Multifunctional Peptide Synthetases

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I. Introduction

The biosynthesis of peptides proceeds either on the complex ribosomal machinery involving various RNA molecules, or in a RNA-independent fashion on protein templates called peptide synthetases.[1] Peptide synthetase systems integrate various enzyme activities both by gene fusion and protein complex formation. Thus multistep synthetic processes are directed by covalent catalysis, therefore avoiding free intermediates. These organizational principles are not only found in the peptide field, but also in polyketide biosynthesis. In this review the current state of research on the enzymatic formation of linear, cyclic, and branched cyclic peptides, as well as peptidolactones, depsipeptides, and acylpeptides is presented. These diverse structures are derived from a limited number of catalytic domains, which are discussed in some detail in the first section. Sets of catalytic domains comprise a functional module containing sufficient information to complete an

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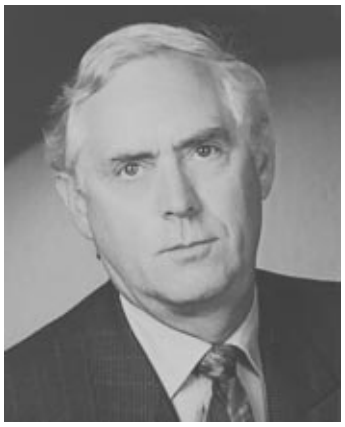
Hans von Döhren was born in Dolgowitz, Germany, in 1948. He graduated from the Technical University Berlin with a degree in chemistry in 1972. There he received his doctoral degree with Horst Kleinkauf in 1977 characterizing the structure of gramicidin S synthetases. As a postdoc he initiated work on the applications of peptide synthetases and multienzyme structure–function analysis. From 1983 to the present, he has been a lecturer in biochemistry. His laboratory is engaged in the study of peptide biosynthesis with the current main topics in the areas of mechanisms of amino acid activation and peptide bond formation, β-lactam antibiotics, and cyanobacterial toxins.

Ullrich Keller, born in 1948 in Heidelberg, Germany, studied chemistry and chemical engineering at the Technical University Berlin. After graduation in 1972 he worked as a research assistant on the structure of phospholipids in the group of W. Kreutz at the University of Freiburg. In 1974 he joined the group of Horst Kleinkauf. He received his doctoral degree in 1977 on the biosynthesis of actinomycins in *Streptomycetes*. He has been a group leader since then, continuing research in the field of acylpeptide lactones and alkaloid cyclopeptides. In 1985 he has got his habilitation in biochemistry.



Joachim Vater was born in Heidenheim, Germany, in 1938. He studied chemistry at the University of Cologne and the Technical University Berlin, where he graduated with a diploma in chemistry in 1965. He then joined the group of H. T. Witt to work in the field of the primary processes in photosynthesis and received his doctoral degree in 1972. As a postdoc he has been working with Horst Kleinkauf on bioactive peptides and photosynthetic CO_2 fixation. From 1979 to the present, he has been a lecturer in biochemistry and group leader. His current interests include the biosynthesis and biotechnological utilization of lipopeptides.

Rainer Zocher was born in Fassberg, Germany, in 1948. He studied chemistry at the Technical University Berlin, where he graduated with a diploma in chemistry in 1976. He then joined the group of Horst Kleinkauf to work in the field of *N*-methylated peptides and received his doctoral degree in 1981. As a postdoc he continued his studies on the biosynthesis of cyclosporins and related compounds. From 1991 to the present, he has been a lecturer in biochemistry and group leader. His current interests include molecular genetics of cyclopeptide producing fungi and plants and the enzymology of taxol biosynthesis.

elongation step in either peptide or polyketide biosynthesis. The key element in peptide formation is the amino acid activation domain, selecting substrate amino acids and forming adenylates. Although equivalent to aminoacyl-tRNA synthetases, both classes of enzymes share no obvious similarities.[2]

Historically, the basic features of both the ribosomal and nonribosomal systems, as well as of polyketide biosynthesis, were elucidated in the 1950s and 1960s. By 1970 ribosomes, RNA, and the required enzymes, as well as multienzymes involved in biosynthesis, had been described and functionally characterized.[3,4] Only in the last decade has the molecular basis of construction, assembly, architecture, and functioning begun to be unraveled, and is now a field of basic research in protein chemistry.[1,5] In the second part of this review the current states of research in various selected model systems are discussed. It is especially intriguing how sequences of 40 reactions (as in the biosynthesis of cyclosporins) are structurally assembled to produce complex peptides.

The products of these biosynthetic processes may be correlated or connected to functions within events of their producer's life cycles[6−8] such as iron starvation followed by induction of iron uptake systems, competing microorganisms triggering the excretion of antimicrobials, or invasion processes directed by the production of host-selective toxins. Such possible functions should be taken into consideration in exploiting natural products by screening procedures or the design of overproducers.

The evolution of many such products may be connected to their functioning in certain processes,[9] particularly mediated by macromolecular receptors or target molecules. Thus functional cycles provide selection mechanisms which may also account for local structural variations encountered in product

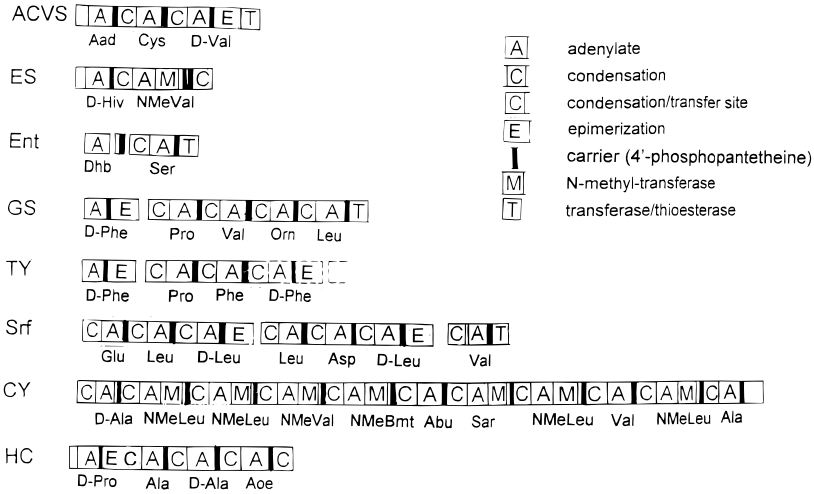


Figure 1. Arrangement of domains in peptide-forming multienzymes:[13] ACVS, ACV synthetase; ES, enniatin synthetase; Ent, enterobactin biosynthetic enzymes EntE and EntF; GS, gramicidin S synthetases 1 and 2; TY, tyrocidine synthetases 1 and 2; Srf, surfactin synthetases 1, 2, and 3; CY, cyclosporin synthetase; HC, HC-toxin synthetase. Amino or hydroxy acid specificity of the domains is indicated. Uncommon abbreviations are as follows: Aad, α-aminoadipic acid; Aeo, 2-amino-8-oxo-9,10-epoxy-decanoic acid; Bmt, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine; Dhb, dihydroxybenzoic acid; D-Hiv, D-hydroxyisovaleric acid; Nme, *N*-methyl-; Orn, ornithine; Sar, sarcosine. (Modified from ref 13.)

families. A future challenge will thus be to gain insight into natural selection and evolution mechanisms to improve our current approaches to target-oriented compound design.

The purpose of this review is to provide an update to our current understanding of enzymatic peptide-forming systems, their exploitation as synthetic tools, and the beginning of the investigation of their biological roles. Some of these aspects have been subjects of recent reviews, and these will be referred to in the respective sections.

II. The Thiotemplate Mechanism

Peptide biosynthesis requires activation of groups involved in bond formation by condensation and protection of reactive side chains to exclude unwanted side reactions. In biological peptide synthesis involves exclusively carboxyl activation, and protection is achieved by noncovalent side-chain binding to protein surfaces. The diversity of amino acid structures may account for the integration of activating and acylating functions in peptide synthetases. In contrast, synthetases of polyketide forming systems, activating acetate, propionate, and butyrate, are separate enzymes, providing CoA and derivatives of aliphatic carboxylic acids, which still have to undergo carboxylation before eventual for C−C bond formation.

Beyond the scope of this review are single-step enzyme systems, forming e.g., glutathione, pantetheine, or muropeptides; these systems form phosphate-activated carboxyl groups and have no covalent intermediates.[1,10,11]

1. The Modular Structure of Peptide Synthetases

The term *module* has been introduced into polyketide biosynthesis to describe a DNA fragment encoding all functions required to complete an elongation step within a sequentially acting multienzyme system.[12] In complete analogy, a peptide synthetase forming a peptide of *n* amino acids would thus be encoded by a DNA segment of *n* modules. Each module combines the catalytic functions for the activation as the corresponding adenylate of an amino, imino, and hydroxy acid, respectively, for the transfer of the activated carboxyl group to the cofactor 4′-phosphopantetheine, optionally to generate the derived thioester, for the epimerization or *N*-methylation of the activated intermediate, as well as for peptide bond formation. Several of these modular structures are illustrated in Figure 1. The integration of modules into multienzymes of various types is not fully understood. As will be seen in section IV, the known peptide-forming modules of biosynthetic systems of eukaryotic origin are completely integrated.

2. Carboxyl Activation

i. Adenylate Formation

Activation of amino, imino, or hydroxy acids is achieved by the formation of mixed anhydrides with adenylic acid (eq 1) (Figure 2). This process is the key reaction in enzymatic peptide biosynthesis. The adenylate forming domains represent the nonribo-

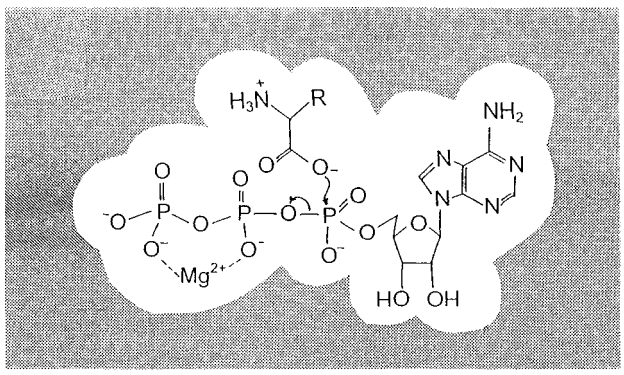


Figure 2. Scheme of adenylate formation in amino acid activation.[1] Carboxyl activation of aliphatic and aromatic acids, hydroxy acids, and imino acids proceeds in the same way. (Reprinted with permission from ref 237. Copyright 1997 Marcel Dekker Inc.)

somal code, since primary selection of substrates is located within this element. The accuracy of the ribosomal code is assured by several interactive measures involving RNA structures. The pure protein template is generally considered less precise than its ribosomal counterpart, and peptide isoforms are an indication of this interpretation. However certain positions in enzymatically formed peptides are highly conserved. Thus, positions 2, 3, and 10 of the cycloundecapeptide cyclosporin are strictly occupied by leucine, while position 8 does not discriminate among leucine, valine, and isoleucine.[14] Likewise, the echinocandin-type of acylcyclohexapeptides efficiently discriminates between proline and hydroxyproline,[15] which is essential for the antifungal action of the compound.[16] In gramicidin S, the proline site is less restrictive and accepts various substituted prolines, but not larger ring structures.[17] In actinomycin even pipecolic acid is accepted.[18] Discrimination control may take place at different steps of the overall process, to assure a particular range of product structure (see sections III.1 and III.2). Each adenylate-forming domain displays a characteristic substrate profile.

In this class of enzymes including acyl-CoA synthetases and insect luciferases the reaction involves binding a Mg^{2+} or Mn^{2+} complex of ATP. Less efficient substrates are dATP and GTP. Ca^{2+} may also replace Mg^{2+} in the reaction.[2] Since the α,β-group of the triphosphate is cleaved, the pyrophosphate−metal complex serves as the leaving group.

$$E + RCOOH + MATP^{2-} \rightleftarrows E\{RCO\text{-}AMP\} + MPP_i^{2-} \quad (1)$$

No detailed mechanism for this reaction has yet been worked out. The reaction is usually studied by the carboxyl substrate-dependent reversal of adenylation with labeled PPi. This reaction detects the adenylate, but requires binding of pyrophosphate. Random attachment of substrates is implied from kinetic data.[2,19] At higher ATP concentrations the adenylate may be transformed to A_2P_4 (eq 2), or when employing polyphosphates (eq 3) or NTPs (eq 4) to adenosine polyphosphates or dinucleotide tetraphosphates, respectively.[20−22]

$$E\{RCO\text{-}AMP\} + ATP \rightarrow E + Ap_4A + RCOOH \quad (2)$$

$$E\{RCO\text{-}AMP\} + P_n \rightarrow E + AP_{n+1} + RCOOH \quad (3)$$

$$E\{RCO\text{-}AMP\} + NTP \rightarrow E + Ap_4N + RCOOH \quad (4)$$

So far these reactions have not been studied in detail with peptide synthetases, and variations can be expected in individual enzymes. It has not been established if an adenylated enzyme is formed in the absence of the carboxyl substrate. The isotope exchange reaction is the most sensitive procedure to detect peptide synthetases, and high rates of exchange are often considered as an indication of efficient formation of adenylate. However, low rates of isotope exchange may indicate an adenylate of enhanced stability. Thus the direct measurement of aminoacyladenylate formation is also desirable.[23]

ii. Adenylate Domains

The adenylate domain has been deduced from alignments with insect luciferase and acyl-CoA synthetases and belongs to the luciferase-tyrocidine synthetase-acyl-CoA synthetase superfamily.[24] It comprises about 500 amino acid residues and is composed of two interacting subdomains.[25−27] The members of this superfamily were identified from genetic data followed by sequence comparison defining highly conserved core sequences.[1,5,28−31] A scheme of the core sequences is given in Figure 3, and motifs have been listed in the legend. Similar motifs have been defined for acyl CoA synthetases.[30,31] Functional assignments of single residues by directed mutagenesis and affinity labeling have been reviewed recently.[5]

Boundaries of the adenylate domains in integrated systems are defined by alignments with other members of the superfamily and correspond to those obtained by limited proteolysis.[25,32]

Dissection studies of gramicidin S synthetase 1 have provided some evidence for its three-domain structure.[29] An enzyme hybrid study indicated the presence of two subdomains within the adenylate

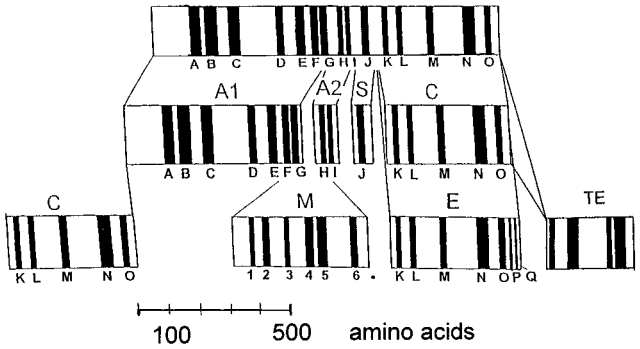


Figure 3. Domain organization of peptide synthetase modules.[13] The black areas represent highly conserved motifs, which permit the identification of these domains from sequence data. A functional module contains at least an activation domain with the two subdomains A1 and A2, the carrier domain S (for thioester), and the condensation domain C. In the case of an epimerization, where C is replaced by the epimerization domain E, an additional condensation domain is needed to complete a module. An optional domain is the *N*-methylation domain M with the motifs 1−6, which is inserted into the activation domain. Finally the thioesterase domain TE assists in chain termination steps by hydrolysis or cyclization. Slightly deviating versions of the motifs have been given, depending on the aligned sequences used[1,5,28,29] (see also sections II.4 and II.5). Currently used motifs are as follows: (adenylate domain) A1, LTxxELxxxAxxLxR; B, AVxxAxAxYVxIDxxYPxXER; C, YTSGTTGxPKG; D, IxxYGxXT; E, GELxIxGxxVAR; F, RxYRTGD, G, GRxDxQVKIRGxR; A2, H; LxxYMVP; I, LTxxGKLxRKAL; (carrier domain S) J, DNFYxLGGxSL; (condensation domain C) K, YPVSSAQKRMY; L, LxxRHEALRTxF; M, HHIIxDGxSxxIL; N, TLYxVLxxxYxVL; O, DIIVGTPxAGRxxP; (epimerization domain E) K, GExLxPIQxWFF; L, LxxxHHDALRMxY; M, HHLVVDGVSWxIL; N, ExNDILLTAxGLAL; O, LEGHGREIL; P, SRTVGWFTSMTP; Q, VPxKGVGYGILxY; R, PxxxFNYLGQF; (*N*-methyltransferase domain M) 1, GxDFxxWTSMYDG; 2, LEIGTGTGMVLFNLxxxxGL; 3, VxNSVAQYFP; 4, ExxEDExLxxPAFF; 5, HVExxPKxMxxxNELSxYRYxAV; 6, GxxVExSxARQxGxLD; thioesterase (TE) motifs have not been evaluated in sufficient detail. (Altered from ref 13.)

domain.[26] The recent elucidation of the crystal structure of the gramicidin S synthetase 1 adenylation domain,[33] which is remarkably similar to the firefly luciferase crystal structure,[27] clearly shows these subdomains. Upon substrate binding and adenylate formation a conformational change is predicted, that involves a rotational movement of the subdomains. Proteolytic studies in the presence and absence of substrates[25] suggest that the two subdomains are involved cooperatively in nucleotide triphosphate binding and stabilization of the (amino)-acyl adenylate intermediates. The mechanism of the following acylation reaction with either free CoA or the pantetheine group of the carrier proteins is still unknown.

The key event of nonribosomal peptide biosynthesis, the selection of an amino (imino, hydroxy) acid, has been tentatively assigned to the region between motifs C and D.[34] The crystal structure of the gramicidin S synthetase adenylation domain activating Phe has led to the identification of several key residues involved in substrate recognition.[33]

Motif C is considered as the signature sequence of this enzyme superfamily. This sequence is only partially visible in the crystal structure.[27] Peptide/specific antibodies raised against this motif have successfully been used to detect various peptide synthetases.[35,36] Adenylation domains with minor terminal extensions (carrying, e.g., in firefly luciferase, a signal sequence, directing the enzyme to the peroxisome) are autonomous in a variety of systems leading to acyl-CoA and aminoacyl-ACP derivatives. In peptide biosynthetic systems autonomous adenylate forming enzymes function in the transfer of aromatic acids, initiating peptide biosynthesis (see section IV.4).

3. Aminoacylation

i. Cofactor Binding

A prerequisite for peptide bond formation is formation of the relatively stable thioester. The discovery of thioesters in peptide biosynthesis lent the name *thiotemplate mechanism* for the nonribosomal peptide/forming systems. The exact nature of these thiol compounds was only recently established as cysteamines of the pantetheine moiety of enzyme-bound cofactors, in complete analogy to acyl esters of carboxylic acids.[1,5,37,38] The cofactor is attached to peptide synthetase by posttranslational modification involving highly specific CoA:4′-phosphopantetheine-protein transferases or holo peptide synthetase synthases.[39,40]

Transfer of an amino, imino or hydroxyacyl moiety to the cofactor involves the cleavage of the adenylate releasing AMP (eq 5) (Figure 4). The numbering of enzyme subsites in eq 5 indicates the adjacent sites 1 and 2 in the first module. The pH dependence of this reaction indicates prior thiolate formation, but the nature of the catalytic groups involved remains to be established.[28]

$$E^{11}\{RCO\text{-}AMP\} + E^{12}\text{-}SH \rightarrow E^{12}\text{-}S\text{-}RCO + AMP \quad (5)$$

The stability of thioesters varies with their structural properties.[41] Sterically favored intramolecular

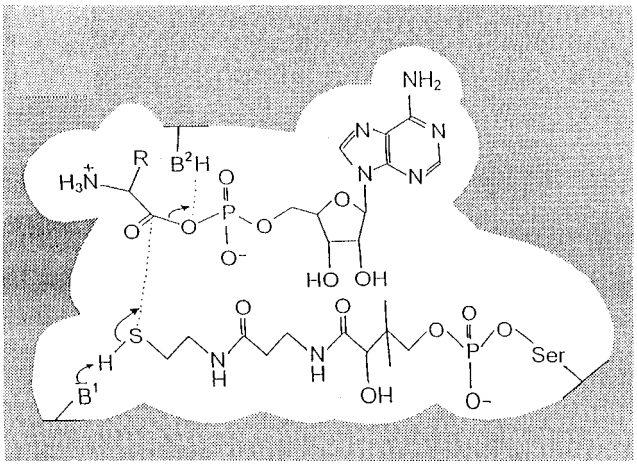


Figure 4. Scheme of the transfer of an aminoacyl residue from the mixed anhydride aminoacyladenylate to a cofactor thiol group (4′-phosphopantetheine).[1] (Modified from ref 237.)

cyclizations may take place, as in the case of ornithine, which may be the cause of unproductive side reactions.[42]

ii. Carrier Domains

Acyl, aminoacyl, and peptidyl carrier domains have been identified by alignments of peptide synthetase sequences with acyl carrier proteins/domains.[5,43,44] They have been termed thioester binding domains,[5] thioester modules,[45] or, in functional analogy, aminoacyl and peptidyl carrier proteins.[44,46] From secondary structure predictions[5,44] and comparison with the NMR-derived spatial structures of ACP from *Escherichia coli*[47] and polyketide-forming ACPs from type II systems,[47,48] a conserved structural organization was suggested. The 4′-phosphopantetheine cofactor is added in a posttranslational modification reaction by highly specific 4′-phosphopantetheine transferases[39,49,50] (eq 6). The attachment site is the highly conserved J motif:

$$\text{apo-E(Ser-OH)} + \text{CoA} \rightarrow \text{holo-E(Ser-O-P-Pan)} + 3',5'\text{-ADP} \quad (6)$$

These transferases that functionalize peptide synthetases have been shown to be part of the biosynthetic gene clusters for enterobactin, surfactin, gramicidin S, bacitracin, iturin, and nosiheptide. In some fatty acid synthases the transferase function may be an integrated domain.[47] The family of known pantetheine-transferases were identified by two consensus motifs found by a refined homology search[39] based on the structure of the ACP modifying transferase from *E. coli*.[50]

Each activation domain is connected to at least one carrier domain, and the mechanism of peptide bond formation is currently described by the multiple carrier model[5,38] (see sections IV.1 and IV. 7). In analogy to type II fatty acid and polyketide synthetase systems, the carrier protein interacts with the respective transferase and condensing enzymes to promote directed peptide bond formation. This implies that the carrier domain, despite its integration between the adenylate and condenation/epimerization domains, has to interact with adjacent con-

densing domains, suggesting highly flexible multidomain structures.

Sequence alignments of carrier proteins and domains lead to a clustering of functionally similar groups.[44] These groups combine those with function in fatty acid synthesis, polyketide synthesis, both type I and II, respectively, and condensations involving L- or D-amino acids and *N*-methylated residues.

4. Modification Reactions

i. Types of Reactions

Modification of amino acid residues may occur (a) before entrance to the catalytic peptide forming cycle, that is prior to activation, (b) at the aminoacyl stage preceding elongation, (c) after elongation at the peptidyl intermediate stage, or (d) following termination as a product transformation reaction. Studies to date have been restricted to epimerization and *N*-methylation. Numerous peptides contain *N*-methylated amino acids in their peptide chains.[51] Biochemical evidence has indicated that in the case of enniatin, cyclosporin, and actinomycin biosynthesis, the *N*-methylation takes place after covalent binding of the amino acid and during step b at the thioester stage,[51] with *S*-adenosyl-L-methionine (AdoMet) as substrate:[52−54]

$$E^1\text{-}S^1A^1 + SAM \rightarrow E^1\text{-}S^1NMe\text{-}A^1 + SAhC \quad (7)$$

There is evidence for several of the various modification routes:

(a) Modified amino acids can be present in the cell extract, and these compounds can serve as substrate for the respective activation domain. Transforming enzymes (e.g., racemases) can be detected and separated from the peptide synthetases.[55]

(b) Unmodified amino acids are substrates, but modified amino acids may be accepted as well. In the case of epimerization, both epimers can be found as covalent enzyme intermediates, although the ratios apparently do not represent true racemates.[56] Epimerization at the aminoacyl stage seems to be limited to reactions initiating peptide synthesis (gramicidin S, tyrocidine, HC toxin); control of stereospecificity is exerted at the level of peptide bond formation.

(c) Epimerization of in-chain residues appears to be the main route of amino acid epimerization. Such peptidyl intermediates have been detected in actinomycin[57] and penicillin[58] biosynthesis.

(d) Examples of product modification have not been observed so far, but presumably will be found, e.g., in glycosylating systems. Modifying enzymes may be targets for disruption, and thus permit knockout as well as transfer of modifying activities.

ii. Epimerization Domains

From the limited information available, the reaction scheme in Figure 5 gives the current tentative view of the epimerization reaction:[59]

$$E^1\text{-}S^1A^1 \rightarrow E^1\text{-}S^1{}_{D,L}\text{-}A^1 \quad (8)$$

$$E^n\text{-}S^nA^nA^{n-1}...A^1 \rightarrow E^n\text{-}S^n{}_{D,L}\text{-}A^nA^{n-1}...A^1 \quad (9)$$

Epimerization and the condensation domains have been analyzed by sequence alignments in some detail

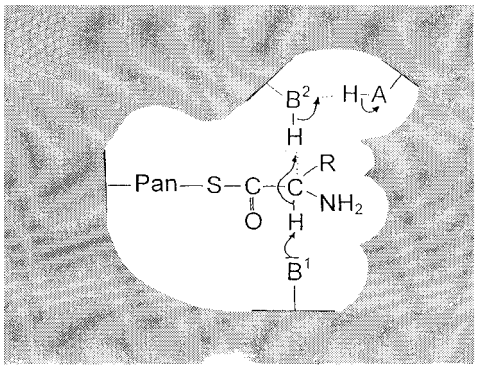


Figure 5. Scheme of epimerization of thioester-bound amino acid residues encountered in gramicidin S and tyrocidine biosynthesis.[1] (Reprinted with permission from ref 237. Copyright 1997 Marcel Dekker Inc.)

by de Crécy-Lagard et al.[60] Some of the motifs have been identified earlier,[45,61] while additional motifs have been described by Stein and Vater[5] and Pfeifer et al.[28] These motifs have been compiled in Figure 3. Motifs similar to the highly conserved M motif (originally termed the spacer motif) HHxxxDG have also been detected in chloramphenicol acetyl transferases and dihydrolipoamide transferases,[60] suggesting the catalytic involvement in peptide bond formation and epimerization of aminoacyl and peptidyl intermediates. Surprisingly, both types of domains are structurally similar despite their different functions. That each type of domain should be monofunctional follows from the inspection of peptide synthetase structural organization deduced from sequence data[60] (Figure 1). Condensation of epimerized intermediates requires the successive action of an epimerizing and a condensing domain, which is achieved by the interaction of multienzymes involving their C-terminal of the epimerization domain and the respective N-terminal of the condensation domain.[5,60] An integrated system adding a D-amino acid, HC toxin synthetase, contains a large epimerization−condensation domain fusion.[62]

iii. N-Methyltransferase Domains

Haese et al.[63] showed that the amino acid-activating domain of enniatin synthetase (Esyn), which catalyzes cyclohexadepsipeptide synthesis from branched chain amino acid and D-2-hydroxyisovalerate, contains an additional stretch of 450 amino acids inserted between the conserved motifs E and F, corresponding to the *N*-methyltransferase domain (Figure 3). No such insertion is seen in the hydroxy acid-activating domain of Esyn. The *N*-methylation domain was also observed in the sequence of the gene of cyclosporin synthetase where it is present in seven of the 11 amino acid activation domains,[64] consistent with the structure of the cycloundecapeptide cyclosporin A which contains seven *N*-methylated amino acids. All methyl transferase domains can be identified by a set of highly conserved motifs.[65] It is striking that no autonomous transferases have been found so far.

5. Peptide Bond Formation

The peptide bond is formed upon the interaction of two carrier domains with an elongation domain, permitting the contact of two thioesters with a

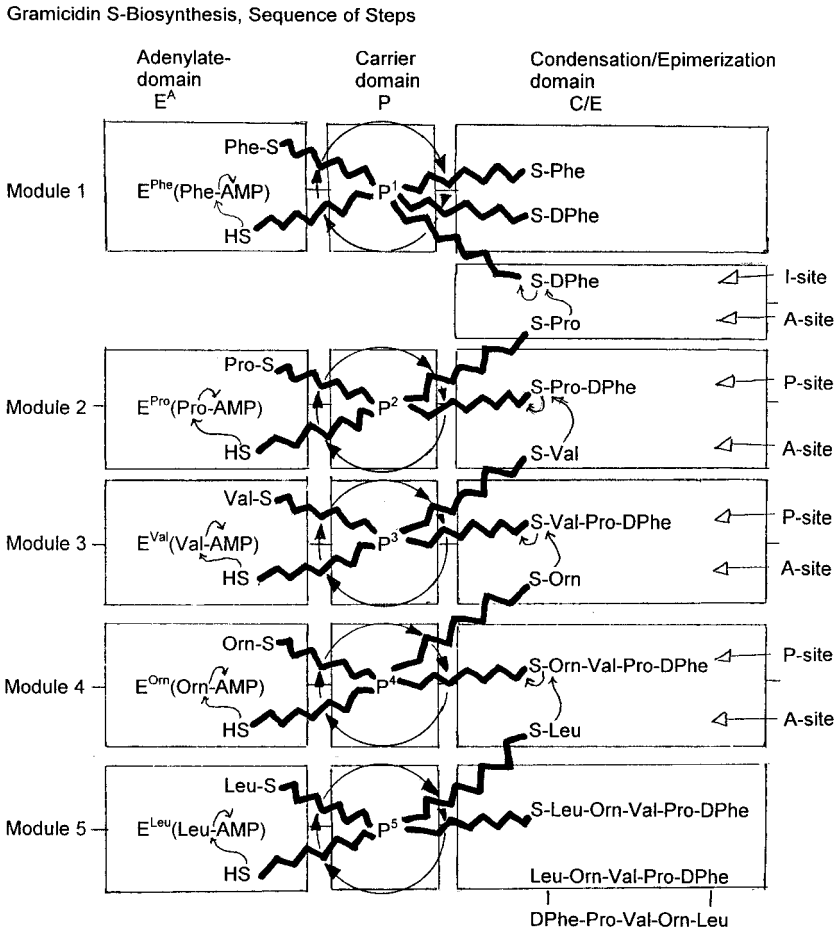


Figure 6. Sequence of reactions and modular organization of gramicidin S biosynthesis. Note the covalent linking of the domains into two multienzymes. So module 1 is formed by the adenylate domain E^{phe}, the carrier domain P^1, an epimerization domain, all three combined in gramicidin S synthetase 1, and the first condensation domain of gramicidin S synthetase 2. Each condensation contains a donor and acceptor (or in analogy to the ribosomal system peptidyl and aminoacyl site), termed I (for initiation) or P (for peptidyl) and A (for aminoacyl) site. The mode of cyclization is still unknown.

respective catalytic site. Initiation thus requires the interaction of adjacent modules, each carrying an aminoacyl thioester. The peptide bond is formed at the condensation site of the first module, the product being generated as a peptidyl thioester at the adjacent module (eq 10). The reaction sequence is illustrated with the gramicidin S biosynthetic sequence in Figure 6.

$$E^1\text{-}S^1A^1 + E^2\text{-}S^2A^2 \rightarrow E^2\text{-}S^2A^2A^1 + E^1\text{-}SH \quad (10)$$

Elongation proceeeds likewise from an aminacyl thioester and a peptidyl thioester:

$$E^n\text{-}S^nA^nA^{n-1}...A^1 + E^{n+1}\text{-}S^{n+1}A^{n+1} \rightarrow E^{n+1}\text{-}S^{n+1}A^{n+1}A^nA^{n-1}...A^1 + E^n\text{-}SH \quad (11)$$

If racemic mixtures of aminoacyl or peptidyl intermediates are present, condensations proceed stereoselectively:

$$E^1\text{-}S^1{}_{D,L}\text{-}A^1 + E^2\text{-}S^2A^2 \rightarrow E^2\text{-}S^2A^2{}_D\text{-}A^1 + E^1\text{-}SH \quad (12)$$

$$E^n\text{-}S^nA^nA^{n-1}...A^1 \rightarrow E^n\text{-}S^n{}_{D,L}\text{-}A^nA^{n-1}...A^1 + E^{n+1}\text{-}S^{n+1}A^{n+1} \rightarrow E^{n+1}\text{-}S^{n+1}A^{n+1}{}_D\text{-}A^nA^{n-1}...A^1 + E^n\text{-}S^nH \quad (13)$$

In the case of misacylation of an L-leucine site with the D epimer, the peptide catalytic cycle was interrupted,[66] implying epimer selection at the catalytic site. In the case of hydroxy acids the formation of ester bonds occurs likewise.[52,53]

6. Termination Reactions

Release of the completed peptidyl intermediates at the final module either leads to linear (hydrolysis, eq 14, or aminolysis, eq 15) or cyclic products (cyclopeptides, peptidolactones, eq 16):

$$E^n\text{-}S^nA^nA^{n-1}...A^1 + OH^- \rightarrow E^n\text{-}S^nH + A^nA^{n-1}...A^1 \quad (14)$$

$$E^n\text{-}S^nA^nA^{n-1}...A^1 + RNH_2 \rightarrow E^n\text{-}S^nH + A^nA^{n-1}...A^1\,NHR \quad (15)$$

$$E^n\text{-}S^nA^nA^{n-1}...A^1 \rightarrow E^n\text{-}S^nH + \textit{cyclo}\text{-}A^nA^{n-1}...A^1 \quad (16)$$

Cyclizations involve terminal amino groups or, internal hydroxy or amino groups (peptidolactones or branched peptides) or proceed by fragment condensations (cyclopeptides and cyclodepsipeptides).

Most if not all peptide biosynthetic clusters contain genes or domains with similarities to thioesterases.[34,67−73] From analogy to fatty acid and polyketide biosynthetic systems[74] these two classes of related enzymes are thought to catalyze the release or cyclization of completed peptide chain intermediates. This function is also supported by their positioning at the C-terminal regions of peptide synthetase multienzymes (according to the sequence of the reactions). The integrated thioesterase of ACV synthetase catalyzes the stereospecific release of the penicillin tripeptide precursor (see section IV.1). In the seven-module surfactin system, insertion of the thioesterase domain behind module four or five releases the corresponding tetra- and pentapeptide, respectively.[75] Since these results were obtained *in vivo*, it remains to be established if the thioesterase domain actually catalyzes lactonization of the shortened lipopeptides. It is evident from Figure 1 that not all synthetases contain an *integrated* thioesterase domain.

7. The Thiotemplate Mechanism

Gramicidin S synthetase is used as a model system to illustrate the thiotemplate mechanism. This cyclic decapeptide antibiotic with potent membrane active properties is produced by some strains of *Bacillus brevis*. It is composed of two identical pentapeptides D-Phe-Pro-Val-Orn-Leu (Figure 6). Its biosynthesis has been investigated in detail in several laboratories.[3,11,77] Gramicidin S synthetase is one of the best-characterized peptide-forming systems. The reaction sequence comprises 16 individual steps and is accomplished by the cooperation of two multifunctional polypeptides (Figure 7). Gramicidin S synthetase 1 (GS1, 127 kDa) is a phenylalanine racemase which activates and racemizes phenylalanine and transfers D-Phe to the condensing multienzyme gramicidin S synthetase 2 (GS2, 508 kDa). GS2 in turn activates L-Pro, L-Val, L-Orn, and L-Leu and catalyzes the elongation reactions leading to the synthesis of two presumably identical pentapeptide intermediates that are assembled head-to-tail by an as yet unknown termination mechanism. Proposals have been made for intramolecular[78] as well as intermolecular cyclization.[79] Early termination of the growing peptide chain can take place by side reactions, such as piperazinedione formation of the D-Phe-Pro dipeptide intermediate[80] or by cyclization of the thioester-bound, C-terminal ornithine of the tetrapeptide D-Phe-Pro-Val-Orn to 3-amino-2-piperidone.[40,42]

Biosynthesis of gramicidin S is encoded by the *grs* operon (Figure 7) which comprises three genes, *grsT*,[67] *grsA*,[67,81] and *grsB*.[73,82] The *grsA* and *grsB* genes code for gramicidin S synthetase 1 and 2. They are composed of one and four modules, respectively, that bear the information for the activation and condensation of Phe, L-Pro, L-Val, L-Orn, and L-Leu, in accordance with the colinearity rule. The *grsT* gene shows a high homology to vertebrate thioesterases of type 2, but its function is still unknown. Upstream of the 5′-end of the *grs* operon of *B. brevis* is located the *gsp* gene encoding the 4′-phosphopantetheine transferase.[83] The function of the *gsp* gene product is the posttranslational modification of the serine residues at each of the thiolation sites of gramicidin S synthetase, thereby converting the apo form of this multienzyme into its holo form. The *gsp* gene is able to complement in trans a defect in the *sfp* gene (*sfp*^{o}), promoting the production of surfactin.

Gramicidin S synthetase activates its substrate amino acids in a two-step process followed by aminoacyl adenylation in the first and aminoacyl thioesterification at specific reactive thiol groups (thiotemplates). A first mechanistic concept for this type of nonribosomal peptide biosynthesis, termed the "Multienzymatic Thiotemplate Mechanism" was presented in the early 1970s,[3,77,84] based mainly on knowledge compiled by the functional characterization of gramicidin S and tyrocidine synthetase. In analogy to fatty acid synthetase it was assumed that specific cysteines at the reaction centers would form the thiotemplate sites and that the thiol group of an intrinsic, central 4′-phosphopantetheine carrier would interact with the thioester-bound substrate amino acids and peptide intermediates at the reaction centers, leading to a step-by-step elongation of the growing peptide chain in a series of transpeptidation and transthiolation reactions.

Recent protein chemical and genetic characterization of gramicidin S synthetase has led to a revision of the original thiotemplate model.[85] In particular, from the gene sequences of gramicidin S synthetases and ACV synthetases, the modular architecture of

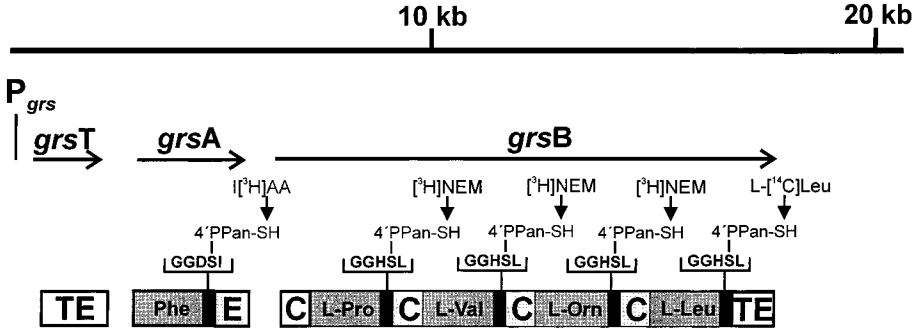


Figure 7. The organization of the gramicidin S biosynthetic system both on the gene and the protein level. The biosynthesis of gramicidin S in *B. brevis* is coded by the *grs* operon comprising three genes *grsT*, *grsA*, and *grsB*. *GrsT* codes for an enzyme homologous to vertebrate thioesterases of type II (TE). *grsA* and *grsB* encode gramicidin S synthetases 1 and 2 (GS1 and GS2), the components of the gramicidin S-forming multienzyme system. GS1 consists of one module for the activation and racemization of phenylalanine. GS2 contains four modules which activate the other four amino acids L-Pro, L-Val, L-Orn, and L-Leu and assemble the cyclic decapeptide product. The affinity labeling of the five 4′-phosphopantetheine cofactors attached to the reactive serine residues of the thiolation motifs of gramicidin S synthetase by iodoacetic acid and *N*-ethylmaleimide is indicated.

Table 1. Structures of Radiolabeled Thiotemplate Site Peptide Fragments of Gramicidin S Synthetase[a-c]

Enzyme	Thio-template	Structure of the radiolabeled fragments from the thiolation sites	Molecular mass calculated: gene	4′PPan-adduct	measured: ESI-MS
GS1	Phe	DNFYALGGDSIK └4′PPan-[^{3}H]NES	1299.4	1764.9	1764.4
GS2	L-Pro	IWEEVLGISQIGIQDNFFSLGGHSLR └4′PPan-[^{3}H]NES	2888.4	3353.8	3352.4
	L-Val	LGGHSLR └4′PPan-[^{3}H]NES	739.0	1204.3	1204.0
	L-Orn	VGIHDDFFTIGGHSLK └4′PPan-[^{3}H]NES	1742.9	2208.3	2208.0
	L-Leu	FELGGHSLKATLL └4′PPan-L[^{14}C]Leu	1385.6	1848.9	1849.1

[a] The molecular masses of the peptides were calculated from the gene derived sequence. [b] The molecular masses of the peptides were calculated as the sum of the molecular masses of the peptide moiety, the 4′-phosphopantetheine substituent which is covalently attached to the serine residue, and the radioactively labeled tracer (NES: N-ethylsuccinimido) bound to the reactive thiol group of the Pan cofactor. [c] Results of the investigation of the active-site peptide fragments by electrospray mass spectrometry (ESI-MS). (Altered from ref 38.)

such multifunctional proteins was convincingly established.[1,5,86] The identification of the thiotemplate sites was achieved by affinity labeling of the thioester binding sites of gramicidin S synthetase by covalent thiolation with substrate amino acids or by alkylation with radioactive thiol inhibitors, such as *N*-ethylmaleimide or iodoacetic acid using the substrate protection technique.[37,38,85,87] The enzyme−substrate/inhibitor complexes were digested both chemically and enzymatically. The active-site peptides were isolated in pure form by multistep HPLC procedures. By peptide sequencing in combination with sequence comparisons with the *grsA* and *grsB* genes a specific, highly conserved motif, LGGH/DSL/I, was detected at the thiolation site of each module. It was shown that the central serine residue at each reaction center was involved in covalent binding of the substrate amino acid, but the thioester binding site could not be identified, because the radiolabel was always lost in the first Edman degradation step. In neither case was a cysteine found in the active-site peptides, as would be expected from the original thiotemplate model. By sequence comparisons with acyl carrier proteins/domains of fatty acid and polyketide synthetases, the H/DSL/I core of the thiolation motif was identified as a binding site for a 4′-phosphopantetheine cofactor.[85] The location of the 4′-phosphopantetheine substituent attached to the serine residue of the thiolation motif was proven by fragmentation data obtained from FAB-MS and collision induced dissociation electrospray MS (Table 1). From the fragmentation pattern of the active site peptides a series of N- and C-terminal sequence ions was obtained determining their structures. The nature of the substituent was supported by amino acid analysis. One mole of each active-site peptide of gramicidin S synthetase contained ~1 mol of β-alanine, a constituent of 4′-phosphopantetheine. From these results evidence was obtained that all five amino acid-activating modules of gramicidin S synthetase are equipped with a separate 4′-phosphopantetheine prosthetic group esterified to the active serine in the H/DSL/I core of their respective thiolation motifs. The cysteamine thiol groups represent the thioester binding sites for the substrate amino acids, instead of cysteines as proposed in the original version of the thiotemplate hypothesis.

On the basis of these results, a multiple-carrier model of nonribosomal peptide biosynthesis was suggested which is in perfect accord with present knowledge.[38] According to this mechanism, the peptide chain is assembled by sequential interactions of the pantetheinyl carriers in a series of transpeptidation steps. The new model allows a much simpler and more straightforward description of the biosynthetic process than the old version, because the assumption of a central carrier and transthiolation reactions for the transport of the peptide intermediates can be omitted. In addition, the charging of a multienzyme with all its peptide intermediates, not understandable by the former hypothesis, can be easily explained by the multiple carrier concept. From the present knowledge of the structure−function relationships of peptide synthetases, three specific sites were implied for the pantetheinyl carrier within an amino acid-activating module which are involved in the biosynthetic process: A charging position for thioester formation with the substrate amino acid as well as a peptidyl acceptor and a peptidyl donor site which would correspond to the A and P sites of peptidyltransferase in the ribosomal system. In one elongation step the following three elementary steps were postulated, as is apparent from Figure 6:

(1) The pantetheinyl carrier of an amino acid-activating domain is charged with its cognate amino acid AA_i. This reaction most probably occurs within the adenylation domain of this module.

(2) The pantetheinyl carrier within this module translocates the activated amino acid to the peptidyl acceptor site. By interaction with the pantetheinyl cofactor of the preceding module which can be thioesterified either by a substrate amino acid or a peptide intermediate, a new peptide bond is formed in a transpeptidation process.

(3) The elongated peptidyl chain covalently bound to the pantetheinyl carrier is translocated to the peptidyl donor site within this module. By interaction with the aminoacylated pantetheinyl cofactor of

Table 2. Selection of Currently Produced Peptides of Commercial Importance

compound	properties	producer organism	structural type
penicillin	antibacterial	*Penicillium chrysogenum*	modified tripeptide
cephalosporins	antibacterial	*Acremonium chrysogenum*	modified tripeptide
clavulanic acid	β-lactamase inhibitor	*Streptomyces clavuligerus*	modified amino acid
Bialaphos	herbicide	*Streptomyces hygrocsopicus*	tripeptide
pristinamycins	antibacterial	*Streptomyces pristinaspiralis*	mixture of a peptidolactone and peptolide
actinomycin	antitumor	*Streptomyces antibioticus*	chromobipentapeptidolacone
viridogrisein	antibacterial	*Streptomyces viridogriseus*	chromobipentapeptidolacone
enniatins	antibiotic	*Fusarium* sp.	cyclohexadepsipeptide
A47934	antibacterial	*Streptomyces toycaensis*	modified heptapeptide
vanocomycin	antibacterial	*Amycolatopsis orientalis*	modified heptapeptide
teichoplanin	antibacterial	*Amycolatopsis teichomyceticus*	modified heptapeptide
ardacin	antibacterial	*Kibdelosporangium aridum*	modified heptapeptide
surfactin	surfactant, antiviral	*Bacillus subtilis*	acylheptapeptidolactone
bacitracin	antibacterial	*Bacillus licheniformis*	dodeca(heptacyclo)peptide
cyclosporin	immunosuppressant	*Tolypocladium niveum*	cycloundecapeptide

the subsequent module the next peptide bond can be formed.

Most probably the region between two adjacent modules contains the site for the interaction between their pantetheinyl carriers, including the peptidyl donor site for the first cofactor and the peptidyl acceptor site for the subsequent swinging arm. In Figure 6 the intermediate steps in the biosynthesis of gramicidin S are shown as an illustration of the multiple carrier model as a new mechanistic concept for nonribosomal peptide biosynthesis at multienzymatic templates.

In the first step the modules of gramicidin S synthetase catalyze the aminoacyl adenylation of their cognate amino acids followed by aminoacylation at the cysteamine thiol groups of the phosphopantetheine carriers at specific charging sites adjacent to the adenylation domains. The energy for this process is provided by the hydrolysis of an ATP α,β linkage resulting in the release of AMP and pyrophosphate PP_i. The elongation cycle starts with the interaction of gramicidin S synthetase 1 (GS1) and gramicidin S synthetase 2 (GS2), forming the initiation complex. Most probably the N-terminal condensation domain of GS2 is involved in this reaction. The pantetheinyl carrier of GS1 transports phenylalanine to the epimerization domain where epimerization occurs. The dipeptide is then formed at the condensation domain by nucleophilic attack of the amino group of L-Pro (P-Pro carrier in its acceptor site) at the thioester activated carboxyl C-atom of D-Phe (P-Phe carrier in its donor site). The product of this reaction is a free thiol group of the P-Phe carrier which binds Phe again and starts a new cycle of elongation. The pantetheinyl carrier of the proline-activating module thioesterified with the dipeptidyl intermediate D-phenylalanyl-L-proline is now translocated to its donor site. By interaction with the L-valyl P-Val carrier in its acceptor site the tripeptide D-Phe-L-Pro-L-Val is formed in a transpeptidation reaction. In a similar manner the tetra- and pentapeptide intermediates D-Phe-L-Pro-L-Val-L-Orn and D-Phe-L-Pro-L-Val-L-Orn-L-Leu are assembled, involving the individual pantetheinyl carriers of the ornithine and leucine activating modules of GS2. Finally the decapeptide gramicidin S is formed by cyclization of two pentapeptide moieties by an as yet unknown mechanism.

III. Applications of Biocatalysts in Peptide Synthesis

1. *In Vivo* Synthesis of Peptides[56,88]

Synthesis of peptides by microbial cultures is carried out commercially, and well-known examples involving multifunctional peptide synthetases are compiled in Table 2. Directed biosynthesis by feeding immediate precursor amino acids has been studied in the cases of enniatins, cyclosporins, ergot peptides, and viridogriseins.[88] A recent example is the formation of the powerful antifungal nonapeptidolactone aureobasidin.

Aureobasidins are products of the black yeast *Aureobasidium pullulans* R106.[89−93] Peptide fractions from fermentation broths have so far yielded 29 structural analogues without precursor feeding (Figure 8). Although the residues in positions 3,5, and 8 have been invariant in nonsupplemented fermentation media, precursor-directed biosynthesis

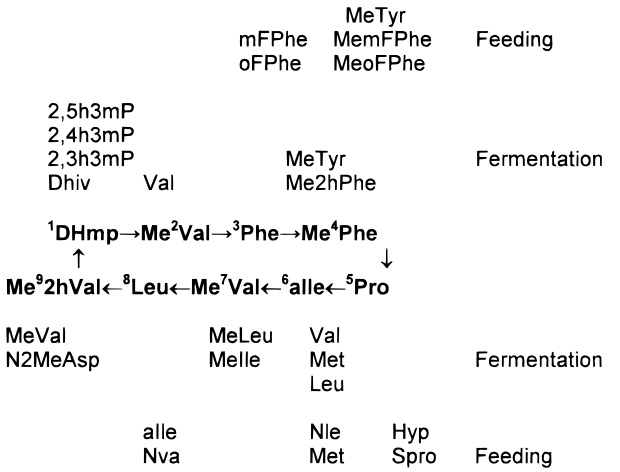


Figure 8. Analogs of the antifungal aureobasidin isolated from fermentations and directed biosynthesis (feeding). Abbreviations used are as follows: 2hPhe, 2-hydroxyphenylalanine; 2,3h3mP, 2,3-hydroxy-3-methylpentanoic acid; 2,4h3mP, 2,4-hydroxy-3-methylpentanoic acid; 2,5h3mP, 2,5-hydroxy-3-methylpentanoic acid; 4hPro, 4-hydroxyproline; aIle, *allo*-isoleucine; Cit, citrulline; D, prefix for D-configuration, used without hyphen; Hiv, hydroxyisovaleric acid; Hmp, hydroxymethylpentanoic acid; Hyp, 4-hydroxyproline; Me, prefix used for *N*-methyl; mFPhe, *m*-fluorophenylalanine; N2MeAsp, *N*,2-methylaspartic acid; Nle, norleucine; Nva, norvaline; oFPhe, *o*-fluorophenylalanine; Spro, thioproline.[32] (Modified from ref 56.)

Scheme 1

$$^{1}\text{DAla}\rightarrow\text{MeLeu}\rightarrow\text{MeLeu}\rightarrow\text{MeVal}\rightarrow{}^{5}\text{MeBmt}$$
$$\uparrow \qquad\qquad\qquad\qquad \downarrow$$
$$\text{Ala}\leftarrow\text{MeLeu}\leftarrow\text{Val}\leftarrow{}^{8}\text{MeLeu}\leftarrow\text{MeGly}\leftarrow{}^{6}\text{Abu}$$

Scheme 2

$$^{1}\text{DAla}\rightarrow\text{MeLeu}\rightarrow\text{MeLeu}\rightarrow\text{MeVal}\rightarrow{}^{5}\text{MeBmt}$$
$$\uparrow \qquad\qquad\qquad\qquad \downarrow$$
$$\text{Ala}\leftarrow\text{MeLeu}\leftarrow\text{Val}\leftarrow{}^{8}\text{MeIle}\leftarrow\text{MeGly}\leftarrow{}^{6}\text{Thr}$$

permitted their substitution.[94] An interesting chemical approach to their modification is a tandem retro-aldol,aldol reaction replacing the amino acid that can contribute the acyl moiety of the lactone bond.[95] This work has demonstrated the importance of the configuration and presence of the tertiary alcohol (2-hydroxy-L-valine) for biological activity. Nonhydroxylated derivatives were inactive against *Cryptococcus*.

Complications of feeding approaches originate from the possible metabolism of the precursors. Thus attempts have been made to replace the initiating D-alanine of cyclosporine (Scheme 1) with D-threonine. Surprisingly, addition of D-threonine led to the formation of *N*-methyl-[8]isoleucyl-cyclosporin[96] (Scheme 2). Presumably the D-threonine is metabolized to L-*allo*-threonine by the nonspecific alanine racemase, providing D-alanine for initiation of cyclosporin synthesis. D-Threonine not being a substrate for cyclosporin synthetase may thus enter the isoleucine pathway. As *in vitro* studies of cyclosporin synthetase have shown, the D-alanine activation site did accept D-alanine, D-serine, and related derivatives, but not D-threonine.[97] Alternatively, as known from the the bacterium *Serratia marcescens*, a specific D-threonine dehydratase may be induced and directly convert D-threonine to L-isoleucine. Site 4 of cyclosporin synthetase, which activates leucine differs from the other leucine-activating sites by its low level of discrimination between leucine and isoleucine. The initial ratio of these amino acids is 5:1 in the medium employed, and the shift in this ratio leads to a cyclosporin analogue devoid of immunosuppressive activity, that, nonetheless, retains affinity to cyclophilin, but no longer binds to calcineurin.[98] Physiological effects on the producer have not been followed, but interestingly the analogue has potential anti-HIV1 activity.

2. *In Vitro* Generation of Peptides[56]

The cell-free biosynthesis or *in vitro* production of peptides requires the efficient handling of enzyme systems. Such enzyme systems have been isolated from microbial cells, and from overexpression of their respective genes. To obtain active enzyme systems, (1) the phase of growth or induction of active production of the metabolite has to be established, (2) a suitable time of harvest permitting the isolation of active and stable enzyme(s) has to be determined, and (3) the conditions for *in vitro* synthesis and stabilization have to be evaluated. Cell-free systems from eukaryotic organisms such as filamentous fungi that actively produce peptides for periods of at least days, and are less sensitive to the time of harvest. Bacterial cultures, such as bacilli, usually have short production phases, and may require harvesting periods of a few hours, requiring cooling of the fermentation broth and high-efficiency flow-through cen-

Table 3. Peptide Synthetases with Established *in Vitro* Activity

peptide	organism	structural type[a]	gene cloned	enzymology
		Linear Peptides		
ACV	*Aspergillus nidulans*	P-3	+	+
	Penicillium chrysogenum		+	+
	Acremonium chrysogenum		+	+
	Streptomyces clavuligerus		+	+
	Nocardia lactamgenus		+	+
ergotpeptides	*Claviceps purpurea*	R-P-3-M	−	+
linear gramicidin	*Bacillus brevis*	P-15-M	−	+
alamethicin	*Trichoderma viride*	P-19-M	−	+
		Cyclopeptides		
ferrichrome	*Aspergillus quadricinctus*	C-6	−	+
gramicidin S	*Bacillus brevis* ATCC 9999	C-(P-5)$_2$	+	+
tyrocidine	*Bacillus brevis* ATCC 8185	C-10	+	+
cyclosporin	*Tolypocladium nivea*	C-11	+	+
mycobacillin	*Bacillus subtilis*	C-13	−	+
		Lactones		
actinomycin	*Streptomyces chrysomall*	R-(L-5)$_2$	−	+
surfactin	*Bacillus subtilis*	L-8	+	+
SDZ90-215	*Septoria* sp.	L-10	−	+
SDZ214-103	*Cylindrotrichum oligosporum*	L-11	(+)	+
		Branched Polypeptides		
polymyxin	*Bacillus polymyxa*	R-P-10-C-7	−	+
bacitracin	*Bacillus licheniformis*	R-P-12-C-7	(+)	+
		Depsipeptides		
enniatin	*Fusarium oxysporum, F. scirpi*	D-6	+	+
beauvericin	*Beauveria bassiana*	D-6	−	+

[a] The abbreviations used are as follows: P, peptide; C, cyclopeptide; L, lactone; D, depsipeptide; E, ester; R, acyl; M, modified. The structural types are defined by the number of amino, imino, or hydroxy acids in the precursor chain. The ring sizes of cyclic structures are indicated in the number following C, L, D, or E, defining the type of ring closure.

Scheme 3

$$^{1}\text{DPhe} \rightarrow {}^{2}\text{Pro} \rightarrow {}^{3}\text{Val} \rightarrow {}^{4}\text{Orn} \rightarrow {}^{5}\text{Leu}$$

$$\uparrow \qquad\qquad\qquad\qquad \downarrow$$

$$^{5'}\text{Leu} \leftarrow {}^{4'}\text{Orn} \leftarrow {}^{3'}\text{Val} \leftarrow {}^{2'}\text{Pro} \leftarrow {}^{1'}\text{DPhe}$$

Scheme 4

$$^{1}\text{DPhe} \rightarrow {}^{2}\text{Pro} \rightarrow {}^{3}\text{Phe} \rightarrow {}^{4}\text{DPhe} \rightarrow {}^{5}\text{Asn}$$

$$\uparrow \qquad\qquad\qquad\qquad\qquad \downarrow$$

$$^{10}\text{Leu} \leftarrow {}^{9}\text{Orn} \leftarrow {}^{8'}\text{Val} \leftarrow {}^{7}\text{Tyr} \leftarrow {}^{6}\text{Gln}$$

trifugation or filtration. The design of continuous culture conditions may be helpful in such cases.[99,100] Several multienzymes can be prepared from lyophilized mycelia or frozen cell materials.[56]

A list of established cell-free systems forming peptides has been compiled in Table 3. Most researchers have employed limiting amounts of radiolabeled precursors to facilitate detection of products by TLC methods. Thus comigration or the occurrence of similar peptides in the case of amino acid analogues have often been the only means of identification. Since definite proof of structural identity was often absent, functional assays have been used instead. Since substrates generally stabilize these fairly unstable proteins, a nonlimiting supply of substrates is advantageous. HPLC/MS methods are now the method of choice for assay. The preparation and stabilization of multienzyme systems may be still difficult, however.

The gramicidin S (Scheme 3) and tyrocidine (Scheme 4) synthetase systems consist of two and three multienzymes, respectively (see section IV.7). For the synthesis of these peptides and their analogues, both partially and completely purified protein preparations were employed.[101] The question of whether *in vitro* systems could compete with fermentation procedures for peptide production[102,103] has been addressed for gramicidin S synthetase. Efficient stabilization of these multienzymes was achieved by polymers like dextran and ficoll, conserving their stability for several days at room temperature.[104,105] A collection of analogues has been produced on a small scale (Figure 9).

The enzyme system forming the linear peptide gramicidin (Scheme 5), which is coproduced with tyrocidine synthetases, still has not been fully characterized. The peptide is N-terminally formylated and carries a C-terminal ethanolamine. Obviously, the termination step involves aminolysis of the nascent pentadecapeptide. Gramicidin is formed upon addition of ethanolamine to the partially purified enzyme fraction.[106]

Kubota has shown that the release of gramicidin involves phophatidylethanolamine.[107]

Bacitracin (Scheme 6) has been shown by Froyshov and colleagues to be synthesized by at least three multienzymes.[108] During the production phase, the enzyme activity was found to be insoluble, but soluble multienzymes could be recovered at the onset of

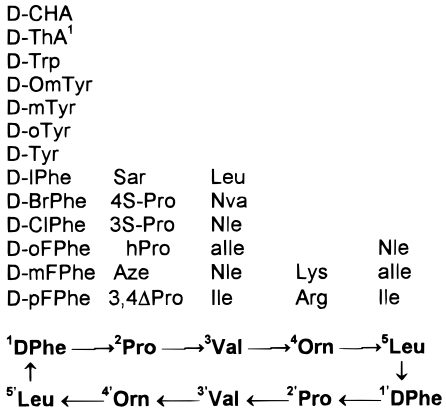


Figure 9. Analogs of gramicidin S obtained by *in vitro* synthesis employing gramicidin S synthetases. Abbreviations used are as follows: 3S-Pro, 3-thioproline; 3,4ΔPro, 3,4-dehydroproline; 4hPro, 4-hydroxyproline; 4S-Pro, 4-thioproline; aIle, *allo*-isoleucine; Aze, azetidine-2-carboxylic acid; hPro, 4-hydroxyproline; mFPhe, *m*-fluorophenylalanine; Nle, norleucine; Nva, norvaline; oFPhe, *o*-fluorophenylalanine; Sar, sarcosine.[32] (Reprinted with permission from ref 13. Copyright 1997 Verlag Chemie.)

Scheme 5

$$\text{fVal} \rightarrow \text{Gly} \rightarrow {}^{3}\text{Ala} \rightarrow \text{DLeu} \rightarrow \text{Ala} \rightarrow \text{DVal} \rightarrow \text{Val} \rightarrow {}^{8}\text{DVal} \rightarrow \text{Trp} \rightarrow \text{DLeu} \rightarrow$$

$$^{11}\text{Trp} \rightarrow \text{DLeu} \rightarrow \text{Trp} \rightarrow \text{DLeu} \rightarrow \text{Trp} \rightarrow \text{EA}$$

Scheme 6

$$^{1}(\text{Ile} \rightarrow \text{Cys}) \rightarrow \text{Leu} \rightarrow \text{DGlu} \rightarrow \text{Ile} \rightarrow \text{Lys} \rightarrow \text{DOrn} \rightarrow \text{Ile} \rightarrow \text{DPhe}$$

$$\uparrow \qquad\qquad \downarrow$$

$$\text{Asn} \leftarrow \text{DAsp} \leftarrow \text{His}$$

peptide synthesis.[109] However, these preparations were only partially active and failed to produce bacitracin. Nevertheless, Wang et al. successfully employed a crude enzyme system from *Bacillus licheniformis*.[101]

The development of eukaryotic cell-free systems from lower fungi has been especially successful by extracting freeze-dried mycelia with buffers containing up to 50% glycerol. This procedure has been employed in the synthesis of cyclic peptides such as cyclosporin, SDZ 214−103 and SDZ 90−215[110] and the linear peptide ACV (see sections IV.1, IV.7, and IV.8). Due to their unusual sizes of about 400−1700 kDa, peptide synthetases can be enriched by salting out at relatively low ionic strength. Thus ammonium sulfate cuts as low as 40% saturation may contain from 10−30% peptide synthetases in the total protein, using high-producing strains as a source.

Peptide synthesis can be carried out by incubation of the relevant synthetases with the constituent amino acids and ATP in solution or in immobilized form.[111] In the case of *N*-methylated peptides *S*-adenosylmethionine must be added.[112] The immediate precursors forming adenylates have to be known, since some enter the cycle in the D configuration (e.g., D-Ala in cyclosporin), or even have to be hydroxylated in advance (D-4-hydroxyproline in neoviridogrisein).[113]

The efficiency of these systems still needs improvement.[56]

Table 4. Enzymatic Peptide Synthesis with ACV Synthetase[a]

product	amino acids supplied	identity by	reference
Aad-δ → Cys → D-Val	Aad, Cys, L-Val	HPLC, MS	Zhang et al.[123]
Aad-δ → Cys → D-Ile	Aad, Cys, L-Ile	HPLC, ESMS, NMR	Baldwin et al.[119]
Aad-δ → Cys → D-aIle	Aad, Cys, L-aIle	HPLC	Zhang et al.[123]
Aad-δ → Cys → D-Abu	Aad, Cys, L-Abu	HPLC	Zhang et al.[123]
Aad-δ → Cys → D-Nva	Aad, Cys, L-Nve	HPLC	Zhang et al.[123]
Aad-δ → Cys → D-Leu	Aad, Cys, L-Leu	HPLC	Zhang et al.[123]
Aad-δ → Cys → D-allylGly	Aad, Cys, L-allylGly	HPLC	Zhang et al.[123]
Aad-δ → Cys → Gly	Aad, Cys, Gly	HPLC	Zhang et al.[123]
Aad-δ → Ser → D-Val	Aad, Ser, L-Val	ESMS	Hadjmalek et al., unpublished
Aad-δ → Hse → D-Val	Aad, Hse, L-Val	MALDI-MS	Hadjmalek et al., unpublished
Aad-δ → Hcy → D-Val	Aad, D,L-Hcy, L-Val	HPLC, MALDI-MS	Zhang et al.,[123] Hadjmalek et al., unpublished
Aad-δ → OMeSer → D-Val	Aad, OMeSer, L-Val	MALDI-MS	Hadjmalek et al., unpublished
CMC-δ → Cys → D-Val	CMC, Cys, L-Val	HPLC, ESMS, NMR	Baldwin et al.[119]
Aad-δ → allyGly → D-Val	Aad, allylGly, L-Val	HPLC, ESMS, NMR	Baldwin et al.[119]
Aad-δ → vinylGly → D-Val	Aad, vinylGly, L-Val	HPLC, ESMS	Baldwin et al.[119]
Cys → D-Val	Glu, Cys, L-Val	HPLC, ESMS, NMR	Shiau et al.[120]
OMeSer → D,L-Val	Aad, L-OMeSer, L-Val	HPLC, ESMS, NMR	Shiau et al.[121]

[a] Abbreviation: CMC, *S*-carboxymethyl-L-cysteine; products just identified by HPLC without synthetic standards should be considered as proposed.

IV. State of Research on Selected Systems

1. Penicillin (ACV Synthetase)

Despite considerable efforts, the study of penicillin biosynthesis still presents complex problems, with new insights emerging from the combined research activities of several laboratories. For about 30 years the tripeptide precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valin (ACV) was thought to be formed in analogy to glutathione.[114] Numerous attempts to isolate the respective peptide synthetases remained unsuccessful. Progress was finally achieved with a commercial strain of *Acremonium chrysogenum*,[115] and with strains of *Aspergillus nidulans* and *Streptomyces clavuligerus*.[116] In addition, complete gene sequence information has been obtained for fungal and bacterial ACV synthetases, and these studies have demonstrated the relatedness of multienzymic peptide synthetases of prokaryotic and eukaryotic origin. At the same time these sequence data have contributed to the revision of the elongation mechanism.[1,5,115] The multiple-carrier model has in the meantime also been verified in integrated polyketide–polypeptide-forming systems, mainly by the sequencing work of Leadlay et al.[116,117]

Since the ACV synthetase field has been reviewed recently,[118] we restrict the discussion to very recent work on enzyme structure and mechanism.

i. Formation of Tripeptide Analogues, Dipeptides, and Order of Reactions

ACV synthetase has been established as a model peptide synthetase,[115] in which the formation of both di- and tripeptides has been demonstrated (Table 4). The surprising formation of C-terminal dipeptides such as Cys-D-Val has led to the proposal of variations of the thiotemplate mechanism.[118,120–122] These studies, however, considered isolated products and did not investigate true biosynthetic intermediates. A model scheme has been proposed[124] based on the multiple carrier model (Figure 7), predicting the thioester intermediates of the three precursor amino acids, the dipeptide Aad-Cys, and the tripeptides LLL-ACV and LLD-ACV (Figure 10). This model takes into account the failure to detect the thioester of D-Val as an intermediate, suggesting that epimerization takes place at the peptide stage. Peptide epimerization has already been demonstrated in the case of actinomycin[33] (see section IV.4). While adenylates and thioesters have been detected,[115,120] Aad-Cys has recently been found after performic acid treatment.[124]

Evidence for the direct incorporation of Aad-Cys into ACV had been obtained,[115] and this seems to proceed by direct activation of the dipeptide at the Cys activation site. The tripeptide intermediate has not yet been isolated. Its proposed rapid release by the C-terminal thioesterase domain apparently prevents the accumulation of significant amounts of this intermediate. As an approach to accumulate the intermediate, the putative thioesterase active site Ser within the motif GxSxG has been mutagenized to Ala, and the altered synthetase has been reintroduced into a deletion strain of *Aspergillus nidulans*.[125] The mutant strain produced penicillin at a rate of about 5% of the rate of the wild-type strain,[126] but surprisingly the isolated synthetase displayed an overall rate of peptide formation of 50%.[127] Product analysis revealed 90% of the tripeptide to be in the L,L,L configuration, thus indicating that the stereospecificity of the thioesterase had been altered by the mutation. Although the tripeptide intermediate has still not been isolated, the product spectrum indicates the L,L,L-tripeptide as an obligatory intermediate.

ii. Energy Requirement for Peptide Synthesis

Nonribosomal peptide formation has an approximate requirement for one ATP per peptide bond, shown by Fujikawa et al.,[128] using a crude tyrocidine synthetase system. Peptide biosynthesis was assayed employing labeled amino acids and measuring as a control ATPase activity in the absence of substrate amino acids.

Other studies using bacitracin and mycobacillin biosynthesis, however, gave a measured consumption of 2 mol of ATP per peptide bond.[129,130] Subsequent work on the gramicidin S system has shown a less-defined energy requirement, with an operational ATP consumption between 1 and 2 mol per peptide bond formed.[102]

$$E1 + A + ATP \rightleftarrows E1(A\text{-}AMP) + PPi \rightleftarrows E1\text{-}S^{p1}\text{-}A + AMP \quad (1)$$

$$E2 + C + ATP \rightleftarrows E2(C\text{-}AMP) + PPi \rightleftarrows E2\text{-}S^{p2}\text{-}C + AMP \quad (2)$$

$$E1\text{-}S^{p1}\text{-}A \rightarrow E2\text{-}S^{p2}\text{-}C\text{-}A \xrightarrow{C1} + E2\text{-}S^{p2}\text{-}C\text{-}A + E1\text{-}S^{p1}H \quad (3)$$

$$E3 + V + ATP \rightleftarrows E3(V\text{-}AMP) + PPi \rightleftarrows E3\text{-}S^{p3}\text{-}V + AMP \quad (4)$$

$$E2\text{-}S^{p2}\text{-}C\text{-}A + E3\text{-}S^{p3}\text{-}V \xrightarrow{C2} E3\text{-}S^{p3}\text{-}V\text{-}C\text{-}A + E2\text{-}S^{p}H \quad (5)$$

$$E2\text{-}S^{p2}\text{-}C^{*}\,A + E3\text{-}S^{p3}\text{-}V \xrightarrow{C2} E3\text{-}S^{p3}\text{-}V\text{-}C^{*} + E2\text{-}S^{p}H \quad (6)$$

$$E3\text{-}S^{p3}\text{-}V\text{-}C\text{-}A \xrightarrow{Ep3} E3\text{-}S^{p3}\text{-}DV\text{-}C\text{-}A \xrightarrow{TE} A\text{-}C\text{-}DV \quad (7)$$

$$E3\text{-}S^{p3}\text{-}V\text{-}C \xrightarrow{Ep3} E3\text{-}S^{p3}\text{-}DV\text{-}C \xrightarrow{TE} C\text{-}DV \quad (8)$$

$$EX(AA1\text{-}AMP) + AA2 \rightarrow EX + AA1\text{-}AA2 + AMP \quad (9)$$

Figure 10. Scheme of reactions catalyzed by ACV synthetase. The synthetase consists of the three modules, E1, E2, and E3. Each module is composed of an activation site forming the (amino)acyl adenylate, a carrier domain which is posttranslationally modified with 4′-phosphopantetheine (S^p), and a condensation domain (C1, C2) or alternatively a structurally similar epimerization domain (Ep). Activation of aminoadipate (A) leads to an acylated enzyme intermediate, where A is attached to the terminal cysteamine of the cofactor ($E1\text{-}S^{p1}\text{-}A$) (1). Likewise activation of cysteine (C) leads to cysteinylated module 2 (2). For the condensation reaction to occur between aminoadipate as donor and cysteine as acceptor, both intermediates are thought to react at the condensation site of module 1 (C1). Each condensation site is composed in analogy to the ribosomal peptide formation of an aminoacyl and a peptidyl site, and in this case of initiation the thioester of A enters the P site, while the thioester of C enters the A site. Condensation occurs and leaves the dipeptidyl intermediate AC at the carrier protein of the second module (3). The third amino acid valine is activated on module 3, and V is attached to the carrier protein 3 (4). Formation of the tripeptide occurs at the second condensation site C2, with the dipeptidyl intermediate entering the P site, and the valyl intermediate the A site (5). The synthesis of dipeptides could be seen either as a side reaction, where the cysteinyl thioester enters the P site of C2, and this minor reaction seems to be favored in the case of *O*-methylserine replacing C (C*), or if an unproductive glutamyl adenylate occupies the first module (6). A similar reaction occurs in the synthesis of polyphenylalanine in the ribosomal system, where the phenylalanyl ester of tRNA enters the P site to initiate peptide synthesis. An alternative process of dipeptide formation is the reaction of an enzyme-stabilized aminoacyl adenylate with the free amino group of an amino acid (9). Here X stands for any of the three domains, and AA1 for any amino acid activated, while AA2 stands for any amino acids providing a free amino group. Such a reaction would indeed completely retain an ^{18}O-label in the respective carboxyl group.[122] Finally epimerization of the tripeptide or dipeptide intermediate occurs at the epimerization site of module 3 (Ep3), and the stereospecific peptide release is controlled by the thioesterase (TE) (7). This release occurs likewise with the dipeptide Cys-D-Val. Since the dipeptide *O*-methylseryl-D,L-valine is released as a racemate, it is apparently not a substrate of the thioesterase, but is hydrolyzed nonenzymatically.

According to the currently accepted model of the thiotemplate mechanism of enzymatic peptide biosynthesis, one α,β-phosphate bond of ATP is consumed for the formation of one peptide bond.[1] However, it has not been determined if the directed transport of intermediates requires additional energy. With fluorescence detection of ACV and simultaneous AMP and ATP determination, ACV formation has been shown to require three ATP for one tripeptide formed.[127] Activation of the third amino acid is required for epimerization of the tripeptide, and this energy is sacrificed by enzymatic hydrolysis of the product. No extra energy is needed for directed transport or release.

iii. Correlation of Domains with Catalytic Activities

From the distribution of activation functions in multicomponent peptide synthetases like gramicidin S, tyrocidine, actinomycin, and surfactin it soon became evident that the order of activation domains corresponds to the amino acid sequence in the peptide product. This has been ascertained by the analysis of multienzyme fragments derived by limited proteolysis or fragment expression (see the respective sections IV.4, IV.6, and IV.7 as well as recent reviews[1,5,12]). Surprisingly, ACV synthetase gave rise to unexpected problems in this respect. While limited proteolysis provided the cysteine-activating second adenylate domain, a fragment of the third adenylate domain was recovered that unexpectedly activated aminoadipate.[32] Expression of fragments containing the first or third adenylation domain as inclusion bodies in *Escherichia coli* led to proteins activating both valine and aminoadipate, respectively (von Döhren et al., unpublished). Since renaturation was involved in their assay, concern was raised on possible structural modifications leading to the alteration of substrate binding sites. Homologous expression of fusion proteins or isolated domains carried out in *Aspergillus nidulans*, however, confirmed the previous observations.[32,36,131] This apparent lack of specificity is not compatible with the fidelity of the synthetase which never produces peptides with valine in the first position, or aminoadipate in the third position. Misactivation might therefore take place in the intact enzyme, or it could be an artifact of fragmentation. Evidence for the first possibility has been obtained by Schwecke et al. showing that a first domain fragment of ACV synthetase from *Streptomyces clavuligerus*, although activating both aminoadipate and valine, exclusively transferred aminoadipate to the thioester site.[132] The second possibility has been verified by Tavanlar,[32] who found that a soluble third adenylate domain of ACV synthetase from *Aspergillus nidulans*, which failed to activate valine, but instead formed adenylates with leucine and aminoadipate. Active sites may thus be distorted in multienzyme fragments, but at the same time proofreading functions may operate in these systems at least, at the level of thioester formation.

2. Phosphinothricyl-alanyl-alanine (Ptt)

Phosphinothricyl-alanyl-alanine (Ptt, also named bialaphos), a tripeptide containing the unusual amino acid phosphinothricin (Pt), is synthesized by strains of *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*[133,134] (Figure 11). The antibacterial activity of Ptt is due to the release of Pt after transport into the bacterial cell through the action of intracellular peptidases.[135] Due to its structural similarity with glutamic acid, Pt acts as an inhibitor of glutamine synthetase leading to glutamine starva-

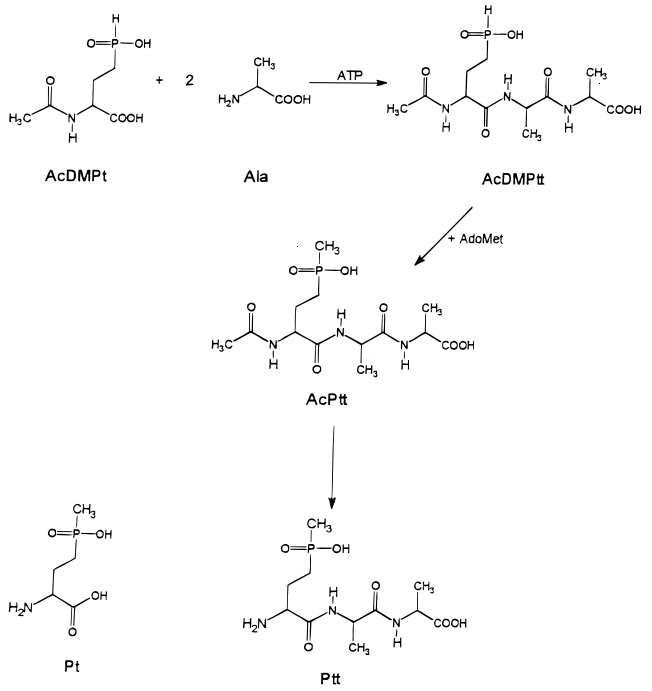


Figure 11. Precursors and assembly of phosphinothricyl-alanyl-alanine (Ptt). *N*-Acetyldemethylphosphinothricin (AcDMPt) is the substrate in the tripeptide assembly. After its condensation with two alanine residues, the resulting AcDMPt-alanyl-alanine (AcDMPtt) is methylated to yield *N*-acetylphosphinothricin-alanyl-alanine (AcPtt). Finally, AcPtt is deacetylated to yield the product Ptt (bialaphos). Phosphinothricin (Pt) acts as inhibitor of glutamine synthetases (see text).

tion.[133] In plants, Pt leads to the accumulation of toxic levels of ammonium ions. Pt resistance genes permit its use as a herbicide.

i. Molecular Cloning and Characterization of Ptt Biosynthesis Genes

The investigation of the accumulation of intermediates in mutants of *S. hygroscopicus* blocked at various steps in the pathway helped to identifiy most of the steps of Pt formation.[136] Cloning by complemention of mutations with plasmid DNA containing fragments of the wild-type strain has helped to identify many of the genes involved in individual steps of Pt synthesis. These genes are assembled in one large gene cluster in *S. hygroscopicus* and *S. viridochromogenes*, respectively.[137−140] Genes controlling condensation of alanine with the immediate precursor of Pt, *N*-acetyldemethyl-Pt (AcDMPt), have been identified by disruption mutants and most of these mutations have been mapped to the bialaphos cluster.[139] These genes were expected to encode enzymes with similarities to peptide synthetases. Sequencing of one particular clone that mapped close to the Ptt-resistance *bar* gene in the bialaphos gene cluster revealed two open reading frames with similarities to thioesterase genes of vertebrates.[65] Wohlleben and co-workers[141,142] have isolated from the same region (near the Ptt-resistance gene *pat*) in the genome of *S. viridochromogenes*, an open reading frame (*phsA*) with similarity to peptide synthetases. The sequence of 660 amino acids contains the highly conserved motifs involved in adenylate and thioester formation. This indicates that the Pt tripeptide synthesis system is composed of more than one enzyme.

ii. Ptt-Synthetase I Is an Acetyl-DMPT-Activating Enzyme

Genetic data had shown that *N*-acetyl-DMPt-Ala-Ala is methylated after its formation and deacetylated to yield the final product Ptt[137] (Figure 11). Accordingly, Hara et al.[138] in their study of mutants of *S. hygroscopicus* impaired in the alanylation step of Pt tripeptide synthesis found that these mutants accumulated *N*-acetyldemethylphosphinothricin (*N*-acetyl-DMPt) An enzyme fraction of *S. viridochromogenes* activated as adenylates *N*-acetyl-DMPt and to a lesser extent acetyl-Pt but not Pt.[143] Glutamic acid or aspartic acid were not substrates, thereby eliminating a possible misactivation of AcPt by an aminoacyl tRNA synthetase specifying glutamate or aspartate. This enzyme, with a native molecular weight of 78 kDa, and 62 kDa in the denatured form, could well be identical with the gene product of *phsA*.

iii. Ptt-Synthetase II Is an Alanine-Activating Enzyme

Elevated levels of an alanine-activating enzyme have been detected in mutants of *S. viridochromogenes* selected for higher levels of Pt tripeptide formation in a strain improvement program[14,38] The enzyme covalently binds L-alanine as its thioester via the adenylate and was shown to contain 4′-phosphopantetheine as cofactor. It was purified and identified by radioactive substrate labeling and autofluorography in SDS-PAGE gels. Depending on the level of proteolytic degradation in the various strains used, in its denatured form the molecular mass of enzyme ranged between 147 and 116 kDa. The enzyme, named Ptt-synthetase II, is clearly distinct from the 80 kDa *phsA* gene product. Contrary to Ptt-synthetase I, Ptt-synthetase II does not activate Pt or AcPt.[143] Attempts to detect a second alanine-activating enzyme from *S. viridochromogenes* were so far unsuccessful. It cannot be ruled out that Ptt-synthetase II is the degradation product of an enzyme harboring the complete set of two alanine-activating domains. These data suggest that Ptt synthesis is catalyzed by a set of at least two peptide synthetases one of which is reponsible for acetyl-DMPt activation while the other contributes one or possibly two alanine residues (Figure 12).

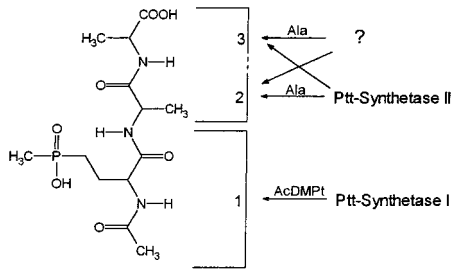


Figure 12. Enzymatic organization of acetyl-demethylphosphinothricyl-alanyl-alanine (AcDMPtt) assembly. Ptt synthetase I activates AcDMPt while Ptt synthetase II activates one of the two alanine residues of the AcDMPt-Ala-Ala product. The crossed arrows and the question mark indicate that it is not known which one of the two alanine residues is activated, because the Ptt synthetase isolated is most probably a proteolytic fragment of a larger enzyme (see text).

3. Ergot Peptide Alkaloids

The ergot peptide alkaloids (also named ergopeptines) are cyclol-structured tripeptide lactams attached at their amino terminal end to the carboxy group of D-lysergic acid (Figure 14). They are produced by the ergot fungus *Claviceps purpurea*. The various ergopeptines differ from each other by substitutions of the peptide portion of the alkaloids in the amino acid positions I and II (Figure 13) which may be occupied by nonpolar amino acids with aliphatic side chains as well as by phenylalanine. By contrast, the proline at position III is invariable, at least in the naturally occurring ergopeptines.[144] The pharmacophore of ergot peptide alkaloids is the D-lysergic acid moiety, which is essential for the various pharmacological activities of these compounds. The various D-lysergic acid amides or peptides and their dihydro derivatives have uterotone, vasodilatory, or vasoconstrictive actions. They induce hypothermia and emesis or control the secretion of the pituitary hormones.[145] These effects are due to structural similarity of the carbon-atom backbone of the ergoline ring system of D-lysergic acid with noradrenaline, serotonin, or dopamine.[146] Variations in the peptide side chains significantly affect the affinities of the alkaloid peptides toward the various noradrenaline, serotonine, and dopamine receptors. Thus the D-lysergic acid peptides and amides have the widest range of pharmacological activities of all microbial drugs used in therapy.

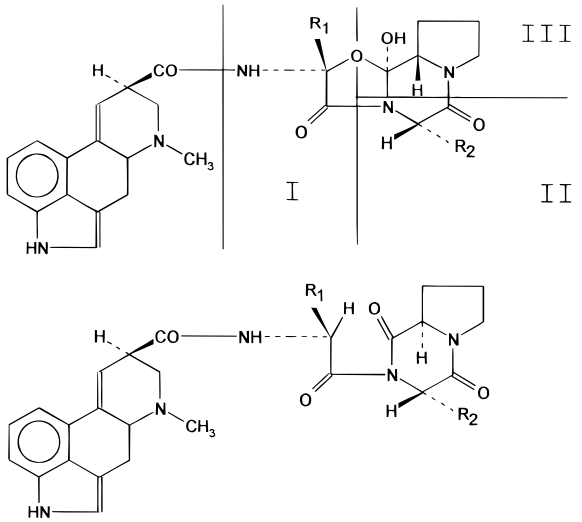


Figure 13. Top: Structure of cyclol-type alkaloid cyclopeptides (ergopeptines). The various ergopeptines differ from each other by substitutions of the amino acids in the variable positions I and II by similar ones. Position III is invariable and contains proline. All ergopeptines contain D-lysergic acid (and occasionally dihydrolysergic acid) attached to the tripeptide portion; they are defined as follows: ergotamines, R_1 = Me, R_2 = Et, benzyl, isobutyl, or isopropyl; ergoxines, R_1 = Et, R_2 = Et, benzyl, isobutyl, or isopropyl; ergotoxines, R_1 = isopropyl, R_2 = Et, benzyl, isobutyl, or isopropyl side groups. Bottom: Structure of alkaloid peptide lactams. D-Lysergyl peptide lactams are the immediate precursors of the ergopeptines. Substituents are the same as in the ergopeptines.

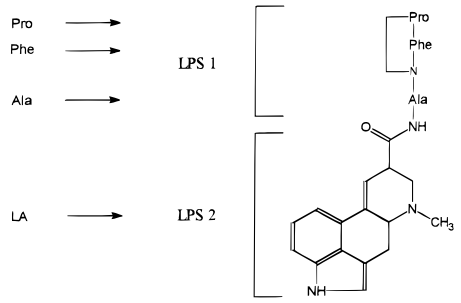


Figure 14. Enzyme organization catalyzing the assembly of alkaloid peptide lactams. D-Lysergyl peptide lactams are synthesized by D-lysergyl peptide synthetase (LPS). LPS 2 activates D-lysergic acid (or dihydrolysergic acid) as thioester via the adenylate. LPS 1 activates the amino acids of the tripeptide portion such as alanine, phenylalanine, and proline in the case of ergotamine.

Numerous studies of *in vitro* and *in vivo* biosynthesis of alkaloid peptides have been performed by academic and industrial groups aiming to widen the pharmacological spectrum of these compounds.[147−150] These studies have helped to clarify the biosynthetic origins of the ergoline ring system of D-lysergic acid and of the amino acids of the peptide portion of these compounds, the latter being derived from the cellular amino acid pool.[151] *In vivo* experiments provided indirect evidence that D-lysergic acid peptides are synthesized by a mechanism resembling the formation of antibiotic peptides of bacterial and fungal origin.[150] However, enzymatic investigations of D-lysergyl peptide assembly were hampered by the apparent instability of the enzyme system responsible for ergopeptine formation in *Claviceps purpurea*. In addition, the question as to which chemical form of the precursor of peptide-bound D-lysergic acid—i.e., either free D-lysergic acid, D-lysergyl Coenzyme A, or a simple clavine—may serve as substrate in the assembly process remained a central controversial issue in these investigations.[152−154] Eventually the development of a cell-free system that incorporated exclusively free D-lysergic acid into the novel noncyclol peptide D-lysergyl-L-alanyl-L-phenylalanyl-L-proline lactam (Figure 14) provided the starting point to study D-lysergic acid peptide formation at the molecular level.[155] D-Lysergyl-L-alanyl-L-phenylalanyl-L-proline lactam has long been considered the immediate precursor of ergotamine.[156] However, this compound and its analogues have never been detected in strains of *Claviceps purpurea* because they appear to be short-lived intermediates in the biosynthesis of various ergot peptide alkaloids.[155,156] The detection of this intermediate under cell-free conditions clarified the proposed biosynthetic pathway and proved the intermediacy of free D-lysergic acid in the assembly process.

i. D-Lysergyl Peptide Formation Is Catalyzed by D-Lysergyl Peptide Synthetase (LPS)

Purification of the D-lysergyl peptide lactam synthesizing activity from a crude extract of ergotamine-producing *C. purpurea* revealed a ~500 kDa multifunctional enzyme complex that harbors all catalytic activities required to assemble D-lysergic acid (or its dihydro derivative dihydrolysergic acid) and the amino acids alanine, phenylalanine, and proline into product at the expense of ATP.[157] The enzyme (named D-lysergyl peptide synthetase, LPS) was shown to consist of two polypeptide chains of 370 kDa (LPS 1) and 140 kDa (LPS 2), respectively. LPS 1 activates the amino acids present in the tripeptide

portion of ergotamine, i.e., alanine, phenylalanine, and proline, as adenylates and subsequently as covalent thioesters. In contrast, LPS 2 exclusively activates D-lysergic acid as a thioester. Both enzymes contain covalently bound 4′-phosphopantetheine cofactor responsible for covalent binding of amino acids and D-lysergic acid and thus appear to synthesize their products by a thiotemplate mechanism (Figure 14).

Synthesis of ergoline ring-containing alkaloids is regulated by tryptophan and phosphate.[158,159] Tryptophan is an inducer of alkaloid production while the presence of phosphate in cultures results in growth-linked repression of alkaloid production. Regulation occurs at the first step of synthesis of the ergoline ring system, the condensation of dimethylallyl pyrophosphate and tryptophan catalyzed by dimethylallyl tryptophan synthase (DMAT).[160,161] In contrast to DMAT, LPS is expressed constitutively in *C. purpurea*. The enzyme is strictly specific for D-lysergic acid (or dihydrolysergic acid) and kinetically regulated by the level of free D-lysergic acid in the cell (see below). As measured under synthesis conditions, the K_m value of D-lysergic acid (1.5 μM) is 1−2 orders of magnitude lower than that for the amino acids constituting the peptide portion.[157] In contrast to its narrow specificity for D-lysergic acid, LPS has broad substrate specificity for amino acids occurring in positions I and II of the natural ergot peptide alkaloids. This may explain the structural diversity in ergopeptines observed in nature. In fact, LPS isolated from the ergotamine-producing *Claviceps* strain used in these studies is able to synthesize *in vitro* various D-lysergyl peptide lactams related to the ergotoxin and ergoxin group of alkaloids.[157] These findings may indicate that the ability of LPS to synthesize different alkaloid cyclopeptides is subject to fluctuations in the concentration of free amino acids in the cellular pools of the various chemical races of the ergot fungus rather than to the simultaneous presence of various isoforms of LPS in the *Claviceps* cell.

ii. Mechanism of Formation of D-Lysergyl Peptide Lactam

Various models for the mechanism of assembly of ergopeptines, elaborated from cell-free and *in vivo* data, have been proposed in the past.[152−154] They suggest unusual direction of peptide chain growth or free precursor peptides in the assembly of the D-lysergyl peptide backbone—such as free D-lysergyl-alanine or D-lysergylvaline reacting with preformed prolyldiketopiperazines—obscuring the reaction mechanism of alkaloid cylopeptide formation.[152−154,162]

Analysis of peptide intermediates accumulating on LPS 1 and LPS 2 in the presence of D-lysergic acid (or dihydrolysergic acid) and the amino acids of the alkaloid peptide chains shed light on the mode of the assembly of D-lysergyl peptides.[163] D-lysergic acid activated by LPS 2 was strictly required for priming peptide formation on LPS 1, to which it is transferred in the presence of the amino acid which occupies position I of the alkaloid cyclopeptide, e.g., alanine in Figure 14. In the absence of D-lysergic acid and in the presence of amino acid substrates, LPS 1 carries only covalently bound amino acid substrates but no peptide intermediates. By contrast, in the presence of D-lysergic acid and amino acids in various combinations, the formation of D-lysergyl mono-, -di-, and -tripeptides was demonstrated (Figure 15). This unequivocally shows that D-lysergyl peptide formation proceeds in the direction from the amino- to the carboxy-terminal end (head growth). The failure of the enzyme to synthesize any covalently bound peptide intermediates in the absence of D-lysergic acid precludes a mechanism of chain growth from the opposite direction. Quantitation of all intermediates accumulating under conditions allowing D-lysergyl dipeptide or tripeptide formation showed that in each case the final (longest) intermediate was the most abundant. This indicates programming of the peptide synthetase complex toward end product. The unequal distribution of intermediates was not the result of a chemical equilibrium between the various intermediates. Apparently the irreversibility of thioester formation as well as of peptide bond formation dictates the direction of peptide growth toward end product. Steric constraints may also play a role in programming by allowing entry of D-lysergic acid into the enzyme complex only after completion of the previous round of alkaloid cyclopeptide synthesis. This kind of regulation of peptide synthesis initiation and elongation may also operate in other nonribosomal peptide syntheses.[164]

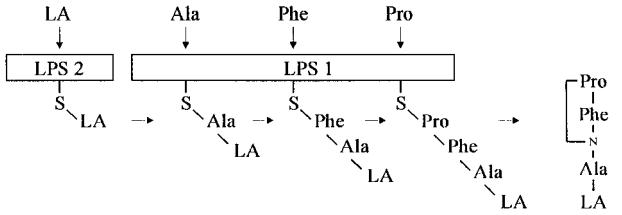


Figure 15. Formation of alkaloid cyclopeptide lactam via covalently enzyme-bound D-lysergyl peptides. D-lysergyl cyclopeptide lactam formation takes place in a successive series of acyltransfers starting with D-lysergic acid activation via the D-lysergyl mono-, -di-, and -tripeptides. In the last step D-lysergyl tripeptide is cyclized to the peptide lactam (e.g., D-lysergyl-alanyl-phenylalanyl-proline lactam). This step is catalyzed by LPS 1 also.

4. Acyl Peptide Lactone Synthetases

Acyl peptide lactones contain peptide lactone rings that are attached via their terminal amino groups (usually belonging to a threonine or a serine residue) to aromatic carboxylic acids. They are almost exclusively produced by streptomycetes.[152,165] These compounds combine nearly all features of peptide structural elements containing L- and D-, as well as *N*-methylated, amino acids. The structures of acylpeptide lactones with aromatic side chains have been reviewed elsewhere.[150,164] Many of these compounds have therapeutic value as antibiotics or antitumor reagents. The enzymatic steps in the biosyntheses of the different aromatic acyl peptide lactones appear to be similar.[164,166] Much of the knowledge of these pathways has been obtained from studies of the biosynthesis of actinomycin. Actinomycin synthetases (ACMSs)[57,167,168] have been established as model biocatalysts for the assembly of various peptide lactones that contain aromatic carboxylic acids attached to a peptide lactone by an amide linkage.

i. Aromatic Carboxylic Acid Activation as the First Step in Acyl Peptide Lactone Biosynthesis

Actinomycins (e.g., actinomycin D) are bicyclic and are derived from a monocyclic precursor that consists of one pentapeptide lactone ring attached to 3-hydroxy-4-methylanthranilic acid (3-HMA). This compound is the precursor of the chromophoric phenoxazinone dicarboxylic acid actinocin that is common to all actinomycins. 3-HMA pentapeptide lactone is a short-lived intermediate because it is rapidly converted to the corresponding actinomycin in a oxidative amino phenol condensation, as shown in Figure 16.

Activation of 3-HMA is catalyzed by actinomycin synthetase I (ACMS I). The 45 kDa ACMS I has been characterized as a 3-HMA-AMP ligase which catalyzes the synthesis of adenylyl-3-HMA from 3-HMA and ATP (Figure 17).[21,169] The enzyme has broad substrate specificity with respect to various structurally related benzenecarboxylic acids. Accordingly, the enzyme incorporates benzenecarboxylic acids with hydroxy groups in the 3 position or a methyl group in the 4 position into novel actinomycin analogues, formed after feeding these carboxylic acids to cultures of *Streptomyces chrysomallus*.[170] Structural analogues of 3-HMA compete with endogenous 3-HMA and become incorporated into material identified as monocyclic acylpentapeptide lactones (actinomycin half molecules) containing the administered analogue. Due to the absence of the *o*-aminophenol structure present in 3-HMA, these compounds were not converted to the corresponding phenoxazinone (actinomycin) and, therefore, accumulated in the cultures. Their formation indirectly proved that actinomycins are directly derived from their monocyclic precursor lactone, i.e., the 3-HMA pentapeptide lactone.

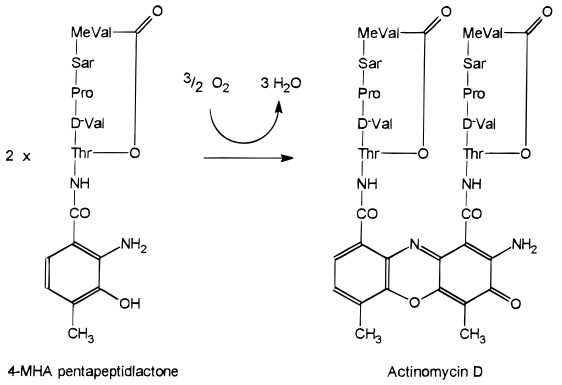


Figure 16. Formation of actinomycin by the oxidative condensation of 3-hydroxy-4-methylanthranilic acid pentapeptide lactones. The bicyclic chromopeptide lactones (actinomycins) arise by the oxidative condensation of two monocyclic 3-hydroxy-4-methylanthranilic acid (3-HMA) pentapeptide lactones. The reaction is catalyzed by phenoxazinone synthase or may proceed nonenzymatically in the presence of molecular oxygen.

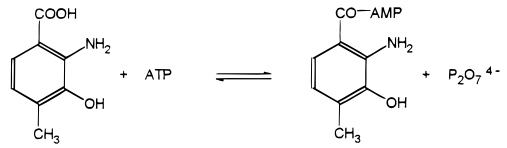


Figure 17. Activation of 3-hydroxy-4-methylanthranilic acid (3-HMA) as adenylate is catalyzed by ACMS I and is the first step in the assembly of 3-HMA pentapeptide lactones.

ii. Priming, Initiation, and First Elongation Steps in Acyl Peptide Lactone Synthesis

Actinomycin synthetase II (ACMS II) is a 280 kDa multienzyme that assembles L-threonine and L-valine into occupying positions 1 and 2 (as D-valine) of the peptide lactone ring of actinomycin by the usual activation reactions of enzymatic peptide synthesis.[167,168] ACMS II has sites responsible for receiving 3-HMA adenylate (formed by ACMS I) and appears to bind 3-HMA covalently by a thioester linkage.[168] In the presence of 3-HMA (or its structural analogue, *p*-toluic acid) initiation of peptide synthesis starts with formation of covalently bound 3-HMA-threonine, similar to the formation of D-lysergyl-alanine in the synthesis of D-lysergyl peptide lactam (see above). 3-HMA-threonine further reacts with covalently bound L-valine to yield a mixture of covalently bound 3-HMA-threonyl-L-valine and 3-HMA-L-threonyl-D-valine (Figure 18).

In contrast to peptide alkaloid formation, initiation of peptide formation by acylation of the first amino acid with the aromatic carboxylic acid (3-HMA or *p*-toluic acid) is less strict. In the absence of aromatic carboxylic acid but with L-threonine and L-valine, formation of L-threonyl-L-valine and L-threonyl-D-valine takes place. However, this occurs with much lower yield than when 3-HMA or a structurally related acid is present. Thus priming affects the extent of dipeptide or acyl dipeptide formation.

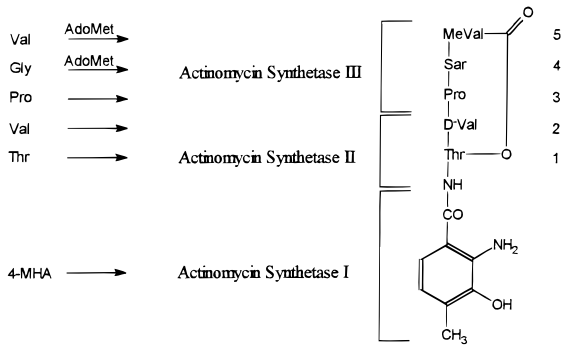


Figure 18. Priming, initiation, and elongation steps in 3-HMA pentapeptide lactone assembly. Actinomycin synthetase II (ACMS II) activates threonine, valine, or isoleucine as thioester via the adenylates (reaction b). In addition, the enzyme is able to receive 3-HMA adenylate formed by ACMS I (reaction a) and to bind 3-HMA as thioester. The enzyme catalyzes initiation primed by 3-HMA by acylating covalently bound threonine with 3-HMA. In the elongation reaction covalently bound L-valine (or L-isoleucine) reacts with 3-HMA-threonine to give a mixture of diastereomeric acyl dipeptides covalently bound to the enzyme. Priming with 3-HMA preferentially leads to the incorporation of L-isoleucine in 3-HMA-threonyl-L-isoleucine and 3-HMA-threonyl-D-*allo*-isoleucine. In the absence of primer, threonine condenses with L-valine to yield a mixture of covalently bound L-threonyl-L-valine and L-threonyl-D-valine (reaction c). In the latter reaction which shows a considerably lower yield than reaction b, the enzyme has strong preference for valine instead of L-isoleucine. This illustrates the importance of priming for programming the substrate specificity of the peptide synthetase. (Reprinted with permission from ref 168. Copyright American Society for Biochemistry and Molecular Biology, Inc.)

Priming also has an effect on programming of the peptide synthetase, because it clearly affects substrate specificity in position 2 of the peptide lactone. If ACMS I and an aromatic carboxylic acid such as *p*-toluic acid is present, leading to the formation of *p*-toluylthreonine, ACMS II prefers incorporation of L-isoleucine to give the acyl dipeptide *p*-toluyl-L-threonine-L-isoleucine, which subsequently is epimerized to the stereoisomer *p*-toluyl-L-threonine-D-*allo*-isoleucine. In the absence of an aromatic carboxylic acid, the enzyme prefers L-valine over L-isoleucine. Thus, acylation of threonine with 3-HMA has a significant influence on the specificity of the enzyme and influence programming of the synthetic events on the peptide synthetase.

iii. Epimerization of Amino Acids in the Peptide-Bound State

Besides activation of threonine and the acylation of threonine with 3-HMA, ACMS II catalyzes activation of L-valine but not of D-valine.[167] L-valine is the precursor of peptide-bound D-valine. It was shown that covalently bound L-valine reacts with enzyme-bound 3-HMA-threonine to yield 3-HMA-L-threonyl-L-valine.[57] Inversion at the carbon C-2 of L-valine in the covalently bound intermediate 3-HMA-L-threonyl-L-valine then takes place by abstraction of a proton which subsequently is released into the solvent. Epimerization proceeds independently of addition of cofactors such as NAD, FAD, or pyridoxal phosphate. Spectral analysis of ACMS II indicates the absence of any tightly bound cofactors on the enzyme. ACMS II exclusively activates the L enantiomer of valine and catalyzes epimerization of valine (or isoleucine) in the peptide-bound state. Stindl and Keller[57] propose an unknown proton acceptor group that is present on ACMS II catalyzing proton abstraction during inversion in a one or two base mechanism. A similar mechanism was proposed for the epimerization of valine in the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine catalyzed by ACV synthetases.[124,127]

iv. Actinomycin Synthetase III, the Third Enzyme Involved in Acyl Pentapeptide Lactone Synthesis

ACMS III is a 480 kDa polypeptide chain harboring all activities responsible for incorporation of the amino acids present in position 3, 4, and 5 of the acyl pentapeptide lactone[167] (Figure 18). Similar to the case of ACMS II, it contains covalently bound 4′-phosphopantetheine cofactors which act as the acceptor groups for amino acid binding in the various peptide synthetase domains of this multienzyme. These cofactors also serve as carrier arms for peptidyl intermediates. Besides its ability to activate proline, glycine, and valine as thioesters, ACMS III methylates thioester-bound glycine and valine at the expense of *S*-adenosylmethionine (Adomet) yielding both sarcosine and *N*-methyl-L-valine, respectively, covalently bound to the enzyme. Thus ACMS III has two distinct *N*-methylation domains (Figure 19). The mechanism of the methylation reactions are the same as in the synthesis of the cyclohexadepsipeptide enniatin produced by *Fusaria*.[112,171] ACMS III is freely dissociable from ACMS II, as is ACMS I, and under *in vitro* conditions ACMS III synthesizes cyclo-

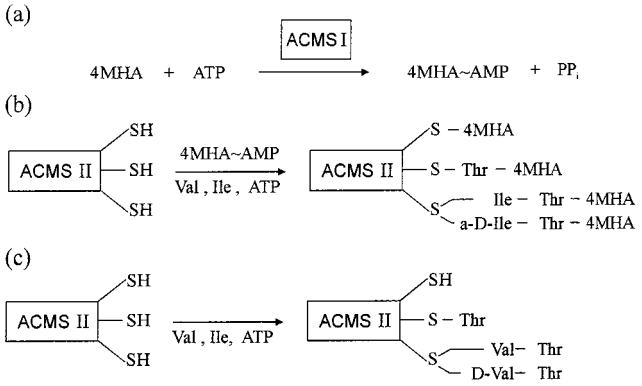


Figure 19. Enzyme organization catalyzing the assembly of 3-hydroxy-4-methylanthranilic acid pentapeptide lactone. 3-HMA pentapeptide lactone is assembled by actinomycin synthetases (ACMSs) I, II, and III. ACMS II activates threonine and valine (or isoleucine) and catalyzes the formation of intermediates up to the 3-HMA dipeptide stage. ACMS III activates proline, glycine, and valine and additionally methylates covalently glycine and valine. Full synthesis of the acyl pentapeptide lactone has been demonstrated. However, it is not known whether the release of product and lactonization is catalyzed by that same enzyme.

(prolylsarcosine) and cyclo(sarcosyl-*N*-methyl-L-valine)[57] (Figure 18).

v. Total Cell-Free Synthesis of Acylpentapeptide Lactone

The ability of the three ACMSs to catalyze the synthesis of *p*-toluic pentapeptide lactone was demonstrated in unfractionated cell extracts from *Streptomyces chrysomallus*.[172] This system rapidly loses activity with half-life of 5−20 h, depending on the quality of the preparations, and has turnover numbers from 0.1 to 1 min^{-1}. The extreme lability of the cell-free system and the fact that a mixture of the purified ACMSs did not catalyze the formation of the peptide lactone could be due to the absence in the purified enzyme of some additional factor(s) involved in acyl transfers or release (lactonization) of the acyl peptide chain.

Much higher levels of enzymatic acyl peptide lactone synthesis were observed in permeabilized cell systems which produced *p*-toluic acid pentapeptide lactone at a rate 1 order of magnitude higher than in the cell-free extract (taking into account differences in the amount of protein in the two systems).[172] This strongly suggests that functional activity of the enzyme system in terms of end product formation is dependent on the structural integrity of the cell, possibly through membrane association of the three ACMSs. Testing the three enzymes individually for adenylate and thioester formation, *N*-methylation, epimerization, and formation of covalently bound intermediates confirmed their functional integrity as well as their ability to synthesize intermediates up to the acyl pentapeptide chain.[57,167,168,172] Apparently, impairment in the correct positioning of the three synthetases under cell-free conditions and/or lack of a crucial factor in enzyme assembly or product release might be responsible for the instability of the system under *in vitro* conditions.

5. *N*-Methylcyclodepsipeptides Enniatin and Beauvericin

Enniatins are cyclohexadepsipeptides produced by various strains of the genus *Fusarium*.[173] As shown in Figure 20, enniatins consist of three residues of a branched chain *N*-methyl amino acid and D-2-hydroxyisovaleric acid (D-Hiv) arranged in an alternating fashion. Enniatins have antibiotic activity against various bacteria, exhibit immunomodulatory properties,[174] and are potent inhibitors of mammalian cholesterol acyl transferase.[175] Enniatins are also well-known for their behavior as ionophors with high specificity for potassium ions.[176] Enniatin-producing *Fusaria* are plant pathogens and the compound was postulated to play a role as a wilt toxin during infection of plants.[177] The structurally related cyclodepsipeptides beauvericin and bassianolide exhibit entomopathogenic properties.[178]

i. Enniatin Synthetase: Structure and Function

Enniatins are synthesized by the multifunctional enzyme enniatin synthetase (Esyn) which was the first characterized *N*-methylcyclopeptide synthetase, isolated from *Fusarium scirpi*.[179] Sequencing of the gene of Esyn has revealed that the enzyme is one single polypeptide chain of 347 kDa.[61] This is consistent with the earlier biochemical investigations which revealed that all catalytic functions of the enzyme were located on one polypeptide chain. The enniatin molecule is formed in an ATP-dependent process from its primary precursors D-Hiv and a branched chain amino acid such as L-valine or L-isoleucine. *S*-Adenosyl-L-methionine (AdoMet) donates the methyl group in the *N*-methylated peptide bonds. As in other peptide synthetases, 4′-phosphopantetheine is present as the prosthetic group. Dissecting the biosynthetic process catalyzed by the individual domains of Esyn revealed the picture of reaction steps shown in Figure 21. Activation of D-Hiv and the branched chain L-amino acids (e.g., L-valine) proceeds through adenylation and thioester formation. The thioesterified amino acid is methylated with AdoMet prior to peptide bond formation and subsequent cyclization reactions. Omission of AdoMet from the *in vitro* system leads to formation of demethyl enniatins.[112,180] Therefore, Esyn and other *N*-methylcyclopeptide synthetases can be considered as hybrid systems between peptide synthetases and *N*-methyltransferases.

ii. Mechanism of N-Methylation

A characteristic property of all *N*-methyltransferases is their sensitivity to inhibition by *S*-adenosyl-

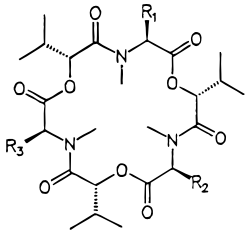


Figure 20. Structures of enniatins and of beauvericin: enniatin A, $R_1 = R_2 = R_3 =$ *sec*-butyl; enniatin A1, $R_1 =$ iso-propyl, $R_2 = R_3 =$ *sec*-butyl; enniatin B, $R_1 = R_2 = R_3 =$ isopropyl; enniatin B1, $R_1 = R_2 =$ isopropyl, $R_3 =$ *sec*-butyl; beauvericin, $R_1 = R_2 = R_3 =$ benzyl.

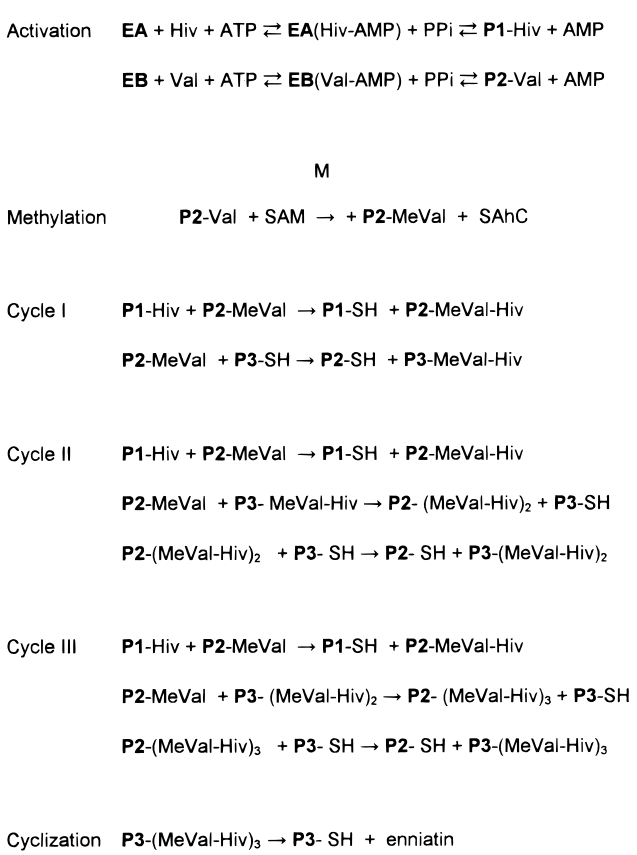


Figure 21. Scheme of partial reactions leading to the dipeptidol intermediate and dipeptidol condensations catalyzed by enniatin synthetase: EA, D-Hivactivation domain; EB, L-Val activation domain; M, methyltransferase; S, carrier domains containing the 4′-phosphopantetheine cofactor; P1, P2, P3 = 4′phosphopantetheine.

L-homocysteine (AdoHCy), the reaction product of the methylation reaction derived from the methyl donor AdoMet. The antibiotic sinefungin, which is structurally related to AdoMet, is a potent inhibitor of a variety of methylases. Billich und Zocher[112] tested the effect of sinefungin and *S*-adenosyl-L-homocysteine on product formation catalyzed by Esyn and found that sinefungin inhibits the methylation reaction of Esyn but allowed synthesis of demethylenniatin even if present in excess. Kinetic analysis showed a competitive inhibition pattern with respect to AdoMet, indicating a direct competition of sinefungin at the AdoMet binding site. In contrast, AdoHCy not only blocked formation of enniatin but also that of the unmethylated product. Kinetic analysis of desmethylenniatin synthesis revealed that AdoHCy is a partially competitive inhibitor, with respect to AdoMet. These results indicate that Esyn must harbor a discrete binding site for the inhibitor AdoHCy but not for sinefungin. Thus, blocking the *N*-methyltransferase function of Esyn by AdoHCy results in the inhibition of peptide bond formation by Esyn (R. Zocher, unpublished data). These data suggest that the active sites for depsipeptide formation and for the methylation step in the Esyn system are not independent of each other, as subsequently confirmed by the analysis of the reaction sequence of Esyn (see below).

iii. Substrate Specificity of Esyn

Due to the relatively broad substrate specificity of Esyn for amino and hydroxy acids, a variety of

different enniatins can be synthesized by Esyn if appropriate concentrations of substrates (depending on the various K_m values) are used (Zocher, unpublished data). Nevertheless, Esyns from different *Fusarium* strains differ in their amino acid specificity, i.e., they display different K_m values for each of the various branched-chain amino acids.[180] For example, the enzyme from the enniatin A producer *F. sambucinum* exhibits high affinity for the substrate amino acids L-Leu and L-Ile. By contrast, Esyn from the enniatin B producer *F. lateritium* preferably accepts L-Val, the constituent amino acid of enniatin B and, therefore, strongly resembles the Esyn from *F. scirpi*. Mutations in the amino acid binding sites of the polypeptide chains may be the reasons for the altered substrate specificity among these synthetases.

iv. Molecular Structure of Esyn

Monoclonal antibodies directed to the multienzyme Esyn were used to map the catalytic sites of the enzyme.[181] They could be divided into three groups based on their influence on catalytic function. Members of group one exclusively inhibited L-Val thioester formation while members of group two interfered with D-Hiv thioester formation. Antibodies of group three inhibited both L-Val thioester and D-Hiv thioester formation as well as the *N*-methyltransferase. From these findings it was concluded that the two domains of Esyn containing the two catalytic binding sites are located very close to each other in the three-dimensional structure of the enzyme. The immunochemical data with group three antibodies indicated that this is also the case for the *N*-methyltransferase site, which is in the vicinity of the acyl and aminoacyl binding sites. Titration of Esyn with radioactive AdoMet revealed binding of 1 mol AdoMet per mole Esyn,[112] indicating the presence of one methyl transferase unit per enzyme molecule. Interestingly, the adenylation reactions for D-Hiv and L-Val were not affected by the monoclonal antibodies, indicating that these reactions have different sites on the multienzyme at some distance from the thiol binding sites, thereby confirming the modular structure of peptide synthetase domains.

v. Structure of the Esyn Gene

Analysis of the sequence of the Esyn gene (*esyn1*) of *Fusarium scirpi* revealed an open reading frame of 9393 bp encoding a 347 kDa polypeptide that contains two highly conserved peptide synthetase domains (designated EA and EB) (see Figure 22).[61] The domain EA lying on the aminoterminal side of the protein could be identified as the D-Hiv binding site and EB as the L-amino acid binding site on the carboxy terminal side. In contrast to domain EA, EB is interrupted by insertion of a 434 amino acid portion (M domain) between motifs E and F (Figure 22), which contains a sequence showing similarity to a motif apparently conserved within a number of methyl transferases.[61] It was possible to express the M segment in *E. coli* and to identify this protein as the methylase of Esyn by its binding properties for AdoMet. Three deletion mutants of this protein were shown to be inactive with respect to AdoMet binding.[182] Similarly, a variety of other functional recombinant Esyn protein fragments could be expressed leading to the identification of the substrate activation sites for D-Hiv and L-Val.[183] A peculiar property of *esyn1* is that it harbors two 4′-phosphopantetheine binding motifs in domain EB, one of which may represent a waiting position for peptidol units during chain growth.

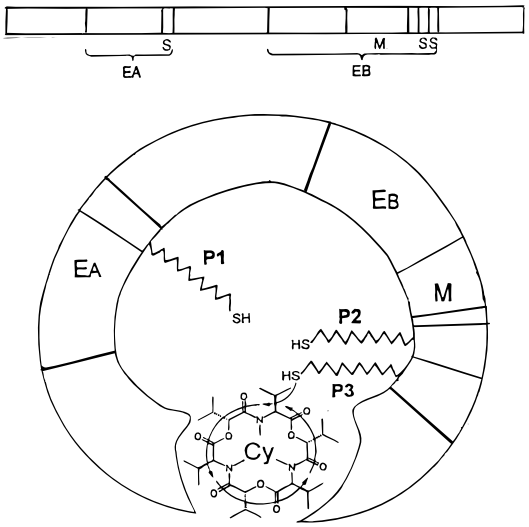


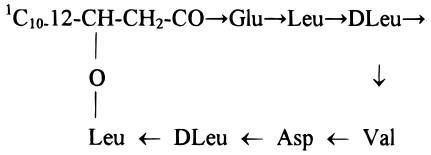
Figure 22. Structure and model of arrangement of catalytic sites of the enniatin synthetase as deduced from the gene sequence and biochemical characterization Cy = cyclization cavity.

The methyltransferase portion of the Esyn gene from *F. sambucinum* was amplified by the polymerase chain reaction (PCR) using primers derived from the highly conserved sequences of the flanking peptide synthetase domain.[63] The deduced amino acid sequence of the product shares high similarity to the 430 amino acid methyltransferase portion of Esyn from *F. scirpi* and cyclosporin synthetase from *Tolypocadium niveum*. As these methylase portions show only local similarity to motifs apparently conserved within methyltransferases, they represent a new class of *S*-adenosyl-L-methionine-dependent methyltransferases.

vi. Mechanism of Depsipeptide Formation

Esyn consists of two peptide synthetase modules EA and EB, but assembles three amino acids and three D-2-hydroxy acids in the final product enniatin. Studies on the mechanism of depsipeptide (enniatin B) formation revealed that the enniatin molecule is synthesized by three successive condensations of enzyme-bound (thioester) dipeptidols.[184] This implies that, like the fatty acid synthases, Esyn contains a specific thiol group (waiting position) that picks up the intermediates of enniatin synthesis, i.e., the dipeptidol, tetrapeptidol, and hexapeptidol, to allow depsipeptide chain elongation (Figure 21). After reaction of the thioester-bound *N*-methyl amino acid with the covalently bound D-Hiv (domain EA), the resulting dipeptidol is transferred to the waiting position and attacked by the hydroxyl group of the subsequently formed dipeptidol, yielding a tetrapeptidol. After transthiolation to the waiting position, the tetrapeptidol is then attacked by a third dipeptidol to form a hexapeptidol which gives rise to enniatin in the final condensation reaction. The

Scheme 7



presence of a second thioester formation domain in the module EB is consistent with this model and may represent the waiting position. The length of the growing depsipeptide chain could be determined by the space provided by a putative cyclization cavity (Figure 22).

vii. Biosynthesis of Beauvericin

Beauvericin is a homologue of the enniatins in which the branched chain *N*-methyl amino acid position always contains *N*-methyl-L-phenylalanine (see Figure 20). Beauvericin synthetase catalyzing beauvericin synthesis from L-Phe and D-Hiv with consumption of ATP and AdoMet has been isolated from the fungus *Beauveria bassiana*.[185] The enzyme strongly resembles Esyn with respect to its molecular size and reaction mechanism. The main differences between both enzymes lie in the substrate specificity. Beauvericin synthetase exhibits a high specificity for aromatic amino acids such as phenylalanine, whereas Esyn is unable to incorporate such compounds.

6. Surfactin

i. Structure and Properties of Surfactin

Bacillus subtilis produces a great variety of lipopeptide compounds, among them surfactin, the iturin family (iturins, bacillomycins, mycosubtilins), and fengycin which are all potent antibiotics. Surfactin[186−191] is a cyclic amphiphilic lipoheptapeptide (Scheme 7).

The lipid portion of surfactin is a mixture of several β-hydroxy fatty acids with chain lengths between 13 and 15 carbon atoms integrated into the heptapeptide ring as a lactone. Therefore, natural surfactin is a mixture of isoforms which differ slightly in their physicochemical parameters due to variations in the chain length and branching of the hydroxy fatty acid components[190−192] as well as by substitutions of amino acid components in their peptide ring.[193−196] These structural features are not determined genetically, but rather depend on the specific *B. subtilis* strain as well as on nutritional and environmental conditions.[197] The three-dimensional structure of surfactin in solution has recently been investigated by 2D NMR spectroscopy and mass spectrometry.[195,198] The model derived from the NMR data shows a saddle topology for the peptide ring atoms. The two polar side chains of L-Glu and L-Asp are arranged in a claw configuration consistent with the ionophore and chelating properties of surfactin.[199,200]

The lipopeptides produced by *B. subtilis* are of industrial interest and increasing biotechnological and pharmaceutical importance. Surfactin is one of the most potent biosurfactants[201−203] and shows antimicrobial,[203−205] antiviral,[195,206] antitumor,[207,208] and hypocholesterolemic activities.[209]

ii. The Surfactin Synthetase Multienzyme System (Figure 23)

The biosynthesis of surfactin was studied in a cell-free system prepared from the wild-type *B. subtilis* ATCC 21332 and the genetically engineered high producer strain *B. subtilis* OKB 105 by Ullrich et al.[210] Surfactin is formed by a nonribosomal mechanism. Lipopeptide biosynthesis required the L-amino acid components and D-3-(hydroxytetradecanoyl)-coenzymeA thioester in combination with ATP and Mg^{2+}. The structure of surfactin synthe-

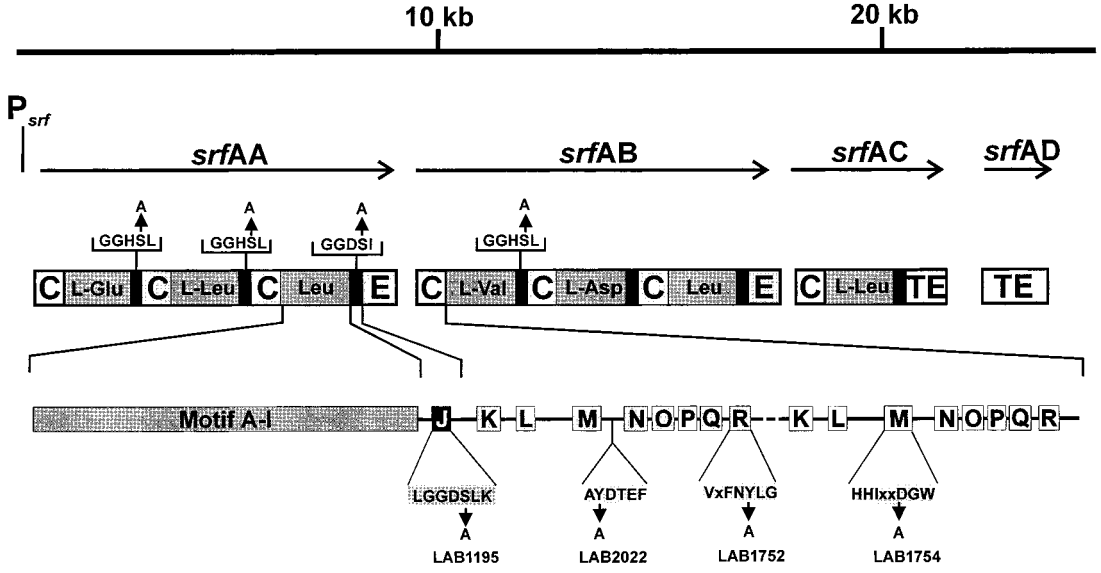


Figure 23. The surfactin biosynthetic cluster. The biosynthesis of surfactin in *B. subtilis* is codified by the *srfA* operon comprising four genes *srfAA*−*srfAD*. *srfAA*−*srfAC* code for surfactin synthetases 2−4 (SRFS 2−4), which catalyze the activation of the amino acid substrates and the assembly of the lipoheptapeptide product. The *srfAD* gene encodes a yet unknown thioesterase enzyme which is homologous to the *GrsT* protein. SRFS2 activates L-Glu and two leucines, SRFS3 activates L-Val, L-Asp and leucine, while SRFS4 contributes the terminal leucine. Every amino acid activating module of such multienzymes contains at least 18 highly conserved consensus motifs A−R, as demonstrated for the third module SrfAA3 of surfactin synthetase 2. In addition, the the N-terminal elongation domain E_N of the first module SrfAB1 of surfactin synthetase 3 is shown. The positions of site-specific mutation of highly conserved amino acid residues in consensus motifs for amino acid thiolation as well as peptide elongation and epimerization of this region are indicated.

sized *in vitro* was confirmed by chromatographic procedures in combination with amino acid analysis. From the crude extract, four enzymes involved in surfactin biosynthesis have been isolated and purified.[211−213] Menkhaus et al.[211] obtained three enzyme fractions E_1, E_2, and E_3 by gel filtration of crude extracts of *B. subtilis* OKB 105. E_1 is composed of two multifunctional polypeptides E_{1A} and E_{1B} which were separated and obtained in purified form by high-resolution anion exchange chromatography on Pharmacia Mono Q. E_{1A} (402 kDa) binds L-Glu and L-Leu in a molar ratio of 1:2, whereas E_{1B} (401 kDa) incorporates L-Val, L-Asp, and L-Leu in a molar ratio of 1:1:1. E_2 (144 kDa) is a leucine-activating enzyme. E_3 is an acyltransferase with a molecular mass of ∼40 kDa that uses β-hydroxytetradecanoic acid-coenzymeA thioester as substrate. E_2 catalyzes the initiation of surfactin biosynthesis by transfer of the β-hydroxy fatty acid to E_{1A} followed by the formation of the hydroxy-L-glutamate intermediate.[211] In the absence of the other surfactin synthetase components, E_3 hydrolyzes the hydroxy fatty acid substrate, thereby functioning as a thioesterase. Because the lipid moiety of natural surfactin represents a mixture of several β-hydroxy fatty acids with chain lengths of 13−15 carbon atoms, the acyltransferase E_3 obviously shows a rather broad substrate specificity. The D(−) enantiomer of the β-hydroxy fatty acid-CoA-substrate is preferred in the biosynthetic process. In contrast the hydrolysis of the L(+) enantiomer is ∼10-fold higher than that of the D form, implying a possible proofreading function of E_3 in the initiation reaction, eliminating the fatty acid substrate in its L(+) form and favoring the D(−) enantiomer in the biosynthetic process. It remains to be clarified whether more than one acyltransferase enzyme is involved in surfactin biosynthesis.

The entire surfactin synthetase multienzyme system was reconstituted by complementation of all four enzyme fractions E_{1A}, E_{1B}, E_2, and E_3.[211]

iii. The Surfactin Biosynthetic Genes—Organization of the srfA Operon

Three genetic loci, *srfA*,[34,214−216] *srfB*,[217] and *sfp*,[39,218,219] have been shown to be essential for surfactin production in *B. subtilis* (Figure 23). *SrfA* and *sfp* are genetically linked, while *srfB* maps at a locus distant from srfA. *SrfB* includes the *comA* and *comP* genes which represent a two-component signal transduction system of the sensor-regulated type involved in surfactin biosynthesis and the competence-development pathway. SrfB functions as the transcriptional activator of the *srfA* promoter.[217,220] Biosynthesis of surfactin is essentially codified by the *srfA* operon which comprises 27 kb. The entire sequence of *srfA* has been determined by Cosmina et al.[34] It contains four open reading frames (Figure 23). Sequence analysis of the first three genes revealed seven homologous amino acid-activating modules each with a size of 600−1000 amino acid residues which are obviously responsible for the activation and condensation of the amino acid substrates of surfactin synthetase. S*rfAA*, *srfAB*, and *srfAC* encode three peptide-forming multienzymes composed of repeated homologous amino acid-activating modules. Each of the first two genes (*srfAA* and *srfAB*) are composed of three such building blocks, while *srfAC* contains only one. At the 3′-end of *srfAC* a thioesterase active site is located that is homologous to the type II thioesterases of fatty acid synthetase multienzyme systems.[34] Similar structural features have also been detected in *acvA*[70−72] and *grsB*[73,82] involved in the biosynthesis of β-lactam peptides and gramicidin S. The gene organization of *srfA* is consistent with the enzymatic studies, corroborating that three multifunctional polypeptides are involved in the assembly of the peptide part of surfactin. The fourth gene, *srfAD*, encodes a 29 kDa protein. It is the counterpart to *GrsT* in gramicidin S biosynthesis[67,220] both showing homology to vertebrate thioesterase II enzymes involved in fatty acid biosynthesis. The function of these proteins is still unknown.

Sfp[39,218,219] maps downstream of the *srfA* operon. It is encoded by a separate transcriptional unit. The region between the transcriptional termination of *srfA* and the *sfp*-gene spans about 4 kb. It contains at least four open reading frames. Mutant studies show that the integrity of this intervening region is not required for surfactin biosynthesis, with the possible exception of a region immediately downstream from the *srfAD* gene. The *sfp* gene is essential for surfactin formation. Frequently the integrity of *sfp* distinguishes surfactin, producing *B. subtilis* strains from nonproducers. For example, in the nonproducing reference strain 168 *sfp* is truncated, while the genetic information of *srfA* is intact. Moving an active copy of *sfp* into the chromosome of this strain restored surfactin production and created a high-producing strain. Recently the function of the Sfp protein was elucidated.[39] Sfp is a member of an enzyme superfamily catalyzing the posttranslational modification of the apo form of peptide synthetases by transfer of 4′-phosphopantetheine from coenzymeA to the reactive serine of their thiolation motifs. Sfp therefore plays an essential role in the biosynthesis of surfactin as a 4′-phosphopantetheine-transferase.

The *srfA* operon is not only required for the biosynthesis of surfactin, but is also essential for efficient sporulation and competence development of *B. subtilis*, a prokaryotic cell specialization process that enables cells growing in minimal medium supplemented with glucose to bind and take up extracellular DNA at the end of the logarithmic phase. The growth stage-specific and nutritional regulation of competence is managed by a complicated signal transduction network involving the cooperation and sequential action of several regulatory gene products. Obviously *srfA* plays an important role in cell differentiation and specialization processes in *B. subtilis*.[215] Initial studies show that the 5′ part of this operon is essential for the regulation of genetic competence, while the complete locus is necessary for both lipopeptide formation and sporulation. A more refined analysis revealed that the minimal genetic element of *srfA* required for the establishment of competence is the DNA segment encoding the valine-activating module *srfAB1*,[34] but the activities of the gene product, as adenylation and thiolation of L-valine, did not affect this process.[221] Finally, from deletion analysis and complementation studies it became obvious that *srfAB1* contains a separate

small open reading frame designated *comS*,[222−224] encoding a polypeptide of 46 amino acids. Introduction of an amber mutation into the comS-encoding frame prevented development of competence, demonstrating that comS is the regulatory element in this prokaryotic specialization process. Recently Venema et al. (personal communication) have shown that ComS is essential for the activation of ComK which is the transcription factor for the late competence genes. Comk is kept inactive during the exponential growth phase by formation of a ternary complex with MecA and ClpC. At the beginning of the stationary phase the expression of srfA is stimulated. Because *comS* is a part of this operon, its expression is also increased. The *comS* gene product destabilizes the ComK/MecA/ClpC complex, leading to an activation of the competence transcription activity of ComK.

iv. Mutational Studies—Analysis of Gene–Protein Relationships: Structure and Functional Organization of Surfactin Synthetase

B. subtilis srf mutant strains deficient in surfactin biosynthesis due to insertions and deletions at various locations of the *srfA* operon were used to correlate the biosynthetic genes with their gene products.[216,225] The components of surfactin synthetase expressed in the mutant strains were isolated, purified, and functionally characterized. From the mutant studies it was clearly demonstrated that the surfactin biosynthetic genes were expressed in such a way that the amino acid-activating modules are arranged colinearly with the sequence of the amino acid components in the lipopeptide product. As a consequence, the first three genes of the *srfA* operon, *srfAA*−*srfAC*, codify E_{1A}, E_{1B}, and E_2 of the surfactin multienzyme system. The expression of the acyl transferase E_3 was not significantly affected in all mutants tested.[225] The amount and activity of E_3 were similar to the wild-type strain, indicating that this enzyme is obviously not encoded by the *srfA* operon. Presumably E_3 is related to the genes responsible for fatty acid metabolism in *B. subtilis*.

By correlation of the genetic and biochemical data, the following functional organization of the surfactin multienzyme system can be derived.[225] Surfactin formation starts by the transfer of the hydroxy fatty acid component from the acyl transferase enzyme (surfactin synthetase 1) to the first peptide-forming enzyme (surfactin synthetase 2) which elongates the hydroxyacyl glutamate intermediate formed in the initiation process to the lipotripeptide β-hydroxyacyl → L-Glu → L-Leu → D-Leu. This intermediate is in turn transferred to surfactin synthetase 3 which forms the lipohexapeptide by addition of the three amino acids L-Val, L-Asp, and D-Leu following in the sequence of surfactin. Surfactin synthetase 4 contributes the residual L-Leu residue in position 7 of surfactin. In addition, this enzyme presumably also catalyzes the cyclization of the complete lipopeptide chain by lactone formation between the terminal leucine and the hydroxyl function of the β-hydroxy fatty acid component in position 1. Recently de Ferra et al.[75] demonstrated that the thioesterase domain located at the C-terminus of this enzyme determines the release of peptidyl intermediates. By gene replacement experiments, new enzymes were constructed fusing the thioesterase domain either to module 4 or 5 of the surfactin synthetase leading *in vivo* to the formation of the lipotetra- and lipopentapeptide, respectively. These results indicate the crucial role of the thioesterase domain in the termination reaction.

Probing of the modular structure of surfactin synthetase was initiated by site-specific mutagenesis of amino acid components in highly conserved consensus motifs of its amino acid-activating modules (Figure 23) specifically with the aim of consolidating the multiple carrier model of nonribosomal peptide biosynthesis[225−227] and to understand the elongation and epimerization steps involved in surfactin formation on the molecular level.[227] In particular, the serines in the thiolation motifs LGGH/DSL/I of the first four modules of surfactin synthetase were mutated to an alanine.[225,226] All these mutants were inactive in lipopeptide formation. In each case the thioester-binding activity of the mutated module was eliminated, while the other thioester formation reactions and all substrate-dependent ATP/PP_i exchange activities were not significantly affected consistent with the presence in surfactin synthetase of multiple 4′-phosphopantetheine cofactors.

Putatively the so-called spacer motif M (Figure 3, see section III.4) plays an essential role in the transpeptidation steps involved in the elongation of the growing peptide chain. Mutations of both histidines and the aspartic acid in the spacer motif HHIMMDGWS in the N-terminal region of surfactin synthetase 3 by alanines abolished surfactin formation, indicating that these structural elements are essential for the elongation process and the interaction between surfactin synthetases 2 and 3.[227] The highly conserved tyrosines in the conserved sequence AYDTEF between motifs M and N and in motif Q (FNYLGQ) of the third module of surfactin synthetase 3 were also replaced by alanines in order to test their putative function as a proton donor/acceptor system in the epimerization of leucine.[227] Analysis of the reactivity of the mutant enzymes and their product formation activities showed that the mutated tyrosine residues are not essential for the epimerization process. The Y → A replacements affected neither the composition nor the reactivity of the surfactin synthetase components isolated from the mutant strains nor the surfactin formation process.

8. Cyclosporin and SDZ 214-103

Cyclosporin is the currently most prominent immunosuppressor drug, acting selectively on T-cells. Its properties and biosynthesis and those of related cycloundecapeptides and -peptidolactones have been reviewed recently in detail.[14,228,229] The system has several important features, which will be considered in perspective: precursor supply, the sequence of reactions, and the implications for the *in vitro* synthesis of peptide libraries.

i. Precursor Supply

As has already been described in section II.2, D-alanine is a direct precursor in cyclosporin formation. The presence of D-amino acids in nonribosomal systems originates from either direct incorporation or epimerization of thioester intermediates (see sec-

tion III.4). Available sequence information indicate the presence of epimerization domains. The direct incorporation of a D-amino acid requires the presence of an amino acid racemase. An alanine racemase has been isolated from the cyclosporin producer *Tolypocladium niveum*.[55] The enzyme is a tri- or tetramer of a 37 kDa peptide, as judged by gel filtration. The enzyme activity was absent in the SDZ 214-103 producer *Cylindrosporum oligospermum*, in which D-alanine is replaced by D-hydroxyisovalerate.

Pyridoxal phosphate has been detected as the exclusive cofactor. The kinetic constants were K_m-(L-Ala) 38 mM and K_m(D-Ala) 2 mM and V_{max}(D-L) 8 nM/min and V_{max}(L-D) 126 nM/min. The substrate specificity also permits isomerization of L-serine, L-2-aminobutyrate, and L-leucine with rates 23, 15, and 13% of that for L-alanine. Both D-serine and D-2-aminobutyrate may replace D-alanine in biosynthesis, while D-leucine has not been assayed *in vitro*.

The second unusual precursor is the amino acid (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (Bmt). Feeding experiments employing C-13-labeled acetate and methionine have revealed the polyketide origin of the Bmt precursor.[230] Offenzeller et al.[231] have demonstrated *in vivo* and *in vitro* the formation of the Bmt-precursor acid. *In vivo* studies with [1-^{13}C,$^{18}O_2$]acetate have shown oxygen retention in the 3-hydroxyl group, suggesting 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoate as the key intermediate. The respective Bmt polyketide synthase utilizes acetyl-CoA, malonyl-CoA, NADPH, and *S*-adenosylmethionine in a sequential mechanism with enzyme-bound intermediates.[232] While activated intermediates up to 3-oxo-4-hexenoic acid can be introduced into the cycle, no CoA derivatives of the following methylation step to 2-methyl-3-oxo-4-hexenoic acid or any further intermediates are accepted. A relaxed specificity for the starter unit was recognized, so that butyryl-CoA was accepted with comparable efficiency to crotonyl-CoA, leading to the saturated analogue of Bmt.

This feature points to the frequent involvement of polyketide products in peptides and the linking of polyketide and peptide biosynthetic enzyme systems as in actinomycin (IV.4), surfactin (IV.6), or terpenoid systems as in ergot peptides (see section IV.3).

ii. Sequence of Reactions

The cyclosporin synthetase gene consists of 11 modules each for activation, elongation, and carrier proteins, and seven *N*-methyltransferase domains, inserted in the second, third, fourth, sixth, seventh, and nineth adenylate domains. A drawing correlating structure and function is given in Figure 24. Cloning of the 45.8 kb gene, which has no introns, was facilitated by an oligonucleotide corresponding to a peptide sequence of the last domain.[64] Various N-terminal sequences derived from endoproteolytically generated fragments of the synthetase have been traced in the derived amino acid sequence. Two sequences have been assigned to alanine binding and *N*-methyltransferase, respectively, and correlated with the C-terminal domain and the insert within the sixth domain. The functional interpretation as depicted in Figure 24 shows 11 adenylate-forming and aminoacylation domains, seven of them containing *N*-methyltransferase domains inserted in the adenylation domains (see section III.5) colinearly with the cyclosporin amino acid sequence. Each aminoacylation domain is thought to be modified with 4′-phosphopantetheine by a respective holoenzyme synthase utilizing CoA.

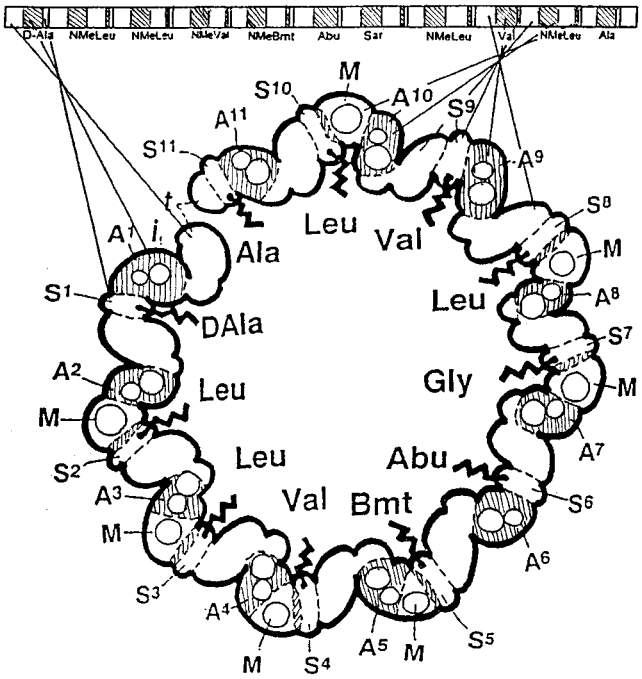


Figure 24. Correlation of gene and protein structure in cyclosporin synthetase.[12] The gene (top) is composed of 11 modular units, composed of either an activation domain (adenylate domain A^i) and a carrier domain (S^i), or additionally have an *N*-methyltransferase domain inserted. The elongation domains have not been assigned. Note that the first D-alanine activating domain A^1 is preceded by a condensation domain. The positioning of this domain is not understood, but suggests its involvement in cyclization. The structural interpretation permits transfer of intermediates only between adjacent functional units, and the putative terminating condensation with the N-terminal condensation domain. The transfer is mediated by 4′-phosphopantetheine posttranslationally attached to each acyl carrier protein domain. This modification increases the unmodified size of 1 689 243 Da by 11 × 340 Da to 1 692 983 Da. (Altered from ref 14.)

In the functional analysis of the multienzyme, the presence of four leucine and two valine binding sites does not permit their separate study. So far adenylate formation has been investigated by the amino acid-dependent reversal, employing labeled pyrophosphate.[238,239] Aminoacylation has been demonstrated with labeled amino acids or, if not available, using labeled *S*-adenosylmethionine to detect the *N*-methylated amino acids. Evidence for the involvement of thiols is based on modification with 4-chloromercuribenzoate or *N*-ethylmaleimide, or the reversible inhibition with 2,2′-dithiopyridine.[234]

To prove the predicted reaction sequence, labeled intermediate peptides were generated, thioesters that were present were cleaved with performic acid, and analysis was carried out by two-dimensional TLC. Only D-alanine could be confirmed as an N-terminal amino acid, and among several peptides D-Ala-MeLeu, D-Ala-MLeu-MeLeu, D-Ala-MeLeuMeLeuMeVal, and the nonapeptide intermediate were identified by cochromatography. These experiments support

Table 5. Cyclosporins Produced by *Tolypocladium niveum* in Different Nutrient Broths

peptide	1	2	3	4	5	6	7	8	9	10	11
CyA	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala
CyB	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Ala**	Sar	MeLeu	Val	MeLeu	Ala
CyC	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Thr**	Sar	MeLeu	Val	MeLeu	Ala
CyD	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Val**	Sar	MeLeu	Val	MeLeu	Ala
CyE	D-Ala	MeLeu	MeLeu	**Val**	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala
CyF	D-Ala	MeLeu	MeLeu	MeVal	**MeBmt**	**Nva**	Sar	MeLeu	Val	MeLeu	Ala
CyG	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala
CyH	D-Ala	MeLeu	MeLeu	**D-MeVal**	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala
CyI	D-Ala	**Leu**	MeLeu	MeVal	MeBmt	**Val**	Sar	MeLeu	Val	MeLeu	Ala
CyK	D-Ala	MeLeu	MeLeu	MeVal	**MedBmt**	**Val**	Sar	MeLeu	Val	MeLeu	Ala
CyL	D-Ala	MeLeu	MeLeu	MeVal	**Bmt**	Abu	Sar	MeLeu	Val	MeLeu	Ala
CyM	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Nva**	Sar	MeLeu	**Nva**	MeLeu	Ala
CyN	D-Ala	**Leu**	MeLeu	MeVal	MeBmt	**Nva**	Sar	MeLeu	Val	MeLeu	Ala
CyO	D-Ala	MeLeu	MeLeu	MeVal	**MeLeu**	**Nva**	Sar	MeLeu	Val	MeLeu	Ala
CyP	D-Ala	MeLeu	MeLeu	MeVal	**Bmt**	**Thr**	Sar	MeLeu	Val	MeLeu	Ala
CyQ	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	Abu	Sar	**Val**	Val	Me**Leu**	Ala
CyR	D-Ala	**Leu**?	MeLeu	MeVal	MeBmt	Abu	Sar	MeLeu	Val	**Leu**?	Ala
CyS	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Thr**	Sar	**Val**	Val	MeLeu	Ala
CyU	D-Ala	**Leu**	MeLeu	MeVal	MeBmt	Abu	Sar	MeLeu	Val	**Leu**	Ala
CyV	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	**Abu**
CyW	D-Ala	MeLeu	MeLeu	**Val**	MeBmt	Thr	Sar	MeLeu	Val	MeLeu	Ala
CyX	D-Ala	**Leu**	MeLeu	MeVal	MeBmt	**Nva**	Sar	MeLeu	Val	MeLeu	Ala
CyY	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Nva**	Sar	MeLeu	Val	**Leu**	Ala
CyZ	D-Ala	MeLeu	MeLeu	MeVal	MeAOA	Abu	Sar	MeLeu	Val	MeLeu	Ala
Cy26	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Nva**	Sar	MeLeu	**Leu**	MeLeu	Ala
Cy27	D-Ala	MeLeu	MeLeu	MeVal	**Bmt**	**Val**	Sar	MeLeu	Val	MeLeu	Ala
Cy28	D-Ala	MeLeu	MeLeu	MeVal	**MeLeu**	Abu	Sar	MeLeu	Val	MeLeu	Ala
Cy29	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	Abu	Sar	**MeIle**	Val	MeLeu	Ala
Cy30	D-Ala	MeLeu	MeLeu	MeVal	**MeLeu**	**Val**	Sar	MeLeu	Val	MeLeu	Ala
Cy31	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	Abu	Sar	**Ile**	Val	MeLeu	Ala
Cy32	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala

well the general scheme of multienzymatic peptide biosynthesis.[1]

Apart from the estimate of cyclosporin formation with a rate of 16 nkatal/mg,[233] no enzyme kinetic data are available. Characterization of the cyclosporin nonproducer *T. niveum* YP582 has shown that only the cyclodipeptide *cyclo*-D-Ala-MeLeu is formed and released.[234,235] No defects in activation and aminoacylation have been found, but to ascertain for example a loss of leucine activation in one of the four leucine-activating domains is a difficult task. Recently Glinski et al.[236] have observed the formation of *cyclo*-L-Ala-MeLeu together with peptide intermediates not terminated by D-alanine. These results indicate possible intermediate intiations of peptide synthesis, comparable to the formation of dipeptides and cyclodipeptides by ACV synthetase and actinomycin synthetase, respectively.

iii. In Vitro Synthesis of Peptide Libraries

So far a total of 32 cyclosporin analogues isolated from fermentation broths have been described.[229] Their location is limited to 20 positional changes (Table 5), which by combinatorial procedures could give rise to 72 000 predictable analogues. Obviously only one or at most two variations are tolerated by the cyclosporin synthetase. At most two *N*-methylations are missing. The rate of synthesis decreases about 10-fold if unmethylated intermediates have to be processed in enniatin synthesis.[112] Similarly, if analogue substrates are poorly accepted, the overall rate will decrease.[180] Dramatic variations in the yield of analogues can be achieved by external feeding (see section II.2). Thus, feeding of L-norvaline doubled the peptide yield and decreased the cyclosporin A content to 9% compared to 91% of the ^{6}Nva analogue cyclosporin G.[236]

The *in vitro* approach, employing cyclosporin synthetase has the advantages that transport and metabolism of precursors does not have to be considered. Thus several analogues have been synthesized which were not available by feeding experiments,[97] including the D-aminobutyrate replacement of D-alanine and the ring-extended 11-β-alanine cyclosporine.[96] Enzymatically synthesized cyclosporin analogues are compiled in Figure 25. Most of the compounds, however, have been only tentatively identified by TLC or HPLC, without actual verification of their structures. The *in vitro* approach uses a synthetase fraction, the precursor amino acids, ATP for activation and *S*-adenosylmethionine for *N*-methylation. If one position is omitted, and the respective amino acid analogue supplied, a new compound indicates the formation of an analogue. This poses problems, if substrate specificities are close. Feeding of norvaline targeted at the variable position 6 also replaces the valines in positions 4 and 9 to some extent. Since position 5 may also be replaced by leucine, substitutions upon feeding Bmt analogues are not necessarily restricted to this site, but could as well replace other positions. Substrate specificities of individual sites vary and these differences, rather than precursor levels, may account for the composition of peptide mixtures isolated from various strains. Comparing the substrate specificities of cyclosporin synthetase with the similar SDZ 214-103, it was demonstrated although not quantified, that substrate discrimination varied in these related multienzymes.[97] The starting activation domain (position 1) of both systems is strictly specific for either D-hydroxy or D-amino acids, respectively.

Table 6. Current State of Research on Peptide Synthetases[a]

peptide	organism	structural type[a]	gene cloned	enzymology
		Linear		
carnosine	rat	P-2	−	+
bacilysin	*Bacillus subtilis*	P-2-M	(+)	(+)
bialaphos	*Streptomyces hygroscopicus*	P-3	+	−
edeine	*Bacillus brevis* Vm4	P-5-M	−	+
anguibactin	*Vibrio anguillarum*	R-P-2-M	+	−
yersiniabactin	*Yersinia enterocolitica*	R-P-3-M	+	−
phaseolotoxin	*Pseudomonas syringae* pv. ph.	P-4-M	(+)	−
ardacin	*Kibdelosporangium aridum*	P-7-M	(+)	−
pyoverdin	*Pseudomonas aeruginosa*	R-P-8-M	+	−
		Cyclopeptides		
cyclopeptin	*Penicillium cyclopium*	C-2	−	+
enterochelin	*Escherichia coli*	P-C-E-3	+	+
HC toxin	*Cochliobolus carbonum*	C-4	+	+
tentoxin	*Alternaria alternata*	C-4	−	+
echinocandin	*Aspergillus nidulans*	R-C-6	−	(+)
microcystin	*Microcystis aeruginosa*	C-7	(+)	−
iturin	*Bacillus subtilis*	C-8	(+)	−
mycobacillin	*Bacillus subtilis*	C-13	−	+
		Lactones		
destruxin	*Metarhizium anisopliae*	L-6	+	(+)
pristinamycin	*Streptomyces pristinaspiralis*	R-L-6	+	+
etamycin	*Streptomyces griseus*	R-L-7	−	(+)
quinomycin	*Streptomyces echinatus*	$(R\text{-}P\text{-}4)_2$	−	+
R106	*Aureobasidium pullulans*	L-9	−	(+)
syringomycin	*Pseudomonas syringae*	R-L-9	(+)	−
syringostatin				
SDZ90-215	*Septoria* sp.	L-10	−	+
		Branched Polypeptides		
nosiheptide	*Streptomyces actuosus*	R-P-13-C-10-M	−	+
thiostrepton	*Streptomyces laurentii*	R-P-17-C-10-M	−	+
		Branched Peptidolactones		
lysobactin	*Lysobacter* sp.	P-11-L-9	(+)	+
A21798A	*Streptomyces roseosporus*	R-P-13-L-10	(+)	+
A54145	*Streptomyces fradiae*	R-P-13-L-10	−	+
tolaasin	*Pseudomonas tolaasii*	R-P-18-L-5	(+)	−

[a] Structural types of compounds are as in Table 3.

Although the procedure is elegant and convincing in the production of, at most, milligram quantities, which is sufficient for many activity tests, it still needs improvement. The synthesis of 11-β-Ala-cyclosporin A for example leads to significant byproducts including cyclosporins A and V or glycine in positions 1 and 11, which are unexpected from the substrates supplied. The enzyme system may contain intermediates which are completed during synthesis, and in case of unfavorable products could even dominate. Yields of synthesis depend not only on stabilities of (a) the multienzyme, (b) ATP, and (c) *S*-adenosylmethionine in the system, but more critically on the tolerance of precursors. Data obtained on the gramicidin S system indicate that rates of synthesis with analogues are additive.[17] Thus if an analogue is incorporated with 10% efficiency, and a second analogue is added having a likewise reduced rate of 10%, the overall rate for the disubstituted peptide will only be 1%. In addition product inhibition may be severe and cause low yields. Synthetic approaches using an excess of ATP often limit the yields employing either labeled amino acids or labeled *S*-adenosylmethionine. This new technique is still an area requiring vast improvement and development, including enzyme stabilization by chaperones, ATP regeneration, and product removal to avoid product inhibition. If small amounts of peptides are needed, and reaction optimization is not considered, *in vitro* synthesis of peptide analogues employing multienzymes is an effective procedure, especially considering the simple one-pot achievement of multistep processes.

9. Other Systems Presently under Investigation

An increasing number of peptide synthetase systems is being investigated. A compilation is given in Table 6. Interest is now being extended from microorganisms to plants and animals, and an increasing number modified ribosomal peptides are being discovered.[13] Besides biological screening methods, genetic techniques including genome sequencing will contribute to the discovery of new peptides.

V. Perspectives

1. Combinatorial Biosynthesis

To approach combinatorial synthesis of peptides with the described biocatalysts, two procedures are obvious. One would be to combine amino acid-introducing elements to generate new peptide-forming systems, while the other utilizes established enzyme systems and exploits their substrate tolerance as a principle of diversity. The latter system has occasionally been used, especially in the synthesis of cyclosporin analogues (section IV.8). Some limits of such approaches are to be expected, since not only the substrate profile of an individual site determines the products possibly formed, but also the functioning of all steps of the respective catalytic

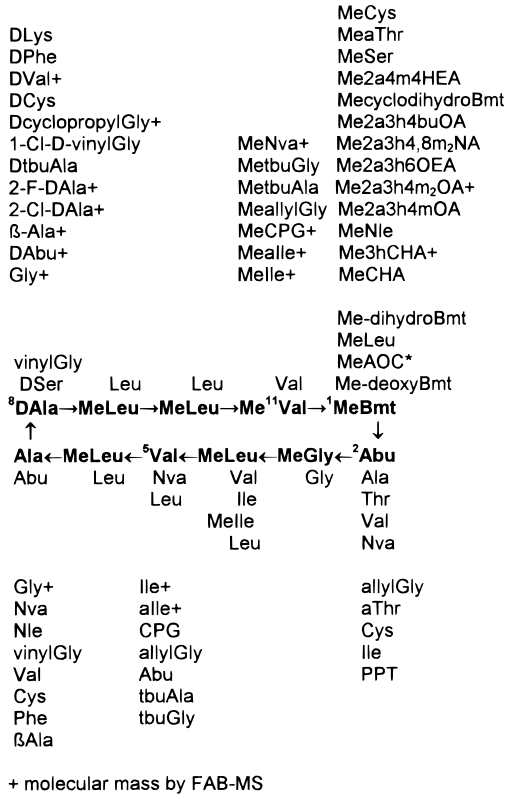


Figure 25. Cyclosporins synthesized *in vitro*.[32] Changed positions are indicated, and generally single replacements have been reported, except for substitutions at positions 5 and 11 showing double replacement, or even triple replacement for 2, 5, and 11 in case of, e.g., Nva or allylglycine. Compounds directly placed at the cyclosporin A structure have also been isolated from fermentations and were available as reference compounds. All other compounds have been described by chromatographic evidence or additional mass spectra (+). Abbreviations used are as follows: Abu, aminobutyrate; allylGly, allylglycine; 2a3h4buOH, 2-amino-3-hydroxy-4-butyloctanoic acid; 2a3h4,8m_2NA, 2-amino-3-hydroxy-4,8-dimethylnonanoic acid; 2a3h6OEA, 2-amino-3-hydroxyoct-6-enoic acid; 2a3h4m_2OA, 2-amino-3hydroxy4,4-dimethylocanoic acid; 2a4m4HEA, 2-amino-4-methylhex-4-enoic acid; AOC, aminooctanoic acid; CHA, cyclohexylalanine; 2-Cl-DAla, 2-chloro-D-alanine; CPG, cyclopropylglycine; cyclopropylGly, cyclopropylglycine; D, D-configuration; 2-F-DAla, 2-fluoro-D-alanine; 3hCHA, 3-hydroxycyclohexylalanine; Me, *N*-methyl; Nle, norleucine; PPT, phosphinothricine; tbuAla, *tert*-butylalanine; tbuGly, *tert*-butylglycine (for details see Lawen and Traber[97]). (Reprinted with permission from ref 13. Copyright 1997 Verlag Chemie.)

cycle has to be considered. The engineering of established enzyme systems is currently under investigation (see section V.2). The generation of new systems utilizing available modules has not been undertaken yet.

2. System Engineering

For most peptides described here, chemical synthesis at the present stage of technology is unsuited for large-scale production. The biotechnical production of certain analogues, if not available by feeding strategies, remains an area of development. The various peptide synthetase genes identified may be a valuable source for amino acid-introducing functional units. Such units may be used as modules in the reconstruction of altered peptide synthetase genes or perhaps in the future in the construction of new peptide synthetases. Reconstruction of genes and reintroduction into defined nonproducers are available techniques for many microorganisms. The successful replacement of amino acid activating modules has been demonstrated in the surfactin system by Stachelhaus et al.[238,239]

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VII. References

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