VOL. 53 NO. 10, OCT. 2000 THE JOURNAL OF ANTIBIOTICS pp. 1008 — 1021

REVIEW ARTICLE

# α-Aminoadipyl-cysteinyl-valine Synthetases in β-Lactam Producing Organisms

## From ABRAHAM's Discoveries to Novel Concepts of Non-Ribosomal Peptide Synthesis

JUAN F. MARTIN

Area of Microbiology, Faculty of Biology, University of León, 24071 León, and
Institute of Biotechnology, INBIOTEC, Science Park of León,
Avda. del Real, n° 1, 24006 León, Spain

(Received for publication July 27, 2000)

The tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) was discovered by ARNSTEIN and MORRIS in *Penicillium chrysogenum* and ABRAHAM and coworkers in *Acremonium chrysogenum*. Other analogous tripeptides and tetrapeptides were later reported in these and other β-lactam producing fungi and actinomycetes. The ACV tripeptide is synthesized by a large non-ribosomal peptide synthetase named ACV synthetase encoded by the 11 kb *pcb*AB gene. This gene has been cloned from the DNA of four different filamentous fungi and two actinomycetes. Detailed analysis of the multifunctional ACV synthetases reveals that they consist of three repeated modules (initially named domains) involved in activation of the corresponding amino acids L-α-aminoadipic acid, L-cysteine and L-valine. Each module consists of functional domains for amino acid activation (A), condensation (C) and thiolation (T). In addition the last module of the ACV synthetase contains an epimerization domain (E) involved in conversion of the L-valine to its D-isomer when the tripeptide is still enzyme linked. There are seven epimerization motifs conserved in the third module of all ACV synthetases. In addition, there is an integrated thioesterase domain in the *C*-terminal region of the ACV synthetases that appears to be involved in the selective release of the tripeptide with the correct LLD configuration. The structure of the ACV synthetase is similar to that of other modular non-ribosomal peptide synthetases of bacterial and fungal origin. This molecular knowledge opens the way for engineering novel tripeptide synthetases that may result in new bioactive compounds.

ABRAHAM and coworkers at Oxford were the first to establish solid basis to understand the biosynthesis of β-lactam antibiotics in fungi[1,2]. They elucidated the role of the tripeptide α-aminoadipyl-cysteinyl-valine (ACV) discovered initially by ARNSTEIN and MORRIS[3] in the biosynthesis of β-lactams[4,5].

Following initial studies on precursor incorporation into penicillin, ARNSTEIN and coworkers observed the formation of the tripeptide ACV[3,6]. This tripeptide was shown to have the configuration δ-(L-α-aminoadipyl-L-cysteinyl-D-valine[7].

### I. Peptides Analogous to ACV

Soon after the discovery of the ACV tripeptide (named

Dedicated to the memory of Sir EDWARD ABRAHAM.

P1) other analogous peptides were found in *P. chrysogenum*, *Cephalosporium acremonium* (renamed *Acremonium chrysogenum*) and *Paecilomyces persicinus*. NEUSS and coworkers[8] described three other peptides in *P. chrysogenum* with the structures:

P2 $\alpha$-aminoadipyl-alanyl-valine
P3 $\alpha$-aminoadipyl-serinyl-valine
P4 $\alpha$-aminoadipyl-serinyl-isodehydrovaline

The formation of these peptides may be explained on the basis of the low substrate specificity of the non-ribosomal peptide synthetase although post-synthetic modifications of some amino acids by "tailoring" enzymes can not be excluded (see below). Later, AVANZINI and coworkers[9,10] reported the presence of a peptide formed by $\alpha$-aminoadipic and valine but not cysteine in several strains of *P. chrysogenum*. It is unclear how this dipeptide may be formed; the authors suggested that it may be synthesized by a different enzyme system following the release of $\alpha$-aminoadipic acid from isopenicillin N after cleavage by the isopenicillin N acyltransferase.

A mixture of ACV related peptides was found in the mycelium of *Acremonium chrysogenum*. The major component (A1) of the mixture was identified as ACV[11,12] but two minor components were tetrapeptides, one (A2) containing $\alpha$-aminoadipic acid, cysteine, valine and glycine and the second one (A3) containing a $\beta$-hydroxyvaline residue in place of valine[11,12].

Later ENRIQUEZ and PISANO[13] identified two tetrapeptides in addition to the standard tripeptide in the cephalosporin producer *Paecilomyces persicinus* (PP peptides).

PP1 $\delta$-($\alpha$-aminoadipyl)-cysteinyl-valine
PP2 Glycyl-$\delta$-($\alpha$-aminoadipyl)-cysteinyl-$\beta$-hydroxyvaline
PP3 Glycyl-$\delta$-($\alpha$-aminoadipyl)-cysteinyl-valine

The *P. persicinus* peptides seem to be identical to those of *A. chrysogenum*. The PP1 tripeptide corresponds to the ACV tripeptide and the PP2 and PP3 tetrapeptides appear to be identical to the A3 and A2 peptides of *A. chrysogenum*, respectively, although the exact sequence of amino acids in the *A. chrysogenum* tetrapeptides have not been reported. ENRIQUEZ and PISANO[13] proposed that glycyl-ACV may be the real precursor for cephalosporin biosynthesis but this hypothesis has not been substantiated further.

It is interesting that a significant proportion of the ACV tripeptide is secreted to the culture broth. This observation was made initially by ADRIAENS *et al.*[14] and confirmed in our laboratory using *P. chrysogenum* strains of low and high penicillin producing ability[15]. Since the ACV tripeptide is not taken back into the cytoplasm[14], the role of the tripeptide in the broth is probably not related to penicillin biosynthesis and it may serve as chemical signals for intercellular communication (pheromones).

## II. $\alpha$-Aminoadipyl-cysteinyl-valine Synthetase: The First Enzyme of the $\beta$-Lactam Biosynthetic Pathway

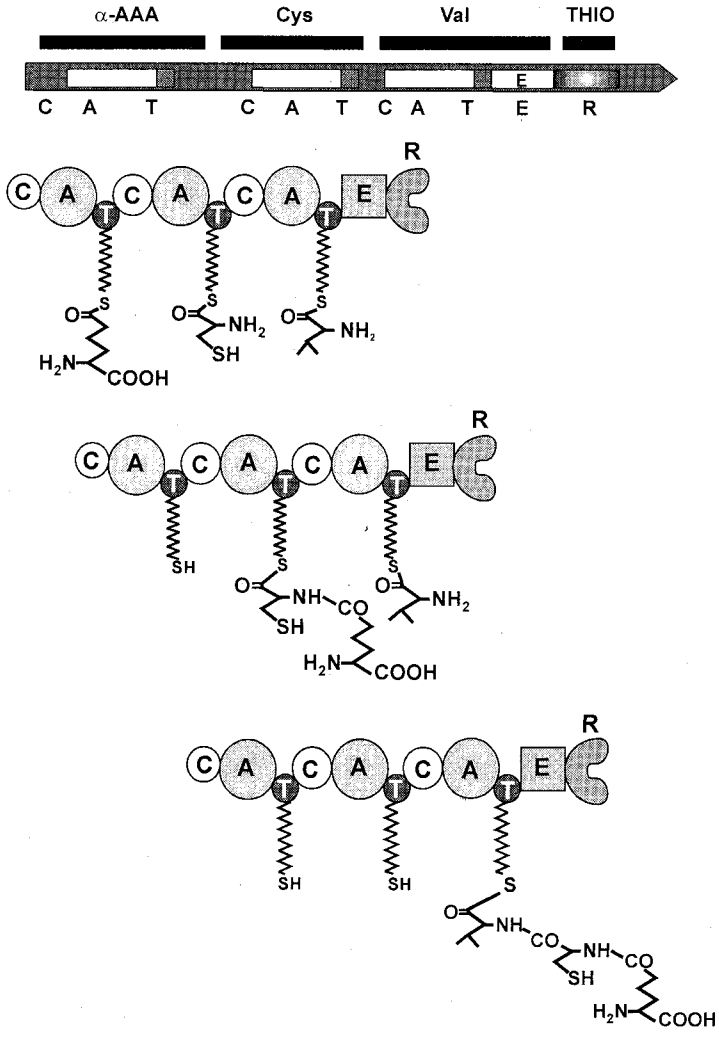
Penicillins, cephalosporins and cephamycins are $\beta$-lactam antibiotics formed by condensation of L-$\alpha$-aminoadipic acid (an intermediate of the lysine biosynthetic pathway in fungi), L-cysteine and L-valine. The three L-amino acids are activated and linked together to form the tripeptide $\delta$-(L-$\alpha$-aminoadipyl)-L-cysteinyl-D-valine. The L-valine molecule is epimerized to the D-configuration during formation of the tripeptide (Fig. 1). Biosynthesis of the ACV tripeptide was initially thought to proceed in two steps forming first the dipeptide AC and then the tripeptide ACV[16]. This led to the proposal of two loci *pcb*A and *pcb*B for the putative genes encoding these enzymes[17].

Later evidence indicated that formation of the ACV tripeptide is catalyzed by a single enzyme named ACV synthetase, which requires ATP (*i.e.* it is a true synthetase) and $Mg^{2+}$ ions[18,19]. Since formation of ACV is the first step committing the amino acids to the $\beta$-lactam biosynthetic pathway, the role of ACV synthetase is of great interest. Recently it has been reported that ACV synthetase activity is limiting for penicillin biosynthesis in wild type *Aspergillus nidulans* strains[20].

Cell-free systems catalyzing ACV synthesis were first described using extracts of *A. chrysogenum*[21,22] and *S. clavuligerus*[23]. The multifunctional ACV synthetase was purified from *Aspergillus nidulans*[24], *A. chrysogenum*[21,25] and *S. clavuligerus*[23,25,26]. The molecular weight of the *A. nidulans* enzyme was originally reported to be 220 kDa, but recent data based on analysis of the cloned gene indicates that its molecular weight is about 400 kDa. For the *A. chrysogenum* enzyme ZHANG and DEMAIN reported that the native enzyme is a dimer of about 800 kDa composed of two identical subunits[21]. The *S. clavuligerus* ACV synthetase appears to be a monomer of about 350 to 400 kDa[23] although these values do not agree with those reported by JENSEN[26] and BALDWIN[25]. The discrepancies appear to be due to the lack of appropriate size markers for the SDS-PAGE of these large proteins.

The tripeptide ACV in a second step, is oxidatively

Fig. 1. Organization of domains of the ACV synthetases and model of elongation of the tripeptide chain. $\alpha$-AAA, cys and val indicate the three repeated modules of the ACV synthetases.




The three classical amino acid activating domains (see text) are now designated as modules and subdivided into functional domains. THIO corresponds to the thioesterase domain downstream of the Val module. A, activation domains; C, condensation domains; T, thiolation domains; E, epimerase domain (occurs only in the valine module); R, Release (thioesterase) domain. Note that elongation of the growing tripeptide chain takes place by activation and transfer of the amino acid to the thiol group of the phosphopantetheine arm linked to the T domain of each module. Epimerization of the L- to the D-valine appears to occur in the tripeptide chain attached to the enzyme (see text).

cyclized by removal of four hydrogen atoms to form the $\beta$-lactam-thiazolidine nucleus of isopenicillin N which is later converted into penicillins containing phenylacetic acid, phenoxyacetic acid or other hydrophobic side chains [reviewed by DEMAIN[5], MARTÍN and LIRAS[27], and AHARONOWTIZ *et al.*[28]]. Further "late" modifications convert isopenicillin N into either cephalosporins or cephamycins[29].

## III. Substrate Specificity of the ACV Synthetases

Studies on the substrate specificity of the ACV synthetases were hampered by the difficulty to obtain pure preparations due to its large size and by its instability[12,18,22,30]. The ACV synthetases can be partially stabilized with glycerol[16,31,32] allowing partial purification of the enzyme.

Table 1. *In vitro* replacement of substrate amino acids by analogues using different ACV synthetases.

| Source of ACV synthetase | Normal substrates | Other amino acids used as substrates | References |
|---|---|---|---|
| *A. chrysogenum* | L-α-AAA replaced by | L-*S*-Carboxymethylcysteine<br>L-Glutamate (very low activity) | 31, 35 |
| | L-Cysteine | L-Allylglycine<br>L-Vinylglycine<br>DL-*O*-Methylserine<br>L-*S*-Methylcysteine | 35 |
| | L-Valine | L-*allo*-Isoleucine<br>L-α-Aminobutyrate | 31, 35 |
| *S. clavuligerus* | L-α-AAA | L-*S*-Carboxymethylcysteine | 33, 34 |
| | L-Valine | L-*allo*-Isoleucine<br>L-α-Aminobutyrate<br>L-Norvaline<br>L-Allylglycine | 33, 34 |
| | L-Cysteine | L-Homocysteine<br>*S*-Methyl-L-cysteine | 33, 34 |
| *A.lactamdurans* (formerly *N. lactamdurans*) | L-α-Aminoadipic acid<br>L-Cysteine | 6-Oxopiperioline-2-carboxylic acid<br>L-Cystathionine | 36 |

The crude ACV synthetase from *A. chrysogenum* showed some degree of substrate unspecificity. L-Carboxymethylcysteine could replace L-α-aminoadipic acid but L-aspartate, adipate or phenylacetate could not. Similarly, L-valine was replaced by L-allo-isoleucine and α-aminobutyrate but not by D-valine, L-isoleucine or glycine (Table 1). Similar results on substrate specificity were observed with the ACV synthetase of *S. clavuligerus*[33,34] (Table 1).

This relative lack of substrate specificity of the ACV synthetase was later confirmed by BALDWIN and co-workers[35]. Using the *A. chrysogenum* enzyme, these authors found that the *S*-carboxymethylcysteine was an effective substitute for α-aminoadipate and both allylglycine and vinylglycine could substitute for cysteine showing that the thiol group of cysteine is not essential for peptide formation. Similarly, L-alloisoleucine but not L-isoleucine substituted effectively for valine. The structure of the alternative peptide products was confirmed by NMR and electrospray-ionization mass spectrometry.

Similarly, the purified ACV synthetase of *Nocardia lactamdurans* can use 6-oxopiperidine 2-carboxylic acid (cyclic form of α-aminoadipic acid) or cystathionine in place of α-aminoadipic acid or cysteine, respectively[36].

## IV. The *pcb*AB Gene Encodes ACV Synthetase

The *P. chrysogenum pcb*AB gene that encodes ACV synthetase was first cloned by DÍEZ *et al.*[37] and simultaneously by TURNER and coworkers[38]. Both groups provided evidence showing that the *pcb*AB is an unusually large gene (11,376 nucleotides) encoding a protein of 3,792 amino acids with a deduced molecular mass of 425,971. The cloned *pcb*AB gene complements a non-producer mutant *P. chrysogenum npe*5 defective in ACV synthetase[39].

Soon after the *P. chrysogenum pcb*AB gene was reported, the *pcb*AB of *Acremonium chrysogenum* was also cloned by hybridization with probes internal to the *Penicillium* gene[40]. A 15.6 kb DNA fragment was shown to complement the *P. chrysogenum npe*5 mutant that is defective in ACV synthetase. The *A. chrysogenum pcb*AB gene contained 11,136 nt encoding a protein of 3,712 amino acids with a deduced molecular weight of 414,791 Da. Similarly, the ACV synthetase gene of *A. nidulans* (named *acv*A) was cloned by MCCABE *et al.*[41] and MONTENEGRO *et al.*[42]. The *pcb*AB has also been found recently in *Penicillium nalgiovense*[43] (LAICH, F. & J. F. MARTÍN,

unpublished data).

Genes encoding ACV synthetases were also cloned from the cephamycin producing actinomycetes *N. lactamdurans*[44] (recently renamed *Amycolatopsis lactamdurans*)[45] and *S. clavuligerus*[46,47]. The *A. lactamdurans pcb*AB gene encodes a protein of 3,694 amino acid with a molecular weight of 404,134 Da. In *S. clavuligerus* the sequence of only a 712 bp of the 3′ end of the ORF is known[46]. In all fungi the *pcb*AB gene is expressed in opposite orientation to the *pcb*C gene (encoding isopenicillin N synthase), the second gene of the penicillin and cephalosporin pathway whereas in bacteria the *pcb*AB gene is arranged in the same orientation as the *pcb*C gene[44].

In spite of its large size, the fungal *pcb*AB genes lack introns. The lack of introns suggests that the ACV synthetase gene is of bacterial origin and it has been transferred horizontally to filamentous fungi[28]. Both the fungal and bacterial genes share a high degree of homology. The three fungal *pcb*AB genes are 71% identical, whereas the bacterial genes show about 48% identical amino acids with the fungal genes. Since the similarity between fungal and bacterial *pcb*AB genes is much higher than that expected on the basis of the separate evolution of procaryotic and eucaryotic microorganisms, this similarity further supports the hypothesis of the horizontal transfer of these genes in soil[28,48,49].

## V. Involvement of the *pcb*AB Gene in β-Lactam Biosynthesis: Evidence for a Single Copy of This Gene

The involvement of the *pcb*AB gene in penicillin biosynthesis was established by gene disruption studies. Disruption of the *pcb*AB gene of *A. chrysogenum*[50] and *A. nidulans*[38] led to the loss of ACV synthetase and cephalosporin or penicillin production ability, respectively. These results unequivocally indicate that formation of ACV is required for penicillin or cephalosporin biosynthesis. Hybridization studies of total DNA of *P. chrysogenum* and *A. chrysogenum* with probes internal to the *pcb*AB gene indicated that there is a single set of the penicillin[51] or cephalosporin (GUTIÉRREZ, S. & J. F. MARTÍN, unpublished) gene cluster in wild type strains of these fungi. High penicillin producing strains contain multiple copies of the *pcb*AB-*pcb*C-*pen*DE cluster reiterated in tandem[52].

## VI. Are the Cephalosporin Tetrapeptides Formed by the ACV Synthetase?

α-Aminoadipic acid - containing tetrapeptides are synthesized in *A. chrysogenum* and *P. persicinus* but not in *P. chrysogenum*. It is unclear if the tetrapeptides are synthesized by the ACV synthetase itself although it seems unlikely since the tripeptide synthetase only contains three amino acid-activating modules. No studies have been performed on the presence or absence of the tetrapeptides in *A. chrysogenum* mutants disrupted in the *pcb*AB gene.

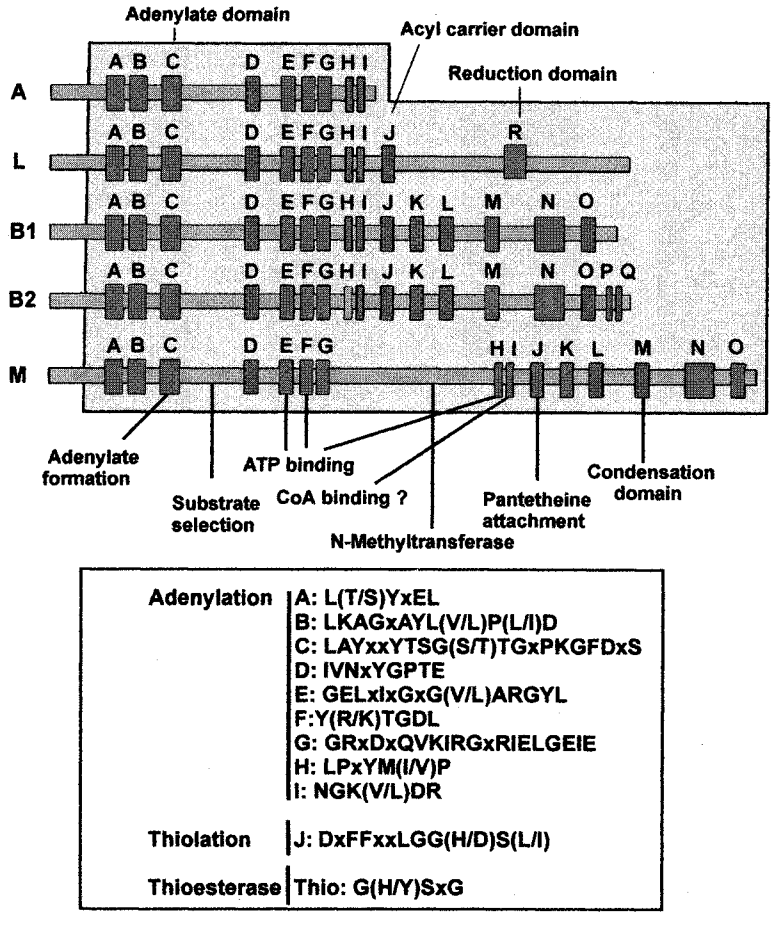
Hybridization studies excluded the possibility of a second peptide synthetase (forming the α-aminoadipic acid containing tetrapeptides) similar to the ACV synthetase since a single hybridizing band was observed in hybridizations of total DNA with a probe internal to the *pcb*AB gene. A possible explanation is that the tetrapeptides are formed by a glycine activating single-module enzyme that might be able to transfer the activated glycine into the ACV tripeptide formed by the ACV synthetase.

## VII. Domains in Peptide Synthetases

The non-ribosomal peptide synthases constitute a group of biosynthetic enzymes of great interest because they catalyze a novel route of peptide bond formation independent of ribosomes[53~56].

Domains similar to those of the ACV synthetase have been found in a large number of enzymes that activate their substrate amino acids as aminoacyl-adenylates, and bind the amino acids covalently as thioesters (Fig. 2). This group includes all peptide synthetases of diverse origins. A region homologous to the repeated domains of ACV synthetases was also found in enzymes not involved in peptide synthesis that activate their substrates with ATP including among others i) the α-aminoadipate reductase (LYS2 gene) of *Saccharomyces cerevisiae*, *P. chrysogenum*[57] and *A. chrysogenum*[58] which activates α-aminoadipate with ATP for reduction to the α-aminoadipic semialdehyde, ii) the EntE protein involved in enterobactin synthesis in *E. coli*, which activate 2,3-dihydroxybenzoate, iii) the acetyl-CoA synthetases of *A. nidulans* and *Neurospora crassa* and iv) the 4-coumaryl-CoA ligase of parsley and the *p*-aminobenzoyl-CoA ligase of *S. griseus* involved in candicidin biosynthesis[59]. These acyl-activating enzymes have a conserved domain of about 550 amino acids (shorter than the 1,000 amino acid domains of peptide synthetases)

Fig. 2. Conserved motifs in the modules of acyl-adenylating enzymes (type A), $\alpha$-aminoadipate reductase of the lysine pathway (type L) and non-ribosomal peptide synthetases (type B1, B2 and M).




B2 modules are longer than B1 and contain epimerization motifs. Type M modules contain a 50 kDa methyltransferase domain inserted between motifs G and H (see text).

(Fig. 2).

All these enzymes have in common their ability to activate the carboxyl group of the corresponding substrate through formation of acyladenylates. MARAHIEL and coworkers have proposed the designation of the ***adenylate-forming enzyme superfamily*** for this group of enzymes[60~62].

The relationship between peptide-synthetases and acyl-CoA ligases is intringuing. Peptidyl macrolactones such as rapamycin or FK-506 originate from both carboxylic acids and amino- or imino acids[63]. Some of the peptide synthetase combine subunits activating amino acids with other that activate organic acid. One of the domains (EA) of enniantin synthetase is involved in activation of the D-hydroxyisovaleric (D-HIV) acid which only requires adenylation whereas the EB domains activate the branched chain amino acid (L-valine and L-isoleucine) forming its *N*-methylderivative and catalyze the formation of the peptide bond[64].

One interesting finding is that the size of the peptide synthetases is directly proportional to the number of domains, which in turn is identical to the number of amino acids that are activated. Gramicidin synthetase 1 (GS1) and tyrocidine synthetase 1 (TY1) with sizes of 126 and 120 kDa activate a single amino acid; enniantin synthetase which activates two residues has a molecular weight of 347 kDa; ACV synthetase and tyrocidine synthetase 2 (TY2) which activate three amino acids have molecular weights of 404 to 425 kDa; gramicidin synthetase 2 (GS2) which activates four amino acids shows a molecular weight of 562 kDa and cyclosporin synthetase which activates 11 amino acids has a calculated molecular weight of

1689 kDa[65].

However not all peptide synthases have all amino acid-activating domains in a single polypeptide. Fungal peptide synthetase are more integrated than their bacterial counterparts. So far, the most complex fungal peptide synthetase is the cyclosporin synthetase that activates 11 amino acids and is a 1.69 million Da protein[65]. The largest known prokaryotic enzyme is the lysobactin synthetase from *Lysobacter* sp. that activates and adds 8 amino acids to a tripeptide precursor, and has an estimated size of 1 million Da[66]. It is not known why multifunctional peptide synthetases are sometimes encoded by a single gene resulting in a large multimodular protein and sometimes (*e.g.* lysobactin) are encoded by two genes forming two peptide synthetases that interact between them.

## VIII. Colinearity Between Peptide Synthetase Modules and the Order of Amino Acids in the Non-ribosomal Peptides

More and more genes encoding peptide synthetases are being sequenced every year. Initial studies on three tryptic fragments of the gramicidin synthetase GS2 which specifically activate proline, ornithine and leucine established the basis of the colinearity rule. The biochemical evidence available indicates that modules 1, 3 and 4 of GS2 activate proline, ornithine and leucine, respectively. The remaining-domain contains the site for valine activation[55]. These results indicate that there is a functional colinearity between the modules in the GS2 protein as deduced from the nucleotide sequence and the order of the amino acids incorporated into gramicidin S.

The largest peptide synthetase cloned so far, the cyclosporin synthetase, contains 11 modules that also appear to adjust to the principle of functional colinearity[65]. There are two types of modules in the cyclosporin synthetase. Type I is very similar to the standard modules (about 1000 aa) found in other multifunctional peptide synthetases. Type II is larger than the first and include a 450 amino acid stretch that corresponds to a region involved in *S*-adenosylmethionine binding as defined experimentally by photoaffinity labeling with *S*-adenosylmethionine after endoproteolytic digestion (Fig. 2). There are four type I and seven type II modules in the cyclosporin synthetase. The order of the eleven type I and type II modules (without or with the methyltransferase activity, respectively) corresponds exactly with the order of non-methylated and methylated amino acids in cyclosporin[65].

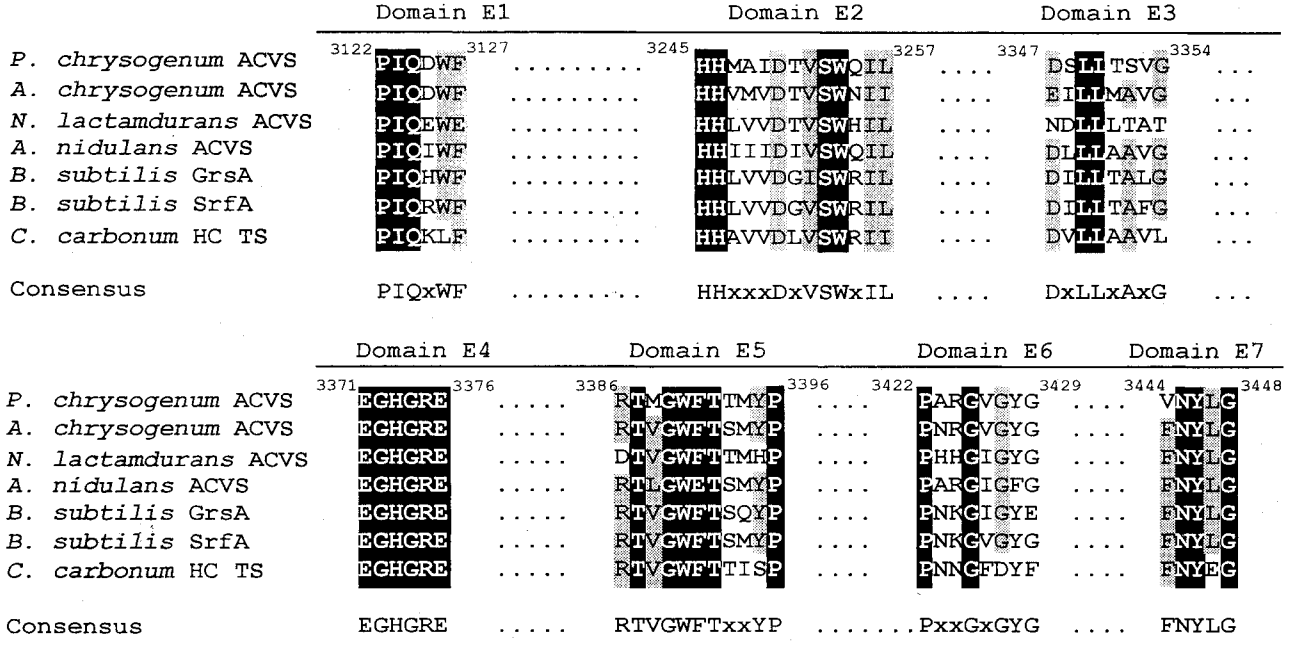
## IX. Amino Acid Condensation in ACV: a Revised Mechanism of Peptide Chain Elongation

The thiol template mechanism for the synthesis of ACV postulates that the first condensation step leads to the formation of the peptide bond between the $\delta$-carboxyl of L-$\alpha$-aminoadipic acid and the amino group of L-cysteine prior to the formation of the bond between L-cysteine and L-valine. However this mechanism has been disputed by SHIAU and coworkers[67,68] who observed that purified preparations of the ACV synthetase form two diasteroisomeric dipeptides, L-(*O*-methylserinyl)-L-valine and L-(*O*-methylserinyl)-D-valine when the enzyme was incubated with $\alpha$-aminoadipate, L-valine and the cysteine analogue L-*O*-methylserine[67]. Later the same authors reported the isolation of the dipeptide $\alpha$-cysteinyl-D-valine from incubations of ACV synthetase with L-cysteine and L-valine. Formation of the L-cysteinyl-D-valine dipeptide was significantly enhanced in the presence of L-glutamate, apparently due to inteference of L-glutamate with the $\alpha$-aminoadipate activation and condensation steps. The efficient formation of the dipeptide L-cysteinyl-D-valine under these conditions suggests that epimerization of the L-valine takes place at the enzyme-bound dipeptide stage. SHIAU and coworkers[69] concluded that the peptide bond between $\alpha$-aminoadipate and cysteine is formed after the L-cysteine-D-valine is synthesized and that thioesterification of valine is not obligatory for peptide bond formation. They proposed a revised mechanism of peptide chain elongation in which formation of the ACV starts with the condensation of L-cysteine and L-valine[67~69]. This result is consistent with the reported utilization of cystathionine (*S*-[2-amino-2-carboxyethyl]homocysteine) by the ACV synthetase of *N. lactamdurans* to form ACV[36].

## X. Specific Motifs and Structural Organization of the Amino Acid Activating Modules of ACV Synthetases

When the ACV synthetases were compared with other peptide synthetases, and with other members of the adenylate-forming enzyme superfamily, several highly conserved amino acid sequences were found[70]. These are named boxes A, B, C, D, E, F, G, H, I and J (Fig. 2). Boxes A to J correspond to the sequences of greater similarity between all peptide synthetases. Motifs A, C, D, E, F, G, H and I were present in all the acyladenylate-forming enzymes and, therefore, seem to be involved in nucleotide

Fig. 3. Conserved motifs E1 to E7 in the epimerization domain (E) of the third (Val) module of the ACV synthetases of *P. chrysogenum*, *A. chrysogenum*, *N. lactamdurans* and *A. nidulans* as compared to the GrsA (GSI) and the SrfA modules of *B. subtilis* and the HC toxin synthetase of *C. carbonum*.




The consensus sequence was proposed by MARAHIEL *et al.*[61]. Identical amino acids in all microorganisms are in reverse type, and other amino acids conserved with respect to the consensus are shaded. The amino acid numbers (in superscript) correspond to the *P. chrysogenum* ACV synthetase.

and carboxylic acid binding to form the acyladenylate.

The J-box (initially know as W box)[70] is present only in the domains of peptide synthetases and in the $\alpha$-aminoadipate reductase (Lys2)[57,58] but not in other adenylate-forming enzymes that do not form peptide bonds. This J-box corresponds to the sequence of a peptide fragment of GS2 containing covalently attached valine isolated after charging the enzyme with this amino acid. This site may, therefore, correspond to the active center for late stages of activation of the amino acid, *i.e.* the site of formation of the activated ester from which the peptide bond is made.

Each module of the ACV synthetase corresponds to about 1,000 amino acids. Most of the conserved amino acid sequences are clustered in a 600 amino acid region, but the active fragments of the GS2 that are able to activate proline, leucine and ornithine have molecular weights of 110~120 kDa that corresponds to 1,000~1,100 amino acids. This implies that the regions downstream from the J-box are also important for functionality of the peptide synthetase modules[61].

The third module (type B2) of the ACV synthetases (Fig. 2) is longer than the first two (type B1) and contains two additional conserved boxes. This region corresponds to the epimerization region of the valine module of ACV synthetases.

Proper characterization of the size and limits of each domain is of extreme interest for peptide synthetase engineering[71~73].

## The Aminoacyl-adenylate Forming Region

Many different enzymes that activate their substrates with ATP as aminoacyl-adenylates have been compared. The sequence SG(S/T)TGXPKG (box C) resembles the glycine-rich loops involved in ATP-binding. The conserved sequence Y(K/R)TGDL (so called TGD box or box F in Fig. 2) is conserved in an extense family of ATPases. This TGD sequence appears to serve as a pocket for binding the ATP nucleotide involved in amino acid activation. *In vitro* directed mutation of the SGTTGXPKG motif (box C) and the TGD motif of the tyrocidine synthetase resulted in a considerable decrease of enzyme activity, particularly in the mutations altering the lysine of box C and the aspartic acid of the TGD box, what indicates that these two motifs

are important for amino acid activation as aminoacyl-adenylates (regions named A in Fig. 1). Further support for this hypothesis has been provided by identifying the sequence of a GS2 mutant that is deficient in proline activation. This mutation altered the glycine of the TGD motif of the proline-activating domain. The consensus motifs of adenylation domains serve for identification of novel amino acid-activating domains in sequences deduced from genome analysis[74].

Recently, a peptide corresponding to the adenylation domain of gramicidin synthetase 1 overexpressed in *E. coli* has been crystallized and the 3D structure resolved at the 1.9 Å[75,76]. The 3D structure of this domain is similar to the structure of the acyl-CoA-forming luciferase and it is likely that the 3D structure is similar in all aminoacyl- and acyl-adenylate forming enzymes.

## Pantetheine-binding Sequences

The stability of the peptide synthetase-substrate complexes at acidic pH but not in alkaline pH and the rapid hydrolysis with peroxyacids led to the conclusion that the amino acids are activated as thioesters. SCHLUMBOHM and coworkers[77] identified the valine (or leucine) binding sequences of the GS2 that contained the sequence LGGHS that correspond to the J box region. This amino acid-binding motif does not contain a cysteine (instead it shows a serine) and there are no conserved cysteines in this region of the peptide synthetases. It seems, therefore, that this sequence binds a thiol-containing phosphopantetheine that may in turn form the thioester with the activated amino acid. This model is very likely to be functional *in vivo* since pantetheine has been found to be present in ACV synthetase[22] and other peptide synthetases[78]. Moreover the LGG(H/D)S motif (box J) is present in all the domains of peptide synthetases which are able to form thioester bonds, but it is absent in adenylate-forming enzymes which are unable to form thioesters. The LGG(H/D)S sequence is known to be a consensus motif of phosphopantetheine-binding in the acylcarrier proteins (ACP) of the fatty acid synthetases and in the polyketide synthetases of a variety of secondary metabolites[79].

If there is a pantetheine binding site in each domain (*i.e.* 3 molecules in the ACV synthetases), the content of this vitamin should be higher than previously reported for different ACV synthetases (0.95 to 1 moles per mole of protein)[22].

## An Acyl-carrier (Thiolation) Region in Each Peptide Synthetase Module

Alignment of the *E. coli* acyl-carrier protein with the modules of the ACV synthetases showed that the homology could be extended for 80~100 amino acids on both sides of the J box for the entire length of the ACP. The tridimensional structure of the ACP protein of *E. coli* has been determined; it is formed by four $\alpha$-helices connected by turns. The pantetheine binding site (Ser 36) is located in one of the turns that binds two of the central $\alpha$-helices. An analysis of the predicted secondary structure of the three ACV synthetase modules around the LGG(H/D)S motif (J box) shows the presence of two amphipatic $\alpha$-helices on both sides of a turn formed by the LGGXS motif. These observations indicate that an "ACP region" or thiolation region (T in Fig. 1) is integrated within each module of the peptide synthetases, as occurs also in polyketide synthases[80].

## An Epimerase Motif at the End of the Third Module of ACV Synthetases

Many non-ribosomally synthesized peptides contain D-amino acids[53,54,80]. The *C*-terminal region of the ACV synthetases of *P. chrysogenum*, *A. nidulans*, *A. chrysogenum* and *A. lactamdurans* located after the end of the third module shows high similarity with the homologous regions of GS1 and TY1 and the third module of the surfactin synthetase 1. Since all those peptide antibiotics contain a D-amino acid in its carboxyl terminal region (D-Phe in gramicidin and tyrocidine; D-leu in surfactin) it was proposed that an epimerization domain of about 365 amino acids is located in this region[81,82]. Epimerization domains of several non-ribosomal peptide synthetases are now known and all contain characteristic signature sequences. The involvement of a basic amino acid in these motifs as a proton donor/acceptor during racemization of phenylalanine has been proposed[82]. These motifs have been found in the V module of ACV synthetases (Fig. 3) and in a HC-toxin synthetase domain epimerizing L-Pro to D-Pro[83]. It is important to note that the homologous sequences in the A and C modules of the ACV synthetase do not adjust or very poorly to the consensus epimerase signatures. Further biochemical analysis of these epimerization motifs are required to confirm that they constitute an authentic catalytic site for amino acid epimerization.

On the other hand, the D-alanine component of cyclosporin is provided by a distinct alanine racemase that may interact with the peptide synthetase[84]. The HC-toxin producer *Cochliobolus carbonum* contains an alanine

racemase involved in the conversion of L- to D-alanine separated from the HC-toxin synthetase[83]. Thus, in this filamentous fungus there is an epimerization domain converting L- to D-proline and a separate alanine racemase.

Condensation Domains

Accumulative sequence information from different peptide synthetases suggest that the so-called condensing domains (C in Fig. 1) are responsible for the condensation of two activated amino acids on adjacent modules, *i.e.* it catalyzes the elongation reaction[85]. No biochemical evidence is available, however, to support this hypothesis.

The condensation domain is about 450 amino acids in length. Its location in the multifunctional peptide synthetases follows two simple rules: i) It is always present between two adjacent activating units located on the same polypeptide, as it is the case with the ACV synthetase; ii) when the two consecutive activating domains are located on separate synthetases, the C domain is found at the *N*-terminus of the amino acid-accepting synthetase[61].

Release of Enzyme Bound Peptides: Involvement of a Thioesterase

With the exception of ACV synthetases and linear peptide synthetases, most other peptide synthetases do not release a peptide with a free terminal carboxyl group. Usually, the carboxyl group of the last amino acid that is bound to the enzyme as a thioester is transferred to the amino group of the same or a different peptide chain giving cyclic peptides (*e.g.* gramicidin S, tyrocidine, cyclosporin) or to a terminal or side-chain hydroxyl group (*e.g.* in the cyclodepsipeptides). In some linear peptides the terminal carboxyl group is released by aminolysis. In the biosynthesis of ACV the carboxyl group is released in the free form.

The carboxyl terminal region of the ACV synthetases of *P. chrysogenum*, *A. nidulans*, *A. chrysogenum*, *A. lactamdurans* (and also the known *C*-terminal region of the *S. clavuligerus* enzyme) shows a motif GXSXG homologous to the amino acid sequence of the active site of the oleoyl-ACP hydrolase (thioesterase I) of vertebrate fatty acid synthetases (THIO box)[37,40]. A region of about 230 amino acids of the ACV synthetases extending from the THIO box to the *C*-terminus of the protein (R in Fig. 1) shows high similarity to the 29 kDa GrsT protein (a putative thioesterase) of the gramicidin S operon and to the *srfn*-TE gene of the surfactin cluster supporting the hypothesis that the THIO region might be functional in the hydrolysis of the thioester bond of the enzyme-bound ACV tripeptide.

Recently the catalytic SHD of the active site of the 142 kDa Ent-F module of the enterobactin synthetase has been mutated *in vitro*. Results established that the THIO box of EntF is both a cyclotrimerizing lactone synthetase (forming the trilactone enterobactin) and an elongation catalyst for ester bond formation[86]. The thioesterase domain of the ACV synthetase has also been modified[87]. Unexpecteadly, the mutant ACV synthetase showed only a 50% reduction of the peptide formation rate, with the stereoisomer LLL-ACV as the dominating product. In summary, the THIO motif may be involved in the control of tripeptide epimerization by selection of the isomer to be released, and in the liberation of the final peptide.

## XI. Future Outlook

A long path has been walked since the initial discovery of the ACV tripeptide by ARNSTEIN and MORRIS[3] and LODER and ABRAHAM[11,12] to the modern concepts on amino acid activation, condensation and epimerization by the ACV synthetase. The modular nature of non-ribosomal peptide synthetases opens the way for engineering the multifunctional peptide synthetases[88,89]. Efficient engineering of peptide synthases requires a better knowledge of the limits between domains and about the essential regions for catalytic functions of each domain[90]. Novel hybrid tripeptide synthases will be constructed and some of them will lead to the formation of $\beta$-lactam and/or thiazolidine nucleus[91] resulting in novel bioactive compounds.

Acknowledgements

This work was supported by grants of the European Union (BIO-CT94-2100 and QLK3-1999-00729). I acknowledge the help of P. LIRAS, S. GUTIÉRREZ, F. FIERRO and J. CASQUEIRO, and the excellent technical assistance of M. CORRALES.

## References

1) ABRAHAM, E. P.: Biosynthesis and enzymatic hydrolysis of penicillin and cephalosporins. University of Tokyo Press, Tokyo, 1974
2) FAWCETT, P. A.; J. J. USHER, J. A. HUDDLESTON, R. C. BLEANEY, J. J. NISBET & E. P. ABRAHAM: Synthesis of $\delta$-($\alpha$-aminoadipyl)cysteinylvaline and its role in penicillin biosynthesis. Biochem. J. 157: 651~660, 1976
3) ARNSTEIN, H. R. V. & D. MORRIS: The structure of a peptide, containing $\alpha$-aminoadipic acid, cysteine and valine, present in the mycelium of *Penicillium chrysogenum*. Biochem. J. 76: 357~361, 1960
4) ABRAHAM, E. P.: Enzymes involved in penicillin and cephalosporin formation. *In* Regulation of Secondary

Metabolite Formation. *Eds.*, KLEINKAUF, H., H. VON DÖHREN, H. DORNAUER & G. NESEMANN, pp. 115~132, VCH, Weinheim, 1986

5) DEMAIN, A. L.: Biosynthesis of β-lactam antibiotics. *In* Antibiotics Containing the β-Lactam Structure. *Eds.*, DEMAIN, A. L. & N. A. SOLOMON, Springer Verlag, New York, 1983
6) ARNSTEIN, H. R. V.; N. ARTMAN, D. MORRIS & E. J. TOMS: Sulfur-containing amino acids and peptides in the mycelium of *Penicillium chrysogenum*. Biochem. J. 76: 353~357, 1960
7) CHAN, J. A.; F.-C. HUANG & C. J. SIH: The absolute configuration of the amino acids in δ-(α-aminoadipyl)-cysteinyl-valine from *Penicillium chrysogenum*. Biochem. J. 15: 177~180, 1976
8) NEUSS, N.; R. D. MILLER, C. A. AFFOLDER, W. NAKATSUKOSA, J. MABE, L. L. HUCKSTEPP, N. DE LA HIGUERA, A. H. HUNT, J. OCCOLOWITZ & J. H. GILHAM: High performance liquid chromatography (HPLC) of natural products. 3. Isolation of new tripeptides from the fermentation broth of *P. chrysogenum*. Helv. Chim. Acta 63: 1119~1129, 1980
9) AVANZINI, F. & P. VALENTI: Peptide presence constituted by α-aminoadipic acid (α-AAA) and valine in *P. chrysogenum* fermentation broth. Abstracts 6th International Ferm Symp. London, Ontario, p. 18, 1980
10) AVANZINI, F. & S. MORELLI: Amino acids and peptides in penicillin industrial fermentation broths by different *P. chrysogenum* strains. *In* Abstracts of the VII International Biotechnology Symposium, volume 2, pp. 368~369, New Delhi, India, 1984
11) LODER, P. B. & E. P. ABRAHAM: Isolation and nature of intracellular peptides from a cephalosporin C-producing *Cephalosporium* sp. Biochem. J. 123: 471~476, 1971
12) LODER, P. B. & E. P. ABRAHAM: Biosynthesis of peptides containing α-aminoadipic acid and cysteine in extracts of a *Cephalosporium* sp. Biochem. J. 123: 477~482, 1971
13) ENRIQUEZ, L. A. & M. A. PISANO: Isolation and nature of intracellular alpha-aminoadipic acid-containing peptides from *Paecilomyces persicinus* P-10. Antimicrob. Agents Chemother. 16: 392~397, 1977
14) ADRIAENS, P.; B. MEESSCHAERTS, W. WUYTS, H. VANDERHAEGHE & H. EYSSEN: Presence of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine in fermentations of *Penicillium chrysogenum*. Antimicrob. Agents Chemother. 8: 638~642, 1975
15) LOPEZ-NIETO, M. J.; F. R. RAMOS, J. M. LUENGO & J. F. MARTIN: Characterization of the biosynthesis *in vivo* of α-aminoadipyl-cysteinyl-valine in *Penicillium chrysogenum*. Appl. Microbiol. Biotechnol. 22: 343~351, 1985
16) BANKO, G.; S. WOLFE & A. L. DEMAIN: Cell-free synthesis of δ-(L-α-aminoadipyl)-L-cysteine, the first intermediate of penicillin and cephalosporin biosynthesis. Biochem. Biophys. Res. Commun. 137: 528~535, 1986
17) MARTIN, J. F.; T. D. INGOLIA & S. W. QUEENER: Molecular genetics of penicillin and cephalosporin antibiotic biosynthesis. *In* Molecular Industrial Mycology: Systems and Applications for Filamentous Fungi. *Eds.*, LEONG S. A. & R. M. BERKA, pp. 149~196, Marcel Dekker, New York, 1990
18) ZHANG, J. & A. L. DEMAIN: Regulation of ACV synthetase in penicillin- and cephalosporin-producing microorganisms. Biotechnol. Adv. 9: 623~641, 1992
19) KALLOW, W.; H. VON DÖHREN & H. KLEINKAUF: Penicillin biosynthesis: energy requirement for tripeptide precursor formation by *delta*-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase from Acremonium chrysogenum. Biochemistry 37: 5947~5952, 1998
20) KENNEDY, J. & G. TURNER: *delta*-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. Mol. Gen. Genet. 253: 189~197, 1996
21) ZHANG, J. & A. L. DEMAIN: Purification from *Cephalosporium acremonium* of the initial enzyme unique to the biosynthesis of penicillins and cephalosporins. Biochem. Biophys. Res. Commun. 169:1145~1152, 1990
22) BALDWIN, J. E.; J. W. BIRD, R. A. FIELD, N. M. O'CALLAGHAN, C. J. SCHOFIELD & A. C. WILLIS: Isolation and partial characterization of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*: evidence for the presence of phosphopantothenate in ACV synthetase. J. Antibiotics 44: 241~248, 1991
23) ZHANG, J. & A. L. DEMAIN: Purification of ACV synthetase from *Streptomyces clavuligerus*. Biotechnol. Lett. 12: 649~654, 1990
24) VAN LIEMPT, H.; H. VON DÖHREN & H. KLEINKAUF: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. J. Biol. Chem. 264: 3680~3684, 1989
25) BALDWIN, J. E.; J. W. BIRD, R. A. FIELD, N. M. O'CALLAGHAN & C. J. SCHOFIELD: Isolation and partial characterization of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*. J. Antibiotics 43:1055~1057, 1990
26) JENSEN, S. E.; A. WONG, M. J. ROLLINS & D. W. S. WESTLAKE: Purification and partial characterization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Streptomyces clavuligerus*. J. Bacteriol. 172: 7269~7271, 1990
27) MARTÍN, J. F. & P. LIRAS: Enzymes involved in penicillin, cephalosporin and cephamycin biosynthesis. *In* Advances in Biochemical Engineering and Biotechnology. *Ed.*, FIECHER, A., pp. 153~187, Springer Verlag, Berlin, Heidelberg, 1989
28) AHARONOWITZ, Y.; G. COHEN & J. F. MARTIN: Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation and evolution. Annu. Rev. Microbiol. 46: 461~496, 1992
29) MARTIN, J. F.: New aspects of genes and enzymes for beta-lactam antibiotic biosynthesis. Appl. Microbiol. Biotechnol. 50: 1~15, 1998
30) ADLINGTON, R. L.; J. E. BALDWIN, M. LÓPEZ-NIETO, J. A. MURPHY & N. PATEL: A study of the biosynthesis of the tripeptide δ-(L-α-aminoadipyl)-L-cyseinyl-D-valine in a β-lactam-negative mutant of *Cephalosporium acremonium*. Biochem. J. 213: 573~576, 1983
31) BANKO, G.; A. L. DEMAIN & S. WOLFE: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV synthetase): a multifunctional enzyme with broad substrate specificity for the synthesis of penicillin and cephalosporin precursors. J. Am. Chem. Soc. 109:

2858~2860, 1987

32) THEILGAARD, H. B.; K. N. KRISTIANSEN, C. M. HENRIKSEN & J. NIELSEN: Purification and characterization of *delta*-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Penicillium chrysogenum*. Biochem. J. 327: 185~191, 1997

33) JENSEN, S. E.; D. W. S. WESTLAKE & S. WOLFE: Production of the penicillin precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by cell-free extracts from *Streptomyces clavuligerus*. FEMS Microbiol. Lett. 49: 213~218, 1988

34) ZHANG, J.; S. WOLFE & A. L. DEMAIN: Biochemical studies of ACV synthetase activity from *Streptomyces clavuligerus*. Biochem. J. 283: 691~698, 1992

35) BALDWIN, J. E.; C.-Y. SHIAU, M. F. BYFORD & C. J. SCHOFIELD: Substrate specificity of L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine synthetase from *Cephalosporium acremonium*: demonstration of the structure of several unnatural tripeptide products. Biochem. J. 301: 367~372, 1994

36) COQUE, J. J. R.; J. L. DE LA FUENTE, P. LIRAS & J. F. MARTIN: Overexpression of the *Nocardia lactamdurans* α-aminoadipyl-cysteinyl-valine synthetase in *Streptomyces lividans*. The purified multienzyme uses cystathionine and 6-oxopiperidine 2-carboxylate as substrates for synthesis of the tripeptide. Eur. J. Biochem. 242: 264~270, 1996

37) DIEZ, B.; S. GUTIERREZ, J. L. BARREDO, P. VAN SOLINGEN, L. H. M. VAN DER VOORT & J. F. MARTIN: The cluster of penicillin biosynthesis genes. Identification and characterization of the *pcb*AB gene encoding the α-aminoadipyl-cysteinyl-valine synthetase and linkage to the *pcb*C and *pen*DE genes. J. Biol. Chem. 265: 16358~16365, 1990

38) SMITH, D. J.; M. K. R. BURNHAM, J. H. BULL, J. E. HODGSON, J. M. WARD, P. BROWNE, J. BROWN, B. BARTON, A. J. EARL & G. TURNER: β-Lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. EMBO J. 9: 741~747, 1990

39) CANTORAL, J. M.; S. GUTIÉRREZ, F. FIERRO, S. GIL-ESPINOSA, H. VAN LIEMPT & J. F. MARTÍN: Biochemical characterization and molecular genetics of nine mutants of *Penicillium chrysogenum* impaired in penicillin biosynthesis. J. Biol. Chem. 268: 737~744, 1993

40) GUTIERREZ, S.; B. DIEZ, E. MONTENEGRO & J. F. MARTIN: Characterization of the *Cephalosporium acremonium pcb*AB gene encoding α-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: Linkage to the *pcb*C gene as a cluster of early cephalosporin biosynthetic genes and evidence of multiple functional domains. J. Bacteriol. 173: 2354~2365, 1991

41) MACCABE, A. P.; H. VAN LIEMPT, H. PALISSA, S. W. UNKLES, M. B. R. RIACH, E. PFEIFER, H. VON DÖHREN & J. R. KINGHORN: δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. Molecular characterization of the *acv*A gene encoding the first enzyme of the penicillin biosynthetic pathway. J. Biol. Chem. 266: 12646~12654, 1991

42) MONTENEGRO, E.; F. FIERRO, F. J. FERNANDEZ, S. GUTIERREZ & J. F. MARTIN: Resolution of chromosomes III and VI of *Aspergillus nidulans* by pulsed-field gel electrophoresis shows that the penicillin biosynthetic pathway genes *pcb*AB, *pcb*C, and *pen*DE are clustered on chromosome VI (3.0 megabases). J. Bacteriol. 174: 7063~7067, 1992

43) LAICH, F.; F. FIERRO, R. E. CARDOZA & J. F. MARTIN: Organization of the gene cluster for biosynthesis of penicillin in *Penicillium nalgiovense* and antibiotic production in cured dry sausages. Appl. Environm. Microbiol. 65: 1236~1240, 1999

44) COQUE, J. J. R.; J. F. MARTIN, J. G. CALZADA & P. LIRAS: The cephamycin biosynthetic genes *pcb*AB, encoding a large multidomain peptide synthetase, and *pcb*C of *Nocardia lactamdurans* are clustered together in an organization different from the same genes in *Acremonium chrysogenum* and *Penicillium chrysogenum*. Mol. Microbiol. 5: 1125~1133, 1991

45) BARREIRO, C.; A. PISABARRO & J. F. MARTIN: Characterization of the ribosomal *rrn*D operon of the cephamycin-producer '*Nocardia lactamdurans*' shows that this actinomycete belongs to the genus Amycolatopsis. System. Appl. Microbiol. 23: 15~24, 2000

46) DORAN, J. L.; B. K. LESKIW, A. K. PETRICH, D. W. S. WESTLAKE & S. E. JENSEN: Production of *Streptomyces clavuligerus* isopenicillin N synthase in *Escherichia coli* using two-cistron expression system. J. Ind. Microbiol. 5: 197~206, 1990

47) YU, H.; H. Y. E. SERPE, J. ROMERO, J. J. R. COQUE, K. MAEDA, M. OELGESCHLÄGER, M. HINTERMANN, P. LIRAS, J. F. MARTÍN, A. L. DEMAIN & J. PIRET: Possible involvement of the lysine E-aminotransferase gene (*lat*) in the expression of the genes encoding ACV synthetase (*pcb*AB) and isopenicillin N synthase (*pcb*C) in *Streptomyces clavuligerus*. Microbiology 140: 3367~3377, 1994

48) PEÑALVA, M. A.; A. MOYA, J. DOPAZO D. RAMON: Sequences of isopenicillin N synthetase gene suggest horizontal gene transfer from prokaryotes to eukaryotes. Proc. R. Soc. London Ser. B 241: 164~169, 1990

49) LIRAS, P.; A. RODRIGUEZ-GARCIA & J. F. MARTIN: Evolution of the clusters of genes for β-lactam antibiotics: a model for evolutive combinatorial assembly of new β-lactams. Internatl. Microbiol. 1: 271~278, 1998

50) HOSKINS, J. A.; N. O'CALLAGHAN, S. W. QUEENER, C. A. CANTWELL & J. S. WOOD: Gene disruption of the *pcb*AB gene encoding ACV synthetase in *Cephalosporium acremonium*. Curr. Genet. 18: 523~530, 1990

51) FIERRO, F.; E. MONTENEGRO, S. GUTIERREZ & J. F. MARTIN: Mutants blocked in penicillin biosynthesis show a deletion of the entire penicillin gene cluster at a specific site within a conserved hexanucleotide sequence. Appl. Microbiol. Biotechnol. 44: 597~604, 1996

52) FIERRO, F.; J. L. BARREDO, B. DIEZ, S. GUTIÉRREZ, F. J. FERNÁNDEZ & J. F. MARTÍN: The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. Proc. Natl. Acad. Sci. USA 92: 6200~6204, 1995

53) KLEINKAUF, H. & H. VON DÖHREN: Nonribosomal biosynthesis of peptide antibiotics. Eur. J. Biochem. 19: 1~15, 1990

54) MARTÍN, J. F.: Nonribosomal peptides. *Ed.*, MEYERS R. A., pp. 207~217, Encyclopedia of Molecular Biology,

VCH, Germany, 1996
55) KLEINKAUF, H. & H. VON DÖHREN: A nonribosomal system of peptide biosynthesis. Eur. J. Biochem. 192: 1~15, 1996
56) VON DÖHREN, H.; U. KELLER, J. VATER & R. ZOCHER: Multifunctional peptide synthetases. Chem. Rev. 97: 2675~2705, 1997
57) CASQUEIRO, J.; S. GUTIÉRREZ, O. BAÑUELOS, F. FIERRO, J. VELASCO & J. F. MARTÍN: Characterization of the *lys*2 gene of *Penicillium chrysogenum* encoding α-aminoadipic acid reductase. Mol. Gen. Genet. 259: 549~556, 1998
58) HIJARRUBIA, M. J.; J. F. APARICIO & J. F. MARTÍN: Characterization of the *lys*2 gene of *Acremonium chrysogenum* encoding a functional (-aminoadipate activating and reducing enzyme. Mol. Gen. Genet. (in press), 2000
59) CRIADO, L. M.; J. F. MARTÍN & J. A. GIL: The *pab* gene of *Streptomyces griseus*, encoding p-aminobenzoic acid synthase, is located between genes possibly involved in candicidin biosynthesis. Gene 126: 135~139, 1993
60) TURGAY, K.; M. KRAUSE & M. A. MARAHIEL: Four homologous domains in the primary structure of GrsB are related to domains in a superfamily of adenylate forming enzymes. Mol. Microbiol. 6: 529~546, 1992
61) MARAHIEL, M. A.; T. STACHELHAUS & H. D. MOOTZ: Modular peptide synthetases involved in non-ribosomal peptide synthesis. Chem. Rev. 97: 2651~2673, 1997
62) LAMBALOT, R . H.; A. M. GEHRING, R. S. FLUGEL, P. ZUBER, M. LACELLE, M. A. MARAHIEL, R. REID, C. KHOSLA & C. T. WALSH: A new enzyme superfamily-the phosphopantetheinyl transferases. Chem. Biol. 3: 923~936, 1996
63) KLEINKAUF, H. & H. VON DÖHREN: Linking peptide and polyketide biosynthesis. J. Antibiotics 48: 563~567, 1995
64) HAESE, A.; M. SCHUBERT, M. HERRMANN & R. ZOCHER: Molecular characterization of the enniantin synthetase gene encoding a multifunctional enzyme catalysing *N*-methyldepsipeptide formation in *Fusarium scirpi*. Mol. Microbiol. 7: 905~914, 1993
65) WEBER, G.; K. SCHÖRGENDORFER, E. SCHNEIDER-SCHERZER & E. LEITNER: The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8 kilobase open reading frame. Curr. Genet. 26: 120~125, 1994
66) LÜCK-KLATTE, B.: Untersuchungen zur biosynthese von lysobactin in *Lysobacter* sp. *S. clavuligerus* 14,067 und charakterisierung von beteiligten multienzymen. Ph.D. Thesis, TU, Berlin, 1994
67) SHIAU, C.-Y.; J. E. BALDWIN, M. F. BYFORD & C. J. SCHOFIELD: δ-L-(α-Aminoadipoyl)-L-cysteinyl-D-valine synthetase: isolation of L-cysteinyl-D-valine, a 'shunt' product, and implications for the order of peptide bond formation. FEBS Lett. 373: 303~306, 1995a
68) SHIAU, C.-Y.; J. E. BALDWIN, M. F. BYFORD, W. J. SOBEY & C. J. SCHOFIELD: δ-L-(α-Aminoadipoyl)-L-cysteinyl-D-valine synthetase: the order of peptide bond formation and timing of the epimerisation reaction. FEBS Lett. 358: 35~40, 1995b
69) SHIAU, C. Y.; M. F. BYFORD, R. T. APLIN, J. E. BALDWIN & C. J. SCHOFIELD: L-δ-(α-Aminoadipoyl)-L-cysteinyl-D-valine synthetase: thioesterification of valine is not obligatory for peptide bond formation. Biochemistry 36: 8798~8806, 1997
70) AHARONOWITZ, Y.; J. BERGMEYER, J. M. CANTORAL, G. COHEN, A. L. DEMAIN, U. FINK, J. KINGHORN, H. KLEINKAUF, A. MACCABE, H. PALISSA, E. PFEIFER, T. SCHWECKE, H. VAN LIEMPT, H. VON DÖHREN, S. WOLFE & J. ZHANG: δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase, the multienzyme integrating the four primary reactions in β-lactam biosynthesis, as a model peptide synthetase. Biotechnology 11: 807~810, 1993
71) STACHELHAUS, T. & M. A. MARAHIEL: Modular structure of genes encoding multifunctional peptide synthetase required for non-ribosomal peptide synthesis. FEMS Microbiol. Lett. 125: 3~14, 1995
72) STACHELHAUS, T.; H. D. MOOTZ, V. BERGENDAHL & M. A. MARAHIEL: Peptide bond formation in nonribosomal peptide biosynthesis. Catalytic role of the condensation domain. J. Biol. Chem. 273: 22773~22781, 1998
73) SCHNEIDER, A.; T. STACHELHAUS & M. A. MARAHIEL: Targeted alteration of the substrate specificity of peptide synthetases by rational module swapping. Mol. Gen. Genet. 257: 308~318, 1998
74) CHALLIS, G. L.; J. RAVEL & C. A. TOWNSEND: Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem. Biol. 7: 211~224, 2000
75) SCHATELHAUS, T. & M. A. MARAHIEL: Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA. J. Biol. Chem. 270: 6163~6169, 1995
76) CONTI, E.; T. STACHELHAUS, M. A. MARAHIEL & P. BRICK: Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S. EMBO J. 16: 4174~4183, 1997
77) SCHLUMBOHM, W.; T. STEIN, C. ULLRICH, J. VATER, M. KRAUSE, M. A. MARAHIEL, V. KRUFT & B. WITTMANN-LIEBOLD: An active serine is involved in covalent substrate amino acid binding at each reaction center of gramicidin S synthetase. J. Biol. Chem. 266: 23135~23141, 1991
78) ISHIHARA, H.; Y. ENDO, S. ABE & K. SHIMURA: The presence of 4′-phosphopantetheine in the bacitracin synthetase. FEBS Lett. 50: 43~46, 1975
79) MARTIN, J. F. & S. GUTIÉRREZ: Molecular genetics of fungal secondary metabolites. *Eds.*, KINGHORN, J. R. & G. TURNER, pp. 214~252, Applied Molecular of Filamentous Fungi, Blackie and Son, Ltd., Glasgow, 1992
80) MARTIN, J. F.; S. GUTIERREZ & J. F. APARICIO: Secondary metabolites. *Ed.*, LEDERBERG, J., Enzyclopedia of Microbiology, Vol. 4, Academic Press (in press), 2000
81) FUMA, S.; Y. FUJISHIMA, N. CORBELL, C. D'SOUZA, M. M. NAKANO, P. ZUBER & K. YAMANE: Nucleotide sequence of 5′ portion of srfA that contains the region required for competence establishment in *Bacillus subtilis*. Nucl. Acids Res. 21: 93~97, 1993
82) STACHELHAUS, T. & M. A. MARAHIEL: Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. FEMS Microbiol. Lett. 125: 3~14, 1995
83) CHENG, Y.-Q. & J. D. WALTON: A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. J. Biol. Chem. 275: 4906~4911, 2000

84) HOFFMANN, K.; E. SCHNEIDER-SCHERZER, H. KLEINKAUF & R. ZOCHER: Purification and characterization of eucaryotic alanine racemase acting as key enzyme in cyclosporin biosynthesis. J. Biol. Chem. 269: 12710~12714, 1994

85) DE CRÉCY-LAGARD, V.; P. MARLIÈRE & W. SAURIN: Multienzyme non ribosomal peptide biosynthesis: identification of the functional domains catalysing peptide elongation and epimerisation. C.r. Acad. Sci. III 318: 927~936, 1995

86) SHOW-REID, C. A.; N. L. KELLEHER, H. C. LOSEY, A. M. GEHRING, C. BERG & C. T. WALSH: Assembly line enzymology by multimodular nonribosomal peptide synthetases: the thioesterase domain of *E. coli* EntF catalyzes both elongation and cyclolactonization. Chem. Biol. 6: 385~400, 1999

87) KALLOW, W.; J. KENNEDY, B. AREZI, G. TURNER & H. VON DÖHREN: Thioesterase domain of *delta*-(1-alpha-aminoadipyl)-1-cysteinyl-d-valine synthetase: alteration of stereospecificity by site-directed mutagenesis. J. Mol. Biol. 297: 395~408, 2000

88) SYMMMANK, H.; W. SAENGER & F. BERNHARD: Analysis of engineered multifunctional peptide synthetases. Enzymatic characterization of surfactin synthetase domains in hybrid bimodular systems. J. Biol. Chem. 274: 21581~21588, 1999

89) MOOTZ, H. D. & M. A. MARAHIEL: Design and application of multimodular peptide synthetases. Curr. Opin. Biotechnol. 10: 341~348, 1999

90) STACHELHAUS, T.; A. SCHNEIDER & M. A. MARAHIEL: Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. Science 269: 69~72, 1995

91) GEHRING, A. M.; I. MORI, R. D. PERRY & C. T. WALSH: The nonribosomal peptide synthetase HMWP2 forms a thiazoline ring during biogenesis of yersiniabactin, an iron-chelating virulence factor of *Yersinia pestis*. Biochemistry 37: 11637~11650, 1998