The Journal of Antibiotics (2013) 66, 401–410

www.nature.com/ja

REVIEW ARTICLE

Origins of the β-lactam rings in natural products

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Naturally occurring β-lactam compounds fall into four basic structural groups, the penicillins/cephalosporins, the clavams, the carbapenems and the monocyclic β-lactams. Biosynthetic studies have clarified the steps involved in the formation of the β-lactam ring for the first three of these groups, but the corresponding process or processes for the monocyclic β-lactams remains obscure. Isopenicillin N synthase is responsible for formation of the β-lactam ring in all penicillin/cephalosporin compounds, and the reaction catalyzed is completely separate from that of β-lactam synthetase, the enzyme responsible for ring formation in all clavam compounds. Conversely, carbapenam synthetase, the enzyme responsible for β-lactam ring formation for all carbapenem compounds, shows clear relatedness to β-lactam synthetase, despite differences in the substrates and the products for the two enzymes. The mechanism of ring formation has not yet been clarified for any of the monocyclic β-lactams, but a third distinct mechanism of β-lactam ring formation seems likely, and this group includes such a diverse collection of structures that even more new ring-forming reactions may be involved.
The Journal of Antibiotics (2013) **66,** 401–410; doi:10.1038/ja.2013.24; published online 27 March 2013

Keywords: carbapenem; cephalosporin; monobactam; nocardicin; penicillin; tabtoxin

INTRODUCTION

The β-lactam class of compounds is arguably the most important family of microbial natural products ever applied to human medicine. With the discovery of penicillin early in the twentieth century, treatment of infectious diseases entered the modern era in which antibiotics control and eliminate infections that would otherwise be intractable. Even now when our reliance upon antibiotics is threatened by the ever-increasing threat of antibiotic resistance, the β-lactam class of compounds accounts for >50% of all antibiotic prescriptions.[1]

Although penicillin was the first β-lactam compound discovered, we now recognize a whole family of natural products, unified by their possession of the distinctive four-membered β-lactam ring. Biosynthetic studies have shown that the β-lactam class of natural products can be divided into at least four different subgroups based on the origin of the β-lactam ring. These groups will be referred to as the penicillin/cephalosporin, the clavam, the carbapenem and the monocyclic β-lactam compounds. Representative structures encompassed by these groupings are shown in Figure 1. Excellent reviews have appeared over the years dealing with the biosynthesis of β-lactam compounds in general,[2,3] or with specific groups of β-lactam compounds in particular,[4–7] and hence this review will focus instead on the reactions involved in the formation of the β-lactam ring in this diverse group of compounds.

THE PENICILLIN/CEPHALOSPORIN GROUP

Penicillin was first observed as an inhibitory activity resulting from growth of the filamentous fungus, *Penicillium notatum*, found as a chance contaminant on a plate culture of *Staphylococcus sp.*, and astutely recognized by Fleming as a prospect worth pursuing.[8,9] Although industrial penicillin production, to this day, is still carried out using fermentations of *Penicillium* fungi, a longer list of producer species has since been identified[10] (Table 1). Species that produce hydrophobic penicillins are all members of the filamentous fungi, but it is now recognized that penicillin compounds are also obligatory intermediates in the biosynthesis of cephalosporin-type compounds. On this basis, a much longer list of species with the capability to produce penicillins can be inferred, and these cross from the eukaryotic to the prokaryotic domains of living organisms.

Like penicillin, the first cephalosporin antibiotic, cephalosporin C, was also discovered by accident, as a trace contaminant in penicillin N preparations from the filamentous fungus, *Acremonium chrysogenum*.[8] More recently, a larger group of species, both eukaryotic and prokaryotic, that produce cephalosporin-type antibiotics with various substituent groups has been identified.[11,12] In the case of the prokaryotic producer organisms, cephamycin-type antibiotics have been identified from a range of *Streptomyces* spp. and other actinomycetes,[13,14] whereas cephabacin-type compounds are found in *Flavobacterium,* Xanthomonas and *Lysobacter* spp.[15,16] Most recently, with the growing availability of genome sequence information for a wide range of organisms, additional producer species have been identified based solely on their possession of characteristic gene sequences, even though actual metabolite production may not yet have been demonstrated. Such presumptive producer species are identified in Table 1 with asterisks. The surprising finding that

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Received 3 January 2013; revised 26 February 2013; accepted 5 March 2013; published online 27 March 2013

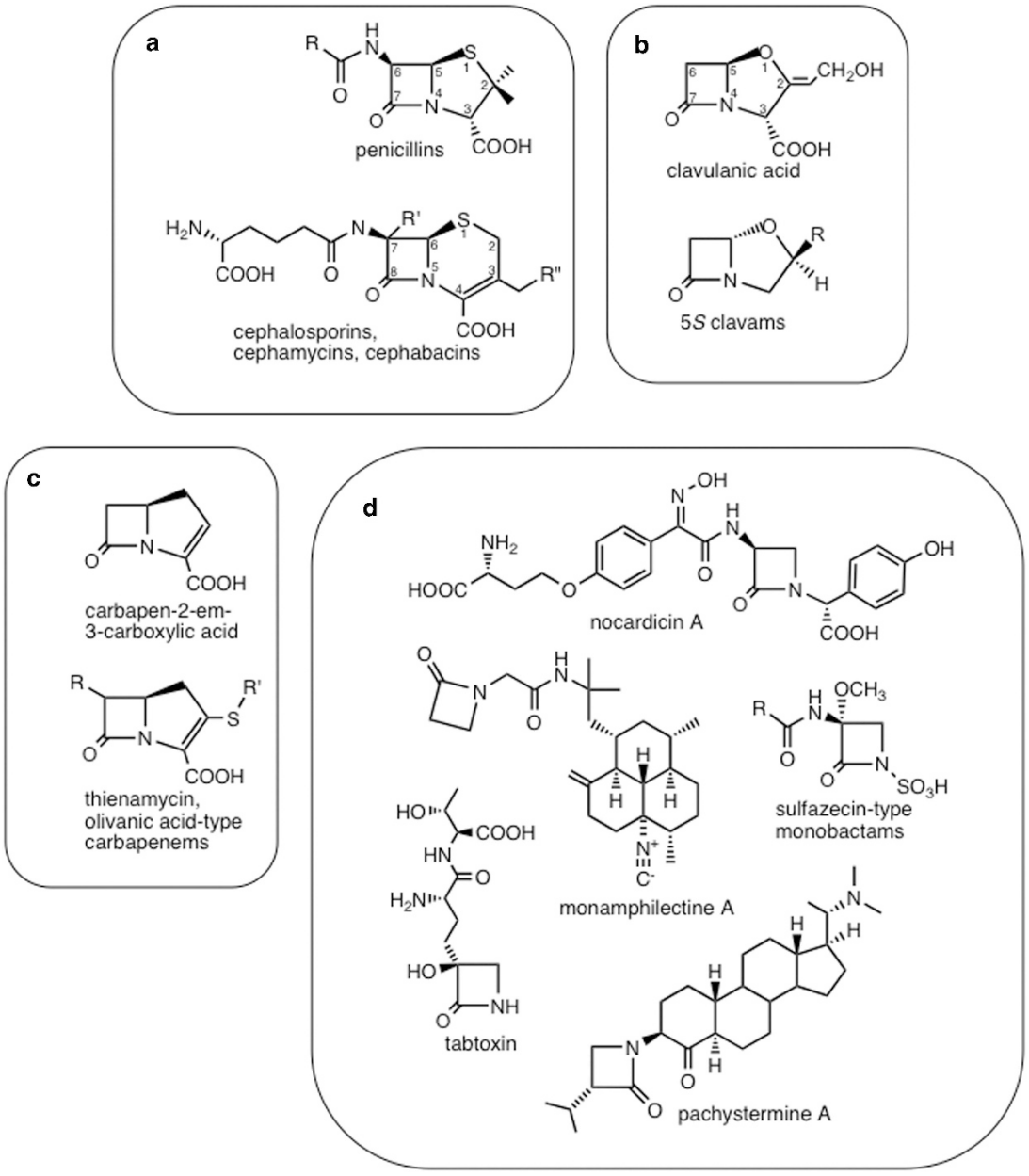

Figure 1 Subgroups of β-lactam compounds. (**a**) Penicillins/cephalosporins; (**b**) clavams; (**c**) carbapenems; and (**d**) monocyclic β-lactams.

penicillin/cephalosporin producer species come from both the eukaryotic and prokaryotic domains of life, along with other unusual features such as the restricted distribution of producing ability to only certain strains within a species, the lack of introns in eukaryotic genes and the anomalous %G + C content of some antibiotic gene clusters, is taken as evidence that the antibiotic-producing ability has crossed evolutionary lines of descent by the process of horizontal gene transfer.[17–20]

Through intensive screening programs using supersensitive indicator strains and β-lactamase induction assays, additional groups of β-lactam compounds, the clavams, carbapenems and monocyclic β-lactams, were discovered in the 1970s and 1980s, and the full range of structural diversity of β-lactam metabolites became apparent.[8] Initially, at least some consideration was given to the possibility that the β-lactam ring structure common to all of these groups might arise from a single β-lactam ring-forming reaction or group of reactions, but that notion was dispelled as the details of penicillin/cephalosporin biosynthesis began to emerge. A single β-lactam ring-forming reaction does give rise to the β-lactam ring in both the penicillins and the cephalosporins, but only because cephalosporins arise from penicillin precursors. Furthermore, the specific characteristics of the ring-forming reaction made it evident that other β-lactam metabolites must result from completely separate synthetic systems.[21,22]

Formation of the β-lactam ring in the penicillin/cephalosporin group of compounds is accomplished by the enzyme isopenicillin N synthase (IPNS). The reaction catalyzed is impressive in its complexity given that IPNS is a relatively small protein (MW ~33 000 Da) that functions in monomeric form.[20,22–29] IPNS belongs to the non-heme iron (II)-dependent oxygenase/oxidase class of enzymes and

Table 1 β-Lactam synthesizing enzymes

Producer species	β-lactam-forming enzyme	Reference or accession number
Penicillins/cephalosporins		
Penicillins		
Penicillium chrysogenum	IPNS	XP_002569113
Aspergillus nidulans		XP_660226
Aspergillus oryzae		XP_001825448
*Trichophyton tonsurans**		EGD99914
*Trichophyton rubrum**		XP_003231418
*Trichophyton verrucosum**		XP_003025275
*Arthroderma benhamiae**		XP_003011671
Epidermophyton	ND	10
Polypaecilum		
Malbranchea		
Aspergillus fumigatus (Sartorya)		
Pleurophomopsis		
Cephalosporins		
Acremonium chrysogenum	IPNS	AAA32674
*Kallichroma tethys**		AAK21903
Xanthothecium (Anixiopsis)	ND	11,12
Arachnomyces		
Spiroidium		
Scopulariopsis		
Diheterospora (Verticillium)		
Cephamycins		
Streptomyces clavuligerus	IPNS	P10621
Streptomyces jumonjinensis		P18286
Streptomyces microflavus (lipmanii)		M22081
Streptomyces cattleya		YP_004915173
Streptomyces griseus		CAA38431
Amycolatopsis (Nocardia) lactamdurans		P27744
*Streptomyces fimbriatus**		AAK11177
*Streptomyces hygroscopicus**		BAB13300
*Streptomyces panayensis**		BAB13299
*Streptomyces viridochromogenes**		BAB13298
*Streptomyces heteromorphus**		BAB13301
*Streptomyces wadayamensis**		AAG31812
*Streptomyces sulfonofaciens**		AAD30553
Cephabacins		
Flavobacterium sp.	IPNS	P16020
Lysobacter lactamgenus		Q48739
Xanthomonas lactamgena		85
Clavams		
Clavulanic acid		
Streptomyces clavuligerus	Bls2	AAC31901
*Streptomyces flavogriseus**	Bls	YP_004921538
*Saccharomonospora viridis**		YP_003135129
*Anoxybacillus flavithermus***		YP_002314584
Streptomyces jumonjinensis	ND	43
Streptomyces katsurahamanus		
5S Clavams		
Streptomyces clavuligerus	Bls1	AAR05435
Streptomyces antibioticus	Bls3	AFH74298
Streptomyces hygroscopicus	ND	86,87
Streptomyces microflavus (lipmanii)		

Table 1 (Continued)

Producer species	β-lactam-forming enzyme	Reference or accession number
Carbapenems		
Carbapenem-3-carboxylic acid		
Pectobacterium carotovorum	Cps	AAD38229
Photorhabdus luminescens		NP_927548
Dickeya zeae		YP_003002585
Pantoea sp.		YP_004118030
*Pelosinus fermentans***		ZP_155372990
Serratia sp.	ND	88
Thienamycin/olivanic acid-type		
Streptomyces cattleya	Cps	CAD18981
Streptomyces flavogriseus		YP_004921141
Streptomyces olivaceus	ND	58
Monocyclic β-lactams		
Sulfazecin-type monobactams		
Gluconobacter spp.	ND	68,89–91
Acetobacter spp.		
Pseudomonas acidophila		
Pseudomonas mesoacidophila		
Chromobacterium violaceum		
Rhizobium radiobacter		
Tabtoxin-type		
Pseudomonas syringae	TblS	AAM77668
Streptomyces sp.	ND	75
Nocardicin A		
Nocardia uniformis	ND	67,80
Actinosynnema mirum		
Nocardiopsis atra		
Microtetraspora caesia		
Pachystermine		
Pachysandra terminalis	ND	92
Monamphilectine		
Hymeniacidon sp.	ND	71

Abbreviations: Bls, β-lactam synthetase; Cps, carbapenam synthetase; IPNS, isopenicillin N synthase; ND, not determined; TblS, tabtoxin β-lactam synthetase.
Enzymes designated as ND represent cases where production of a β-lactam metabolite has been reported in the literature, but the β-lactam ring-forming enzyme has not been identified genetically or biochemically.
*Predicted based on sequence deposit.
**Partial cluster only, suggests no β-lactam product could be formed.

catalyzes the cyclization of the linear tripeptide, δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (ACV), into isopencillin N (Figure 2a). This class of enzymes uses molecular oxygen as cosubstrate, but IPNS is unusual because other members of this class typically also require 2-oxoglutarate as a co-substrate whereas IPNS does not. In the course of the IPNS reaction, four protons are extracted from ACV and combined with the two oxygen atoms from molecular oxygen to give two molecules of water. Neither of the oxygen atoms from molecular oxygen persists in the final product, which is another unusual feature of IPNS that distinguishes it from most others in its class.

The IPNS group of enzymes was also found to form an unprecedented structural family. Based on X-ray crystallographic

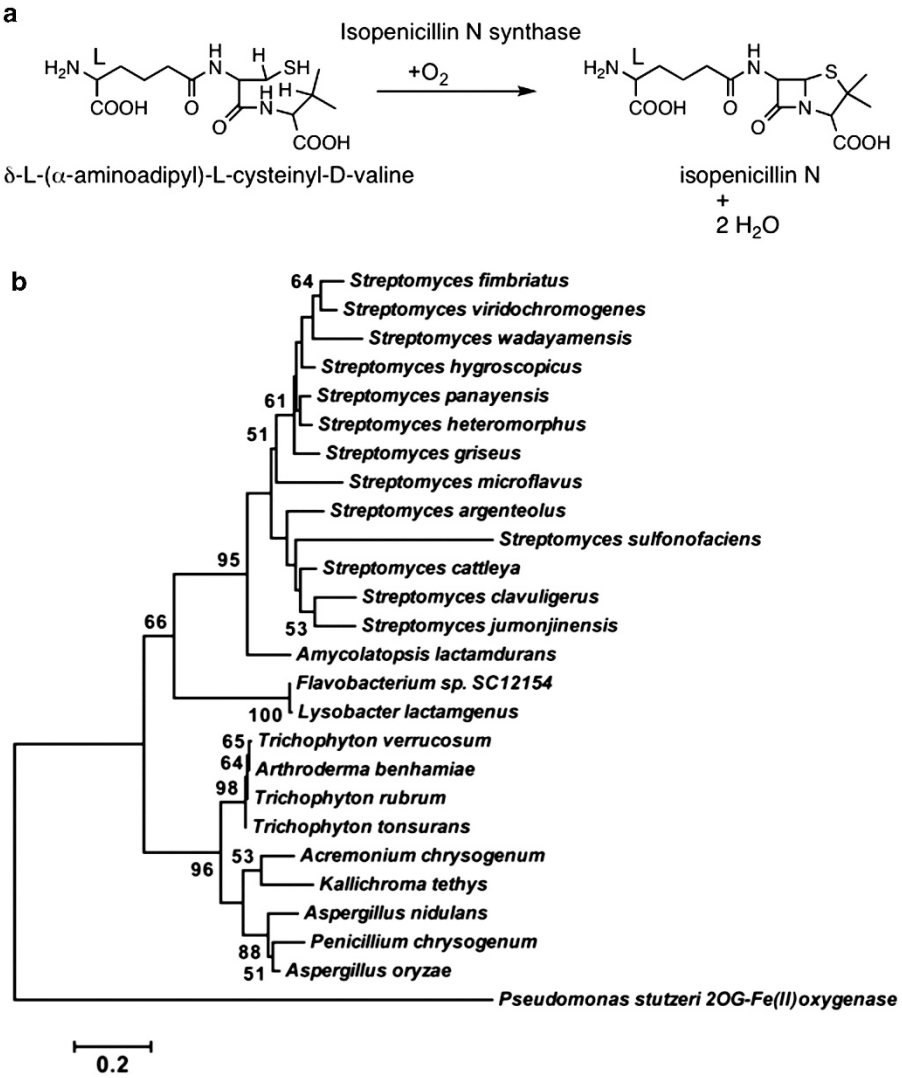

Figure 2 Isopenicillin N synthase. (**a**) The isopenicillin N synthase (IPNS) reaction. (**b**) Phylogenetic relationships between IPNS from different organisms based on protein maximum likelihood method using the Jones–Taylor–Thornton matrix and a constant amino-acid substitution rate.[30] Accession numbers for the IPNS sequences used are listed in Table 1. The sequence for a putative non-heme iron (II)-dependent oxygenase from *Pseudomonas stutzeri* (accession number: YP_001173562) was included as an out-group. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (at least 50% in 1000 replicates) are shown next to the branches.[31] The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Multiple sequence alignments were conducted using ClustalW. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using a Jones–Taylor–Thornton model, and then selecting the topology with superior log likelihood value. All analyses were conducted using the MEGA5 software.[32]

structural studies of *Aspergillus nidulans*, IPNS crystallized with natural and unnatural substrates, the enzyme was shown to contain a single iron atom bound into the active site through coordination with His 214, Asp 216, His 270 and Gln330 residues as well as two water molecules.[22,24] Of these residues, Gln330 is distinctively conserved in all IPNS enzymes and serves to distinguish them from other enzymes of this family.[22,24] The IPNS reaction is predicted to initiate with binding of ACV via its thiol group to the iron atom, displacing Gln330 and one water molecule. This binding allows molecular oxygen to bind to the other face of the iron atom, displacing the second water molecule. The reaction then proceeds through an initial step in which the β-lactam ring is formed with removal of two protons to yield a monocyclic β-lactam intermediate that remains covalently attached to the active site of IPNS. Subsequent formation of isopenicillin N involves the participation of a ferryl intermediate that drives the removal of the second pair of protons and closure of the thiazolidine ring.[33] All of the various hydrophobic penicillin, cephalosporin, cephamycin and cephabacin end products found in nature arise from isopenicillin N through subsequent modifications of this basic penicillin intermediate. The biosynthesis of β-lactam-type compounds in general is remarkable for the involvement of non-heme iron-dependent oxygenase/oxidases in various catalytic steps in the biosynthesis of penicillin/cephalosporins, clavams, carbapenems, and tabtoxin, but IPNS is the sole enzyme of this type responsible for the formation of a β-lactam ring. Conversely, formation of the β-lactam ring in all other groups of β-lactam metabolites occurs by mechanisms that do not involve non-heme iron-dependent oxygenase/oxidase-type enzymes.

When the amino-acid sequences of known and predicted IPNS enzymes are used to group them based on similarities, they roughly

form two clusters (Figure 2b). The first cluster contains enzymes from Gram-positive and Gram-negative bacteria, whereas the second cluster contains those of fungal origin. Within the bacterial cluster, the enzymes from *Streptomyces* spp. form their own large subcluster, consistent with the hypothesis that the IPNS-based mechanism involved in the formation of the β-ring originated in the actinomycetes before being disseminated to other producer organisms.[34] Only partial sequences are available for some of the genes shown in Figure 2b, but enough to categorize them as IPNS. However, closer examination of the predicted protein from *Streptomyces sulfonofaciens* has shown an Asp216Gly substitution (based on the numbering scheme of the *A. nidulans* protein). Therefore, if focused sequence analysis of this species confirms the Asp216Gly substitution, it will be interesting to determine if the IPNS protein from *S. sulfonofaciens* behaves like previously characterized IPNS enzymes or if it has markedly different characteristics.

THE CLAVAM GROUP

Following the precedent set by the penicillins and cephalosporins, the discovery of clavulanic acid provides another example of the importance of serendipity in natural product discovery. *Streptomyces clavuligerus,* the clavulanic acid producer species, was first isolated in a screen for producers of β-lactamase-resistant β-lactam metabolites.[35] Clavulanic acid is well known and widely used for its β-lactamase inhibitory properties, but it was the production of cephamycin C, a moderately β-lactamase-resistant cephalosporin-type metabolite, that led to the initial isolation of *S. clavuligerus*. The discovery of clavulanic acid only came later when *S. clavuligerus* was included as a known cephamycin-producing control species in a new screen for β-lactamase inhibitor compounds.[36] The anomalous behavior of *S. clavuligerus* as compared with other known cephamycin producers in this screen led to the eventual discovery of clavulanic acid.

Clavulanic acid has no useful antibiotic activity in its own right, but it inhibits serine β-lactamases by a mechanism akin to that seen in the inhibition of cell wall biosynthetic transpeptidases by β-lactam antibiotics. The β-lactam antibiotics are substrate analogs of the terminal D-alanyl-D-alanine residues in the substituent peptide substrates of peptidoglycan transpeptidases. They covalently acylate the active site serine residues of transpeptidases to inactivate the enzymes.[37,38] The architecture of the active site region in these enzymes is such that water molecules cannot access the acyl intermediate, and hence the enzymes are irreversibly inactivated. β-Lactamases are evolutionarily related to transpeptidases, and hence they bind β-lactam antibiotics in much the same way. However in the case of β-lactamases, water molecules can penetrate the active site region to hydrolyze the acyl intermediate, releasing active enzyme and hydrolyzed inactive antibiotic.[39,40] Clavulanic acid also binds and acylates the active site of β-lactamases, but then the fate of the clavulanic acid acylated β-lactamase differs depending on the particular enzyme involved. For sensitive β-lactamases, the clavulanic acid molecule rearranges and may fragment and covalently attach to other residues in the active site region to leave the β-lactamase irreversibly inactivated.[41,42] For this reason, when used in combination with otherwise susceptible β-lactam antibiotics, clavulanic acid restores their effectiveness for treatment of diseases caused by β-lactamase-producing organisms.

Since the initial isolation of *S. clavuligerus*, a handful of other clavulanic acid-producing species have been identified, all *Streptomyces* spp.[43] Furthermore, *S. clavuligerus* itself has been found to produce at least four other clavam metabolites, which differ from clavulanic acid in having 5*S* stereochemistry (the 5*S* clavams) compared with the 5*R* configuration of clavulanic acid[44,45] (see Figure 1 for numbering), and still other *Streptomyces* spp. have been identified that produce only 5*S* clavams.[43] Despite their structural similarity to clavulanic acid, none of the 5*S* clavam metabolites has β-lactamase inhibitory activity. Instead, they function as antimetabolites of methionine biosynthesis in prokaryotes, and some also inhibit eukaryotes, but by an unknown mechanism, unrelated to methionine biosynthesis.[46]

Like the penicillin/cephalosporin antibiotics, clavulanic acid and the 5*S* clavams share a single β-lactam ring-forming reaction, and clavulanic acid and the 5*S* clavams are alternative end products of a biosynthetic pathway that branches after this step.[47,48] The β-lactam ring-forming reaction in clavam biosynthesis is catalyzed by the enzyme β-lactam synthetase (Bls), and the details of the reaction are completely distinct from those of IPNS[49–51] (Figure 3a). The Bls reaction begins with activation of the substrate, carboxyethylarginine, by aminoadenylation using ATP, followed by closure of the β-lactam ring to give the monocyclic intermediate, deoxyguanidinoproclavaminic acid, with concomitant release of pyrophosphate (PPi) and AMP.[52–54] Therefore, the closure of the clavam β-lactam ring occurs by amide bond formation and is essentially a reversal of the β-lactam cleavage reaction caused by β-lactamases.[51] This reaction is very different from β-lactam ring formation by IPNS, where the β-lactam amide linkage is preexisting in the ACV substrate. In clavam β-lactam biosynthesis, the energetic cost of β-lactam ring formation is paid by ATP hydrolysis, and unlike IPNS, the monocyclic β-lactam intermediate formed by Bls is released from the enzyme. Closure of the fused oxazolidine ring occurs in a subsequent step catalyzed by a separate enzyme. However, it is worth noting that the enzyme responsible for the eventual closure of the oxazolidine ring, clavaminate synthase, is a non-heme iron (II)-dependent oxygenase with some biochemical similarities to IPNS, but with no ability to form β-lactam rings.

Bls is an example of an ATP/Mg^{2+}-dependent amidotransferase-type enzyme and shows greatest similarity to class B asparagine synthetases. Like asparagine synthetase, both enzymes catalyze amide bond formation, but Bls uses only carboxyethylarginine and ATP as substrates, whereas asparagine synthetase uses aspartic acid, glutamine and ATP. As a result, asparagine synthetase catalyzes an intermolecular transamidation, transferring the amide group from glutamine onto aspartic acid, whereas Bls catalyzes an intramolecular amide bond formation with the resultant closure of the β-lactam ring. These differences are reflected in the structures of the two proteins.[55,56] Both enzymes have a two-domain structure, but X-ray crystallographic studies have shown that the asparagine synthetase structure forms an enclosed tunnel connecting the N-terminal glutaminase site to the C-terminal site of acyladenylation and amide bond formation, and thereby provides a channel for the ammonia cleaved from glutamine to travel to the site of amide bond formation. In Bls, where cleavage of glutamine is not involved, no such tunnel is evident. These differences are also reflected in characteristic patterns in the amino-acid sequences of the proteins that allow the much more numerous asparagine synthetases to be distinguished from Bls.[56] Asparagine synthetase has a strictly conserved cysteine residue at its N terminus that is missing in Bls. Conversely, Bls has a Lys 443 residue that is implicated in activation of carboxyethylarginine and strictly conserved in all Bls enzymes.[54] On the basis of these characteristic sequence differences, it is possible to recognize Bls encoding genes in genome sequences from organisms not otherwise known to produce clavam metabolites, and when these genes are clustered together with additional biosynthetic genes, the organisms can be presumed to

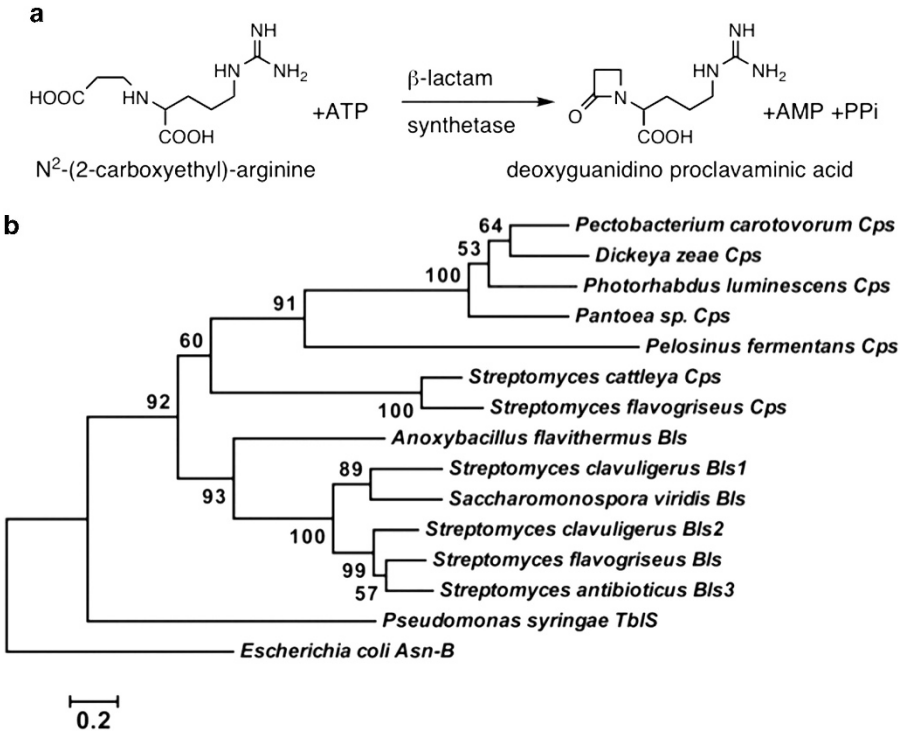

Figure 3 β-Lactam synthetase. (**a**) The β-lactam synthetase reaction. (**b**) Phylogenetic relationships between β-lactam synthetases (Bls), carbapenam synthetases (Cps), tabtoxin synthetase (TblS) and asparagine synthetase B (Asn-B) based on protein maximum likelihood method as described in Figure 2b. Accession numbers for the Bls, Cps and TblS sequences used are listed in Table 1. Asn-B from *Escherichia coli* was included in the analysis (accession number: P22106) and was placed at the root of the tree to obtain the displayed topology. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (at least 50% in 1000 replicates) are shown next to the branches.[31] The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

produce clavam metabolites. These presumptive clavam producer organisms are indicated in Table 1 with asterisks. *Anoxybacillus flavithermus*, a firmicute, is of particular interest in this list as the first example of an organism from outside of the genus *Streptomyces* to contain clavam biosynthetic genes.[57] Inspection of the genes surrounding *bls* in the *A. flavithermus* genome, shows only a total of four clavulanic acid biosynthetic genes flanked on either end by transposase-encoding genes, suggesting that they originated through lateral gene transfer and that clavam biosynthesis is unlikely in this species. However, the %G + C content for *bls* and its surrounding cluster of genes is 33.8%, even lower than the 41.8% G + C found for the *A. flavithermus* genome as a whole, and in marked contrast to the 73.5% G + C of its closest *bls* homolog from a *Streptomyces* spp. If this cluster of genes has been acquired by horizontal gene transfer from a *Streptomyces* spp. into *A. flavithermus*, the extensive amelioration of G + C content may suggest that the genes are expressed, and that the transfer event was very distant. Such a scenario is also suggested by the predicted protein sequence of Bls from *A. flavithermus*, as the protein clusters distantly with the Bls proteins from *Streptomyces* spp. (Figure 3b).

THE CARBAPENEM GROUP

The same screening procedure for β-lactamase inhibitors that led indirectly to the discovery of clavulanic acid also resulted in isolation of the olivanic acids.[8,58] However, olivanic acids, unlike clavulanic acid, are carbapenem-type molecules (Figure 1). Curiously, the producer species, *Streptomyces olivaceus*, like *S. clavuligerus*, also produces both a cephamycin antibiotic and a second β-lactam compound with strong β-lactamase inhibitory activity, but in this case, olivanic acid, as opposed to clavulanic acid. Using similar β-lactamase inhibitor assays, or screens based on supersensitive indicator strains, >40 different carbapenem-type compounds have since been identified from a range of producer species. The simplest of the carbapenem compounds, carbapenem-3-carboxylic acid, is produced by several species of enterobacteriaceae whereas *Streptomyces* spp. produce more complex carbapenem structures like olivanic acid and thienamycin. Table 1 does not attempt to list all known carbapenem producer species, and readers are instead directed to the comprehensive review by Hamed *et al.*[3]

Thienamycin, produced by *Streptomyces cattleya*,[59] is a carbapenem compound and an excellent β-lactamase inhibitor like the olivanic acids, but it is also a powerful antibiotic in its own right. *S. olivaceus*, *S. clavuligerus* and *S. cattleya* all produce cephamycin C in addition to their β-lactamase inhibitory β-lactam product, suggesting that this simultaneous production of β-lactam antibiotics together with β-lactamase inhibitors is more than just coincidence, and must have relevance in nature. In addition, the IPNS proteins from *S. clavuligerus* and *S. cattleya*, which are responsible for the formation of cephamycin C β-lactam ring, also cluster together based on the similarities of their amino-acid sequences, but the evolutionary significance of this observation is not clear at this point (Figure 2b). Interestingly, inspection of the published genome sequence for *Streptomyces flavogriseus*, previously reported as an epi-thienamycin producer,[60] shows that it carries both a thienamycin-type carbapenem gene cluster and a complete clavulanic acid gene cluster[7] but no penicillin/cephalosporin cluster, showing yet a different combination

of the multiple β-lactam biosynthesis gene clusters to be found in some *Streptomyces* producer species.

Carbapenem biosynthesis has not been as widely investigated as either penicillin/cephalosporin or clavam biosynthesis, but the gene clusters from the carbapenem-3-carboxylate producer, *Pectobacterium carotovora*, and the thienamycin producer *S. cattleya*, have been isolated and analyzed.[61,62] In both cases, genes encoding enzymes distantly related to Bls, *carA* for *P. carotovora* and *thnM* for *S. cattleya*, are found within the clusters. Although CarA and ThnM share only limited sequence identity with clavam-type Bls (Bls2 is 31% identical to ThnM over 393 amino acids (aa), and 27% identical to CarA over 416 aa), and with each other (31% identical over 277 aa), biochemical characterization has shown that they all are related to asparagine synthetases and catalyze similar reactions.[63–65] Thienamycin has a much more complex biosynthetic pathway than does carbapenem-3-carboxylic acid, and initial studies suggested that the details of the β-lactam ring-forming reactions catalyzed by ThnM and CarA might differ. However, the most recent studies indicate that both ThnM and CarA employ the same substrate, carboxymethylproline, and catalyze closure of the β-lactam ring to give carbapen-3-am-carboxylic acid, and hence both enzymes are carbapenam synthetases (Cps) (Figure 4).[65] As carboxymethylproline already contains a preexisting saturated five-membered ring, the action of Cps generates a bicyclic carbapenam product, which contrasts with the action of Bls in clavam biosynthesis where β-lactam ring closure gives a monocyclic β-lactam product and precedes closure of the oxazolidine ring. Although crystallographic studies have not yet been conducted on ThnM, structural analysis of CarA[66] has been undertaken, and parallels with the Bls structure are clear. Unlike Bls that crystallizes as a dimer, CarA crystallizes as a tetramer, but otherwise, both enzymes show the same two domain structure and lack the functional N-terminal glutaminase active site that distinguishes Bls-type enzymes from the related asparagine synthetases. The C-terminal synthetase active site of CarA also shows conservation of the ATP-binding site and substrate-binding pocket seen in Bls2, but with changes consistent with the different substrates for the two enzymes. These structural features are reflected in the phylogenetic relationships between the various proteins, where Cps proteins form a cluster separate from the Bls enzymes based on their amino-acid sequences, but the Cps enzymes from *Streptomyces* spp. also form a subcluster separate from the Gram-negative carbapenem producers (Figure 3b). This might suggest a more distant evolutionary relationship as indicated by the differences and the complexities of the carbapenems produced by the *Streptomyces* spp. as compared with their Gram-negative counterparts.

THE MONOCYCLIC β-LACTAMS

The diverse group of monocyclic β-lactams is produced by a wide range of organisms and is unified only by possession of a β-lactam ring. Some monocyclic β-lactams, notably nocardicin A[67] and simple monobactams such as sulfazecin and isosulfazecin,[68] function like classical β-lactam antibiotics in that they inhibit cell wall biosynthesis. However, other members of this group, such as tabtoxin, the pachystermines and monamphilectines, have no cell wall-directed activities and function instead as plant-specific toxins, cytotoxins or antimalarial agents, respectively.[69–71] The pachystermines and monamphilectines also represent rare examples of β-lactam compounds derived from higher eukaryotes. The pachystermines are products of the higher plant, *Pachysandra terminalis*. However, the derivation of the monamphilectines from marine sponges, organisms with intimately associated commensal bacteria, still leaves open the possibility that production of these monocyclic β-lactam compounds, in whole or in part, may be of prokaryotic origin.[72]

As a group, the monocyclic β-lactams are biochemically the least well characterized of the β-lactams, and the mechanism of β-lactam ring formation has not been established for any of these compounds.

Tabtoxin

Tabtoxin (Figure 1) is not a plant toxin in its own right, but it is hydrolyzed in planta to form tabtoxinine-β-lactam, a powerful glutamine synthetase inhibitor.[69] As such, it is representative of a group of natural products, also including the natural herbicide, bialaphos, that have Trojan horse-type properties. Their true functions are not evident in their original forms, but are unmasked to reveal antibiotic or toxic properties when they become activated upon arrival at their intended target.[73,74] Tabtoxin is produced by *Pseudomonas syringae* and related species, where it causes wildfire disease of tobacco, but early reports indicate that very similar peptide conjugate β-lactam products are also produced by *Streptomyces* spp.[75] Whatever little biosynthetic information is available for tabtoxin, however, is derived from studies on *P. syringae*. No detailed studies of tabtoxin biosynthesis have yet been reported, but feeding studies suggest that the skeleton of the molecule is derived from threonine and tetrahydrodipicolinic acid, an intermediate of lysine biosynthesis.[74,76,77] Intriguingly, however, the carbonyl carbon atom of the β-lactam ring apparently arises from the C1 pool.[78] Although the nature of the β-lactam ring-forming reaction has not yet been established, the gene cluster responsible for tabtoxin production has been isolated and partially characterized from *P. syringae*.[79] A gene encoding a putative *S*-adenosyl methionine-dependent methylase is present, consistent with the proposed C1 origin of the β-lactam carbonyl atom, but more strikingly, a second gene, *tblS*, encodes a protein showing clear similarity to Bls, ThnM and CarA. The predicted TblS also clusters distantly with the Bls and Cps proteins based on its amino-acid sequence (Figure 3b). This suggests that, despite its monocyclic nature and the C1 source of the β-lactam carbonyl carbon atom, β-lactam ring formation in tabtoxin biosynthesis proceeds in a manner analogous to that of the clavams and carbapenems.

Nocardicin A

As was the case for tabtoxin, biosynthetic studies on nocardicin A (Figure 1) production have not yet clarified the nature of the β-lactam ring-forming reaction for this product. However, gene clusters responsible for nocardicin A formation have been identified from *Nocardia uniformis* and from *Actinosynnema mirum*,[80] and considerable effort has been expended in determining the roles that their protein products play in biosynthesis. Unlike the situation for tabtoxin, no gene encoding a protein resembling a Bls-type enzyme is evident within the cluster. Furthermore, a complete genome sequence is available for a nocardicin-producing strain of *A. synema*,[81] and BLAST searches with Bls2, ThnM and TblS showed only asparagine synthetases as homologs with no evidence for Bls-type proteins anywhere in the genome. Similarly, searching with the IPNS from *S. clavuligerus* showed no close homologs.

The nocardicin A peptide backbone arises from the action of a nonribosomal peptide synthetase (NRPS) that assembles constituent amino acids for further tailoring by accessory proteins.[82] The nocardicin A peptide synthetase is complex in structure, encoded by two genes, *NocA* and *NocB*, which comprise five modules, two more than can be accounted for by the tripeptide origin of nocardicin, and yet all five modules are essential for nocardicin

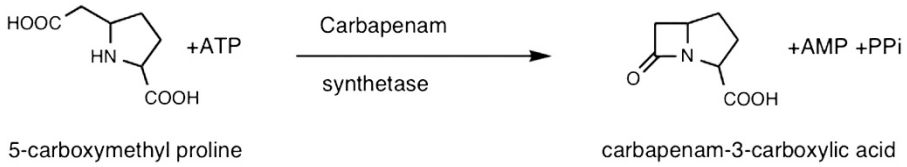

Figure 4 The carbapenam synthetase reaction.

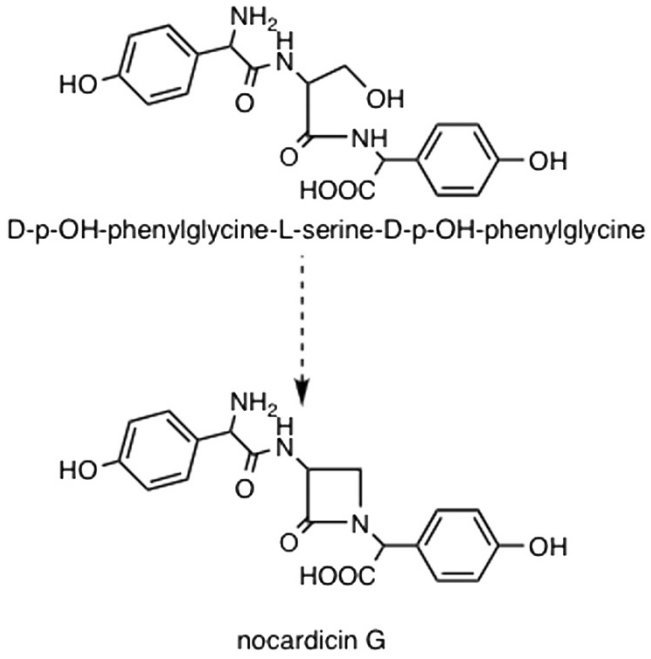

Figure 5 The proposed nocardicin β-lactam ring-forming reaction.

formation. Two possible scenarios for β-lactam ring formation have been proposed: one, that the NocA/NocB peptide synthetase itself may contain one or more domains that catalyze the ring closure or, alternatively, that one or more genes responsible for ring closure may be located outside of the recognized nocardicin gene cluster. Whichever is the case, nocardicin biosynthesis is proposed to proceed via formation of a D-*p*-OH-phenylglycine-L-serine-D-*p*-OH-phenylglycine tripeptide by NocA/NocB with simultaneous or subsequent ring closure involving activation of the serine β-OH and cyclization to form the four-membered ring (Figure 5).[80] The nature of the NRPS reaction further suggests that the amide bond of the nascent β-lactam ring is formed during peptide synthesis, and that ring closure will therefore involve a reaction quite different from that of Bls. With these provisos in mind, it seems likely that a third distinct mechanism of β-lactam ring formation exists for nocardicin A synthesis.

Simple monobactams

The simple monobactams such as sulfacezin and isosulfazecin (Figure 1) are the most common, but least well understood of the microbial monocyclic β-lactam compounds in terms of the biochemistry of their production and, furthermore, no information is available on the genes encoding biosynthetic enzymes. Some of the producer organisms (Table 1) are strains of species for which genome sequences are available, but antibiotic production is not necessarily a uniform property of all members of a species, and hence until a known monobactam producer is sequenced, only limited conclusions can be drawn. BLAST analysis of available genome sequences for *Acetobacter* and *Gluconobacter* spp. as well as for *Chromobacterium violaceum* has not shown any evidence for genes encoding close homologs of IPNS or Bls enzymes. Similarly, as these simple monobactams have O-methoxyl groups reminiscent of the C7 methoxyl groups of the cephamycin antibiotics, available genome sequences were searched for CmcH (the distinctive oxygenase-type hydroxylase responsible for initiating methoxyl group formation in *S. clavuligerus*) homologs[83] and no examples were found. Searching these same genome sequences for NocA (one of the NRPSs possibly involved in nocardicin A β-lactam ring formation) homologs yielded peptide synthetases, but several of these showed greatest similarity to enzymes of siderophore biosynthesis and, for others, there was no indication that they specifically resembled nocardicin synthetases any more than other NRPSs. Therefore, until more biosynthetic and genetic information is available, no firm conclusions can be drawn, but it seems possible that a fourth mechanism for β-lactam synthesis may be at work in these producer species. On the other hand, feeding studies showed that the β-lactam carbon atoms of simple monobactams are derived from serine, and that during formation of the β-lactam ring, the oxidation level of serine is unaltered. This is consistent with ring formation involving displacement of an activated serine OH,[84] and hence in that regard nocardicin and the simple monobactams may share some similarities in their mechanisms of β-lactam ring formation.

Pachystermines and monamphilectines

The most recently identified monocyclic β-lactam products, the pachystermines and monamphilectines (Figure 1), represent unknown territory in terms of their biosynthesis. The bulk of these molecules is clearly steroid or alkaloid, respectively, in nature, but the origins of the β-lactam substituent groups have not yet been examined in any detail.

CONCLUSIONS

Despite the fairly restricted distribution of β-lactam-producing ability in nature, it is intriguing to see that at least two, and likely three or more, distinct routes for the synthesis of this chemically unusual structure have evolved. Although primary metabolism is characterized by a unity of biochemical processes across all domains of living organisms, the biosynthesis of β-lactam compounds provides yet another example of the striking versatility of secondary metabolism. Even in the actinomycetes, where production of different subgroups of β-lactam compounds can co-occur in a single species, completely separate routes for production of the different versions of this strained ring structure have evolved, rather than elaboration of new products as offshoots from one basic biosynthetic template. In addition, the occurrence of the different mechanisms involved in the formation of the β-lactam ring in prokaryotes and eukaryotes raises questions about their origin, dissemination and evolution. As of now, there are no Bls- or Cps-based pathways known to exist in fungi or other eukaryotes. Perhaps they are just yet to be found, as the number of

eukaryotic genome sequences in the databases is dwarfed by those of prokaryotes. On the other hand, knowledge of the existence of IPNS-based mechanisms in both bacteria and fungi might suggest that the Bls- and Cps-based mechanisms are simply more recent in terms of evolutionary timelines and have not yet crossed domain boundaries, as has been proposed for IPNS.[34]

In the more than 80 years since their first discovery, a great deal of effort has been directed toward understanding the biosynthesis of β-lactam compounds, a testament not only to the practical importance of these compounds, but also to the complexity of the processes involved. Significant progress has been made on many fronts, but questions still remain that will continue to challenge researchers well into the future.

DEDICATION

This article is dedicated to the memory of Leo C Vining. Leo was a central figure in Microbiology in Canada throughout his academic career and for many years after his official retirement. He was an ardent and committed researcher, a dedicated teacher and a generous and considerate colleague. Although Leo was best known for his many contributions to the understanding of chloramphenicol biosynthesis, and more recently for his discovery of jadomycin, he also maintained a lifelong interest in the biochemistry of β-lactam antibiotic production.

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