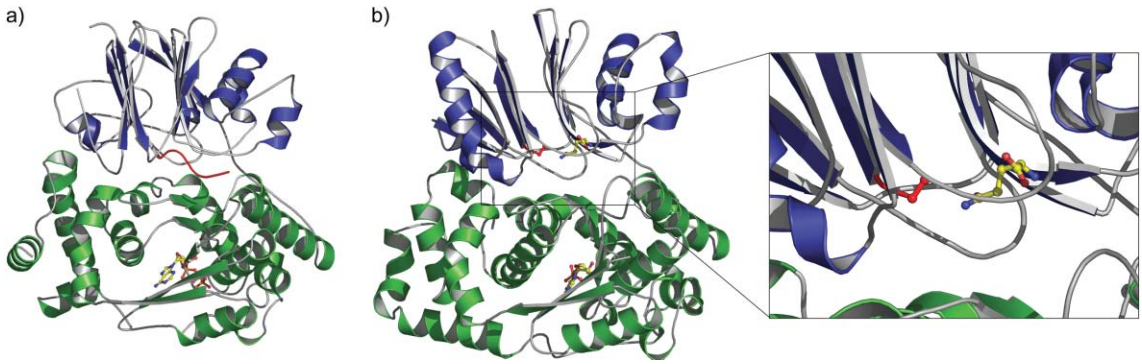

Fig. 10 Comparison between the structures of: (a) β-LS, with ATP shown in yellow (PDB ID 1MB9);[31] (b) Asn-B, with AMP and glutamine shown in yellow (PDB ID 1CT9),[28] illustrating the differences between N-terminal regions (highlighted in red). A close-up view highlighting the proximity of the N-terminal residue of Asn-B (the wild-type N-terminal cysteine has been mutated to alanine) and the glutamine substrate is shown.

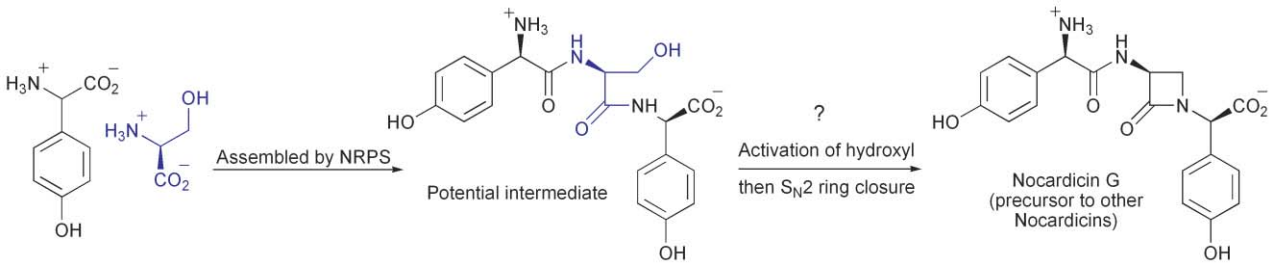

Fig. 11 Proposed pathway for β-lactam formation in nocardicin biosynthesis.[33]

(5*R*)-carbapenem-3-carboxylate in *P. carotovora*: a desaturation which resembles that catalysed by CAS, and an unusual epimerisation at the unactivated C-5 position.[47] The unprecedented nature of the epimerisation may have contributed to some confusion over the, now resolved,[48–50] stereochemical assignments of intermediates in carbapenem biosynthesis. *In vitro*, (3*S*,5*R*)-carbapenam-3-carboxylate can be observed as an intermediate, or shunt metabolite, providing evidence that epimerisation precedes desaturation.[47,51]

In some organisms the endpoint of the carbapenem pathway is the (5*R*)-carbapenem-3-carboxylate.[52] Due to its lack of functionality at C-2 and C-6, this carbapenem is not medicinally useful, but as its biosynthesis directly involves only three enzymes it serves as a useful model system for more complex carbapenems, *e.g.* thienamycin (Fig. 1). Comparison of the thienamycin and (5*R*)-carbapenam-3-carboxylate gene clusters,[53,54] together with labelling studies[55] implies that the first two steps in its biosynthesis involve analogous, if not identical reactions to those involved in the production of the (3*S*,5*S*)-carbapenam-3-carboxylate during biosynthesis of (5*R*)-carbapenem-3-carboxylate.[20] However, it is less clear if the CarC-mediated carbapenem forming step is conserved, possibly indicating C-2 or C-6 functionalisation at a relatively early stage in the pathway.

In cephalosporin/cephamycin biosynthesis, the 2-OG oxygenase DAOCS catalyses the ring expansion of the thiazolidine ring of the penicillins to the 6-membered dihydrothiazine ring of the cephalosporins. A related 2-OG oxygenase, deacetylcephalosporin synthase (DACS) hydroxylates this product. In some species these functions are performed by similar but distinct enzymes, whilst in others a single bifunctional DAOCS/DACS enzyme catalyses both reactions (for review see reference 14).

The first reported crystal structures of enzymes from the 2-OG oxygenase family were of IPNS[9] and DAOCS[56,57] and together with those for CAS and CarC[58] they reveal a common double-stranded β-helix (DSBH) core structure. Structures have been obtained in the presence of Fe(II), 2-OG and various substrates and intermediates and together with spectroscopic analyses,[59] reveal that the enzymes bind their iron cofactor *via* a conserved 2 His, 1 Glu/Asp motif located on the DSBH (Fig. 12). However there are significant variations in the binding site for the prime substrates, and to a lesser extent, for the 2-OG co-substrate. As well as providing mechanistic information, these structures have enabled mutagenesis studies aimed at altering the selectivity of the enzymes. For example, truncating the C-terminus of the bifunctional DAOCS/DACS can bias the selectivity of the enzyme towards substrates with hydrophobic side chains, *e.g.* penicillin G, so producing hydrophobic, and thus more readily extractable, cephalosporins.[60,61]

In the catalysis by 2-OG oxygenases, hydrogen abstractions are proposed to be mediated by an Fe(IV)=O ferryl species generated by reaction of dioxygen and 2-OG with the enzyme–Fe(II) complex (for reviews on mechanism see references 62–64). Evidence for the ferryl intermediate has been reported in the case of taurine dioxygenase.[65,66] Spectroscopic and crystallographic studies on a number of 2-OG oxygenases have

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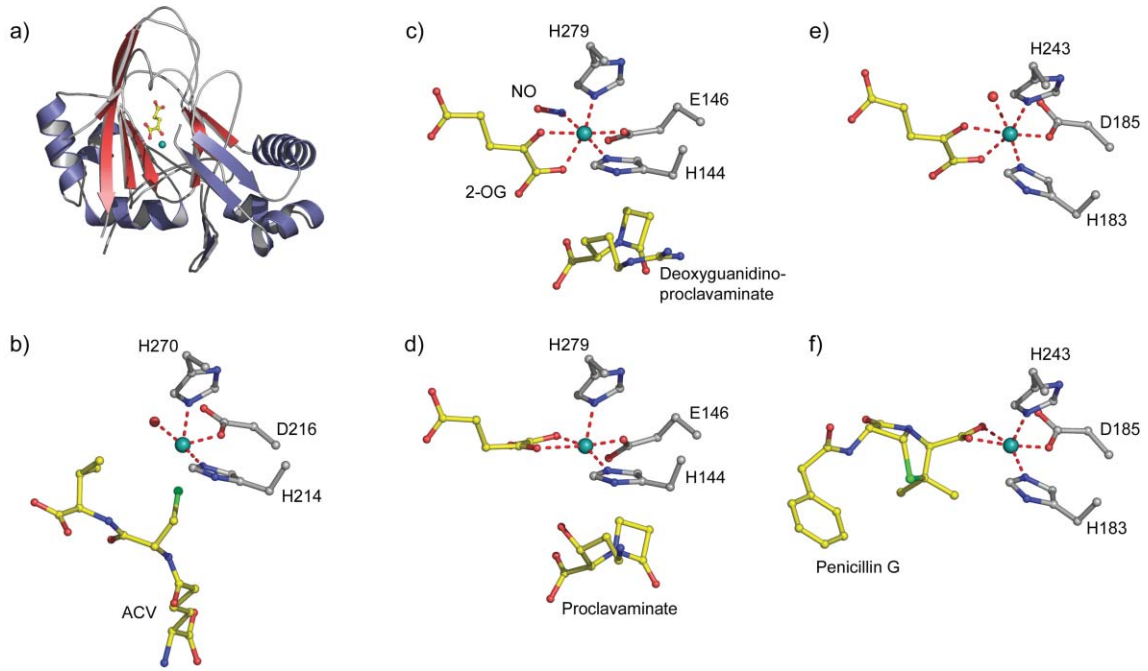

Fig. 12 Views from the crystal structures of Fe(II)-dependent oxidases/oxygenases. Fe is shown in green, conserved active site residues are shown in grey, substrates and 2-OG are shown in yellow. (a) CarC, the double-stranded β-helix is highlighted in red (PDB ID 1NX4); (b) IPNS active site with ACV (PDB ID 1BK0); (c) CAS active site with NO and substrate for hydroxylation (PDB ID 1GVG); (d) CAS active site with substrate for oxazolidine ring formation (PDB ID 1DRT); (e) DAOCS active site with 2-OG (PDB ID 1RXG); (f) DAOCS active site with penicillin G (PDB ID 1UOF).

indicated that the reaction proceeds *via* binding of 2-OG to the iron in a bidentate manner *via* its 1-carboxylate and 2-oxo groups (for review see reference 62). Binding of substrate appears to trigger the iron for binding of oxygen either by, or concomitant with, loss of a complexing water. Oxidative decarboxylation then generates the ferryl intermediate and carbon dioxide, and the enzyme is primed for catalysis. Conversely, recent crystal structures of DAOCS bound to a penicillin substrate suggest that it may follow an unusual mechanistic pathway; rather than formation of a ternary complex these studies suggest that succinate and carbon dioxide products are released from the enzyme prior to penicillin binding [compare (e) and (f), Fig. 12].[67]

In the case of 'simple' hydroxylations, such as those catalysed by CAS and DACS, a radical rebound mechanism, with initial abstraction of hydrogen by the ferryl followed by rapid hydroxyl transfer, appears most likely, although labelling studies infer that the lifetime of any radical intermediate is short (Fig. 13). For some reactions, *e.g.* the oxidative rearrangement of penicillin N to DAOC, a radical mechanism probably occurs (Fig. 14a).[68–70] Modelling studies also suggest that a radical mechanism occurs during the oxidative epimerisation of (3*S*,5*S*)-carbapenam to (3*S*,5*R*)-carbapenam,[71] which proceeds with loss of the C-5 hydrogen (Fig. 14b).[47] The lack of obvious general acid/base catalysts at the active site of DAOCS[57] and CarC[58] has led to

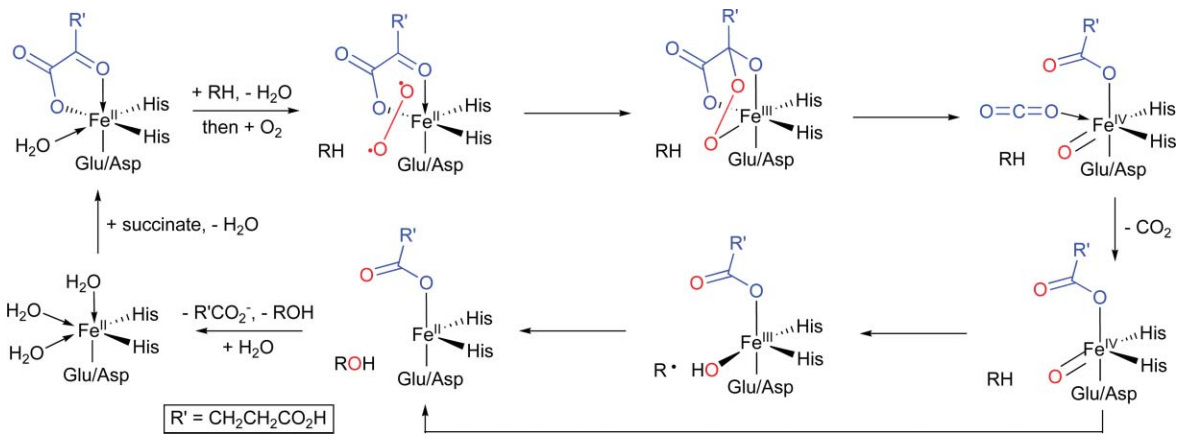

Fig. 13 Outline proposed mechanism for a typical hydroxylation reaction as catalysed by a 2-OG oxygenase.

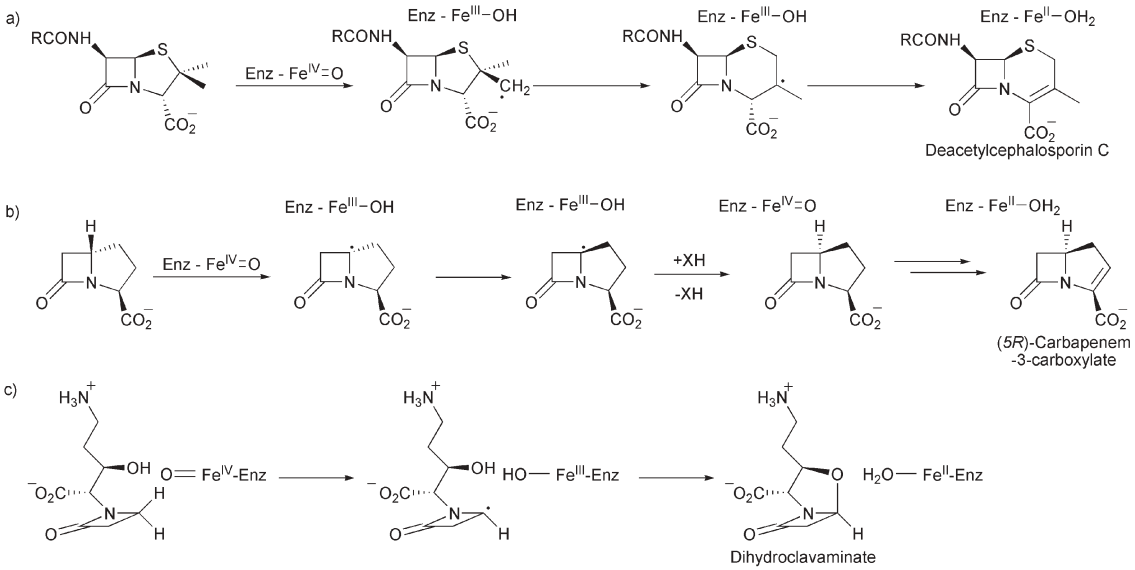

Fig. 14 Proposed outline mechanisms for: (a) the DAOCS catalysed penicillin ring expansion; (b) the CarC catalysed epimerisation; (c) the CAS catalysed ring closure.

the proposal that iron based oxygen-derived species mediate desaturations as well as the DAOCS catalysed rearrangement.

The involvement of 2-OG oxygenases in catalysing chemically challenging reactions in many biosynthetic pathways may not be coincidental. It has been proposed that the non-haem iron environment provides a more flexible environment both for catalysis and the evolution of new reactions than, for example, the haem based oxygenases/oxidases. One reason for this is that the haem enzymes are unable to use their iron cofactor to provide acid/base catalysis, or to coordinate the substrate, as once oxygen is bound it has no accessible vacant coordination sites. A penalty the 2-OG oxygenases may pay is that at least some of them are prone to oxidative damage.

One outcome of the studies on 2-OG oxygenases in β-lactam biosynthesis was the provision of knowledge that was used to identify 2-OG oxygenases involved in other biologically important systems. In addition to antibiotics, the 2-OG oxygenases are involved in many biosynthetic pathways including those leading to collagen, carnitine, plant metabolites (gibberellins, flavonoids) and the metabolism of fatty acids in humans (oxidation of the fatty acid side chain of chlorophyll). Recent studies have also identified roles for them in the repair of methylated DNA[72] and the hypoxic sensing mechanism in animals. In the latter case, bioinformatic searches informed by the structural data were combined with genetic inactivation studies to identify 2-OG oxygenases that regulate the activity of a transcription factor (hypoxia inducible factor) *via* post-translational hydroxylation.[73]

The introduction of structural diversity in β-lactam biosynthesis and the role of epimerisation

In each of the families of bicyclic β-lactams, sets of related compounds are produced by variations in a common biosynthetic pathway. Diversity in β-lactam biosynthesis is achieved both by branching from a common intermediate, and in a linear fashion by a range of reactions including oxidations, epimerisations, and rearrangements.

Branching from a common intermediate is exemplified by acylation of 6-aminopenicillanic acid for the penicillins and deacetylcephalosporin C for the cephalosporins.[14] In the case of the clavams, clavaminic acid acts as a branchpoint between the pathways leading to (3*R*,5*R*)-clavulanic acid and the (5*S*)-clavams, such as (5*S*)-clavam-2-carboxylate.[74] For the functionalised carbapenems the branchpoint(s) are less clear, but the range of bicyclic derivatives at the C-2 and C-6 positions implies that there is likely to be at least one branchpoint after formation of the bicyclic nucleus.[14,75]

A common feature of the β-lactam biosynthesis pathways is the introduction of structural diversity *via* epimerisation. The remarkable oxygenase-catalysed epimerisation that occurs in the biosynthesis of (5*R*)-carbapenem-3-carboxylate has been discussed above (Fig. 14b). During penicillin biosynthesis, the stereochemistry of the L-valine precursor is inverted in the biosynthesis of the L,L,D-ACV tripeptide. The precise mechanism of this epimerisation is uncertain, as for those catalysed by other peptide synthetases,[76] but probably occurs when the valine, or peptide intermediate, is linked to the enzyme as a thioester derivative.[77] An epimerisation also occurs in the conversion of isopenicillin N to penicillin N during penicillin/cephalosporin biosynthesis. In bacteria this reaction is catalysed by a pyridoxal phosphate dependent enzyme, isopenicillin N epimerase, and presumably proceeds *via* an imine intermediate as precedented for other amino acid epimerases/racemases;[78] there is evidence that an alternative mechanism operates in fungi.[79] A pyridoxal phosphate dependent epimerase in nocardicin biosynthesis also catalyses the conversion of the L-homoserinyl side-chain of isonocardicin A to the D-homoserinyl side-chain of nocardicin A.[80]

The clavulanic acid pathway begins with (2*S*)-arginine and proceeds in six steps catalysed by three enzymes to (3*S*,5*S*)-clavaminic acid (Fig. 4a).[10] However, clavaldehyde

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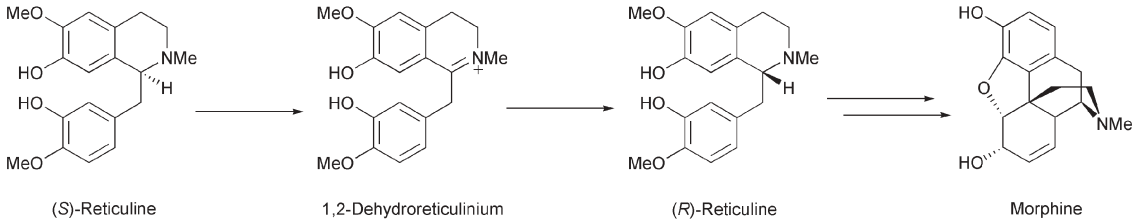

Fig. 15 Part of the morphine biosynthesis pathway in *Papaver somniferum*, detailing the stereochemical inversion step.[85]

the final intermediate, and clavulanic acid itself both have the (3*R*,5*R*)-stereochemistry which confers their β-lactamase inhibitory properties. (3*R*,5*R*)-Clavaldehyde is converted to clavulanic acid in an NADPH dependent reaction, but its (3*S*,5*S*)-enantiomer has not been observed.[81,82] The mechanism of the requisite double epimerisation involved in converting clavaminic acid to clavaldehyde is unknown. However in the oxidative deamination step, the aldehyde oxygen of clavaldehyde is derived from dioxygen,[83] potentially in a reaction mediated by the product of *orf10* of the gene cluster, which apparently encodes for a P450 oxygenase.[84]

Unusual epimerisations/inversions in secondary metabolism are not limited to the β-lactams but also occur for example in morphine biosynthesis in the opium poppy *Papaver somniferum*. Reticuline is synthesised as its (*S*)-enantiomer before being converted by an oxidation–reduction sequence into the (*R*)-enantiomer, a direct precursor of morphine. An iminium species is generated from (*S*)-reticuline, giving 1,2-dehydroreticulinium which is then stereospecifically reduced to (*S*)-reticuline by an NADPH-dependent reductase (Fig. 15).[85]

Modification of a core cyclic template by functionalisation, *e.g.* by amide bond formation, is a commonly used technique in synthesis for generating collections of compounds for pharmaceutical and other applications. However, transformations involving the type of unusual epimerisations and heterocyclic rearrangements exemplified in β-lactam biosynthesis are largely unexplored in synthesis as the reagents required to effect them have not yet been developed. One possibility is to use the biosynthetic enzymes, possibly in mutated form, in combination with an array of substrates. The ability of 2-OG oxygenases and related enzymes to accept alternative substrates, best exemplified by the range of heterocycles produced by IPNS when using tripeptide substrate analogues with alternatives to valine, presents them as candidates for a 'combinatorial' biosynthesis approach (see, *e.g.*, reference 7).

Conclusions and future prospects

Technical advances in molecular and structural biology have enabled many of the recent advances in our understanding of the β-lactam biosynthesis pathways and enzymes. As well as differences between the penicillin/cephalosporin, clavam and carbapenem pathways the results have revealed unexpected similarities, *e.g.* in the use of β-lactam synthetases in the clavam and carbapenem pathways and the common use of 2-OG oxygenases in the biosynthesis or modification of bicyclic β-lactams. These observations raise the possibility either that particular types of enzymes are suited to β-lactam biosynthesis and/or that (some of) the pathways have arisen from a common evolutionary origin(s).

The continuing role played by synthetic chemistry in biosynthetic studies should not be undervalued. Potential intermediates and mechanistic probes have to be synthesised, sometimes *via* non-trivial routes. The synthetic challenges presented are illustrated by clavulanic acid – for which despite its importance and small size (eight carbons, two chiral centres) there is no reported asymmetric synthesis. Although blocked mutants can provide access to intermediates, in the absence of appropriate resources this approach often does not provide large enough quantities for chemical analyses, especially with labile compounds such as the bicyclic β-lactams. Thus, it is important that the introduction of new techniques does not come at the expense of synthetic expertise.

Preferred projects for our group involve interesting chemistry and are of biological/medicinal importance. From our perspective, the challenges provided by clavam and carbapenem biosynthesis have met these criteria. Moreover, the study of reactions of interest from a chemical perspective has had unexpected applications in areas of biology, including transcriptional regulation and fatty acid metabolism, well beyond those we had envisaged at the onset of the work.

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Notes and references

† A difference between the (5*R*)-carbapenem-3-carboxylate pathway and that of the penicillins/cephalosporins, is in the substrate stereoselectivity of the enzymes involved in β-lactam formation. IPNS does not convert L,L,L-ACV to a penicillin.[77] In contrast, two of the enzymes of the carbapenem pathway display a notable lack of substrate stereoselectivity. CarC converts all four possible stereoisomers of carbapenam-3-carboxylate to carbapenem-3-carboxylate[51] and CarA converts at least three of the possible stereoisomers of carboxymethylproline to carbapenam-3-carboxylates[27] (without change in stereochemistry). CarB, however, only converts the L-enantiomer of glutamate semi-aldehyde to (2*S*,5*S*)-carboxymethylproline, and so may in part determine the stereochemical course of the pathway.[23]

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