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QUARTERLY

The paper is dedicated to Professor Włodzimierz Ostrowski

Applications of peptide synthetases in the synthesis of peptide analogues

Horst Kleinkauf and Hans von Döhren

Institute of Biochemistry and Molecular Biology, Technical University Berlin, Germany

Enzymatically formed peptides show positional variations as well as highly conserved amino acids. In the cases of gramicidin S, tyrocidine, linear gramicidins, enniatins, echinocandins and viridogrisein *in vivo* and *in vitro* studies indicate substrate selection at the level of amino acid activation as a major control step. Evidence for proof-reading steps beyond activation has been obtained in penicillin and cyclosporin biosynthesis. Activated substrate analogues may promote the formation of side products such as dipeptides and cyclodipeptides. Modifications of intermediates, such as N-methylation, influence the rates of peptide synthesis. These control steps pose limitations for the application of such enzyme systems in the production of peptide libraries. They may originate from a target oriented evolution of these synthetases.

While high fidelity of the ribosomal peptide forming system is assured by various proof reading mechanisms, the nonribosomal systems display a certain degree of variability. Unlike gene-encoded defined peptides, the peptides synthsized in nonribosomal system form families, frequently isolated from microorganisms. Ribosomal peptide families originate by gene duplication and mutational alteration of genes, exemplified e.g. in the

Abbreviations: 1Cl-D-vinylGl, 1-chloro-D-vinylglycine; 2a3h4buOA, 2-amino-3-hydroxy-4-butyl-octanoic acid; 2a4mHEA, 2-amino-4-methyl-hex-4-enoic acid; 2a3h4mOA, 2-amino-3-hydroxy-4-methyl-octanoic acid; 2a3h4,8m2NA, 2-amino-3-hydroxy-4,8-dimethyl-nonanoic acid; 2-Cl-DAla, 2-chloro-D-alanine; 2-F-DAla, 2-fluoro-D-alanine; 2,3,hTyr, 2,3-dihydroxy-L-homotyrosine; 2,3h3mP, 2,3-hydroxy-3-methyl-pentanoic acid; 2,4h3mP, 2,4-hydroxy-3-methyl-pentanoic acid; 2,5h3mP, 2,5-hydroxy-3-methyl-pentanoic acid; 3h4mPro, 3-methyl-4-hydroxy-L-proline; 3hCHA, 3-hydroxy-cyclohexyl-alanine; 3hC14, 3-hydroxy-tetradecanoic acid; 3hPic, 3-hydroxy-picolinic acid; 4,5hOrn, 4,5-dihydroxy-L-ornithine; 3hpro, 3-hydroxy-L-proline; 3,4Pro, 3,4-dihydroxy-proline; 4hPro, 4-hydroxy-proline; aDhyp, allo-4-hydroxy-D-proline; βAla, β-alanine; Abu, aminobutyric acid; ACV, δ-L-(α-aminoadipoyl)-L-cysteinyl-D-valine; allylGly, allyl-glycine; AOC, amino-octanoic acid; Bmt, (4R)-4-[(4E)-2-butenyl]-4-methyl-L-threonine; C14, tetradecanoic acid; Cit, citrulline; CPG, cyclopropyl-glycine; cyclodihydroBmt, cyclodehydro-(4*R*)-4-[(4*E*)-2-butenyl]-4-methyl-L-threonine; cyclopropylGly, cyclopropyl-glycine; D, prefix for D-configuration used without -; Dbu, diaminobutyric acid; fIle, formyl-isoleucine; fVal, formyl-valine; fOrn, formyl-ornithine; Himv, 2-hydroxy-4-methyl-valeric acid; Hiv, hydroxy-isovaleric acid; Hmp, hydroxy-methyl-pentanoic acid; hPro, 4-hydroxy-proline; Hyp, 4-hydroxy-proline; Lac, lactic acid; Me, prefix used for N-methyl; mFPhe, *meta*-fluoro-phenylalanine; Nle, norleucine; NMe, prefix used for N-methyl-; Nva, norvaline; oFPhe, *ortho*-fluoro-phenylalanine; Phg, phenylglycine; PPT, phosphinothricine; Sar, sarcosine; tbuAla, t-butyl-alanine; tbuGly, t-butyl-glycine; vinylGly, vinyl-glycine.

case of amphibian opiod peptides (Bevins & Zasloff, 1990). At first sight peptide families have been taken as an indication that amino acid selection in nonribosomally protein encoded peptides proceeds at the respective substrate binding sites. Selection would thus be limited to one protein surface and account for the structural variations observed. Investigations of the substrate specificities of various peptide synthetases have shown, however, certain restrictions of amino acid replacements, which closely correlate with the variations in natural families (Kleinkauf & von Döhren, 1997). These restrictions may be related to a target oriented evolution of peptide biosynthetic pathways (Kleinkauf & von Döhren, 1994) and, in addition, point to the presence of control and proof reading mechanisms of the enzymatic systems.

PEPTIDE SYNTHETASES

Peptide forming enzyme systems can be grouped into single step and multistep systems (Table 1) (Kleinkauf & von Döhren, 1990). While glutathione biosynthesis has been studied in detail as a single step system (Meister, 1995), the multistep systems are still incompletely understood. Besides their restricted availability, kinetic analysis of enzymes with six to 29 substrate binding sites and possibly several products is a difficult undertaking. Classical studies on the glutathione system have shown the replacement of substrates, providing clues to the respective active site topography (Kleinkauf & Koischwitz, 1978). In multistep systems the classical studies on the Bacillus antibiotics gramicidin S, tyrocidin and linear gramicidin point to a more complex situation (Kleinkauf & von Döhren, 1996) (Fig. 1).

PEPTIDE FAMILIES SHOW RELAXED SPECIFICITY ONLY AT DEFINED POSITIONS

For a long time analogues of gramicidin S remained undetected, and the control of fidelity of gramicidin synthesis appeared to be strict. Only *in vitro* synthesis by the isolated enzyme or feeding of cultures permitted replacement of Val (Leu) or Orn (Lys, fOrn, Cit). These results seem to indicate that the effective amino-acid concentration at the respective cell site is restricted by the available precursor concentration. So while ^{3}Abu-gramicidin S is now known (Nozaki & Muramatsu, 1987; Thibault *et al.*, 1992), ornithine can be replaced only by feeding of analogues (Haring, 1985). On the other hand, efforts at feeding with analogs of aromatic amino acids have shown that each position in tyrocidin and linear gramicidin displayed a different substrate profile (Kleinkauf & von Döhren, 1996) (Fig. 1).

In their studies on the synthesis of the cyclohexadepsipeptide enniatin (Fig. 2), Zocher *et al.* have characterized depsipeptide synthetases from various strains of *Fusarium* differing in their product profile (Pieper *et al.*, 1992). One suspected possible reason for these differences were different pools of branched chain amino acids controlled by differing metabolic situations. It was dem-

Table 1. Peptide forming enzyme systems

	Single step systems	Multistep systems
Types of peptides made	linear peptides	linear and cyclic peptides
	branched peptides	peptidolactones and depsipeptides
Length of peptides made	2 to 5, or polymers	3 to 48
Activation of carboxyl groups	phosphate or adenylate,	adenylates
	peptide activation	
Intermediates	free intermediates	enzyme bound intermediates

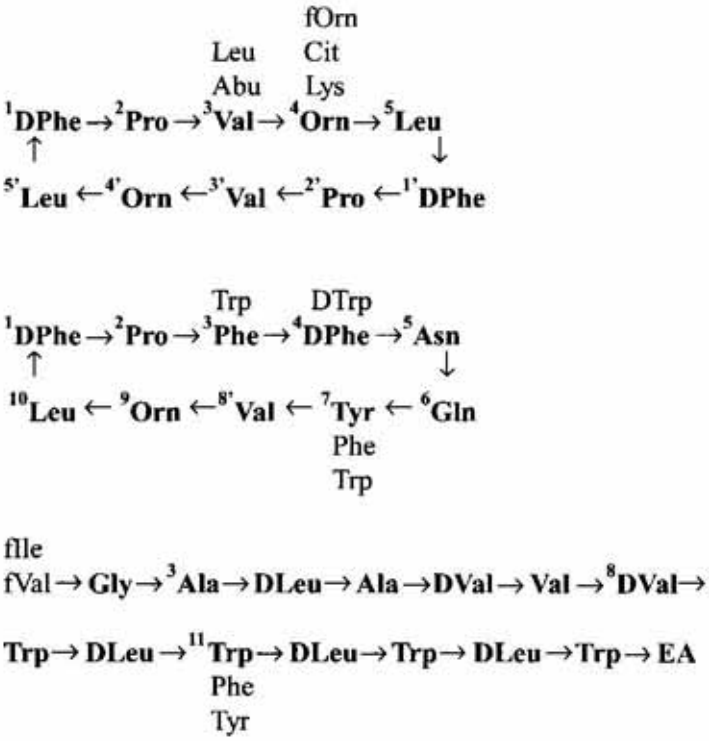

Figure 1. Analogues of gramicidin S (1), tyrocidine (2) and linear gramicidin (3) isolated from cultures (Kleinkauf & von Döhren, 1996).

Note that due to the symmetry of gramicidin S in principle both mono- and disubstituted analogues may be found. Analogues of tyrocidine have similar specificities in positions 3 and 4, while Phe is restricted in position 1, and substitution of Tyr is accepted only in position 7. Analogues of linear gramicidin have only Trp in position 11 and Val in position 1 as variables.

onstrated that, since the multienzymes differred in their affinities for valine, leucine and isoleucine, respectively, primary selection at the branched chain amino acid binding site directed the product pool. Cloning and sequencing of the adenylate forming regions of enniatin synthetases revealed specific positional differences in their peptide sequences (Doller *et al.*, 1996).

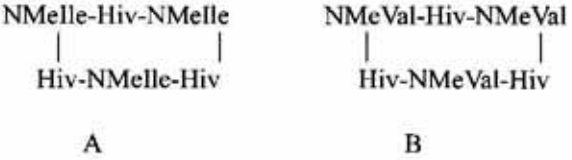

Figure. 2. Structures of enniatin A–C.

Besides these, various mixed products are known, and new enniatins containing Ala, Nva, Abu, Ser and Thr have been generated by *in vitro* synthesis (Krause *et al.*, 1996).

These examples clearly demonstrate that natural peptide families do not display a uniform pattern of amino acid substitution, as would be expected if selection occurred only by uniform selection from a certain pool.

POSITIONAL VARIATIONS MAY BE RELATED TO BIOLOGICAL FUNCTIONS

Positional specified variations in amino acid selection can be also exemplified by various proline-containing peptides. Thus the proline residue in gramicidin S may be replaced by a set of analogues including azetidine-2-carboxylic acid, sarcosine, hydroxyproline, various chloro-, methyl- and thioprolines, 3,4-dehydroproline, but not pipecolic acid (Kleinkauf & von Döhren, 1981). The antifungal cyclopeptide echinocandin (Fig. 3) contains a hydroxyproline residue, which, however, is not replaceable by proline *in vivo* (Adefarati *et al.*, 1991) and *in vitro* (Truglowski & von Döhren, unpublished data). Chemical studies have clearly shown the hydroxy group to be essential for antifungal activity involving inhibition of 1,3-β-glucan synthesis (Zambias *et al.*, 1992).

In the Actinomycete *Streptomyces griseoviridus* the peptidolactone viridogrisein (Fig. 4) containing 4-*allo*-hydroxy-D-proline may be altered to the D-proline analogue neoviridogrisein II by supplementing cultures with proline (Okumura, 1990). The

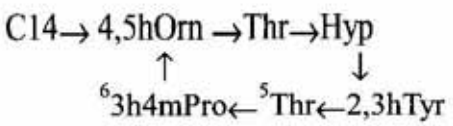

Figure 3. Structure of echinocandin B.

This type of acylcyclopeptide is produced by a variety of fungi in slight structural alterations with respect to the acyl side chain, variation of ^{5}Thr to Ser or Asn, and ^{6}3h4mPro to 3hPro, 4hPro and 3,4hPro. Useful antifungals were obtained by exchange of the acyl side chain and various modifications of hydroxyl groups.

nonhydroxylated product is an even more efficient antibacterial agent in certain applications.

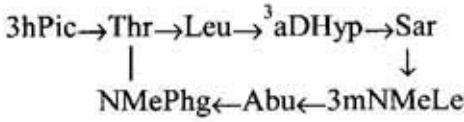

Figure 4. Structure of viridogrisein.

allo-D-Hyp in position 3 may be changed to D-Pro by feeding Pro to fermentations. Presumably the hydroxylation of L-Pro is the rate-limiting step, and the enzyme system accepts L-Hyp as a direct precursor. At high Pro concentrations it is accepted despite lower affinity and epimerized by the synthetase (Okumura, 1990).

These results suggest that peptide structures and thus their biosynthetic enzyme systems have adapted to their interactions with specific target molecules. So substrate specificities with regard to natural precursors may guide structure-function studies at least to naturally relevant receptors or interacting molecules.

EVIDENCE FOR PROOF-READING STEPS BEYOND ACTIVATION

Clues for the existence of additional controls besides primary substrate selection have come from the work on penicillin biosynthesis. The tripeptide precursor with the structure Aad-Cys-DVal is formed on ACV synthetase, a multienzyme containing three activation domains (Aharonowitz *et al.*, 1993). To correlate amino acid selection with domain structures, individual domains have been isolated by limited proteolysis or fragment expression. While the middle adenylate domain specifically activated Cys (Tavanlar, 1996), the N- and C-terminal domains were found to form both Aad and Val adenylates (Schwecke *et al.*, 1993; Etchegaray, 1997; Tavanlar, 1996). The correlation thus remained unsolved, until Schwecke *et al.*, (1993) showed that only Aad did form an intermediate thioester at domain 1. This points to a misactivation of Val, which is unexpected since it has never been detected in this position of tripeptides isolated from penicillin producers or formed by enzymatic synthesis.

Similar misactivations are known for Glu in ACV synthetase, which is activated but not incorporated into the peptide. Instead, it enhances the formation of Cys-DVal, apparently promoting initiation of peptide synthesis at position 2 (Shiau *et al.*, 1995). In gramicidin S, phenylserine was found to be activated presumably at the phenylalanine binding site, but no peptide was formed (Kleinkauf & von Döhren, 1981). These observations suggest controls following the adenylate formation, which may prevent thioester formation or modification of the thioester (e.g. methylation or epimerization), and thus will lead to adenylate hydrolysis as a proof reading reaction, or will interrupt peptide synthesis.

Controls operating at the peptide bond formation step concern also wrong configuration, since a D-Leu thioester intermediate at the L-Leu binding site in gramicidin S synthetase inhibited completion of peptide synthesis (Saxholm *et al.*, 1972).

ONLY A LIMITED NUMBER OF ALTERNATIVE STEPS IN PEPTIDE ELONGATION ARE TOLERATED, GENERALLY NOT MORE THAN TWO

Besides the primary selecting surfaces of substrate binding sites, additional controls may be relevant for peptide synthetic performance. Thus, a comparison of the so far isolated 32 analogues of the powerful immunosuppressor cyclosporin (Kallen *et al.*, 1997) (Table 2) leads to the striking observation that each analogue has at most two alterations. All possible alterations would statistically permit the synthesis of more than 72000 analogues. The preference for certain structures points at first place to

Table 2. Cyclosporins produced by *Tolypocladium niveum* in different nutrient broths (Kallen *et al.*, 1997)

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	**MedBmt**	**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	MeLeu	**Leu**	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	MeLeu	**Leu**	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	MeLeu	**Leu?**	MeVal	MeBmt	Abu	Sar	MeLeu	Val	**Leu?**	Ala
CyS	D-Ala	MeLeu	MeLeu	MeVal	MeBmt	**Thr**	Sar	**Val**	Val	MeLeu	Ala
CyU	D-Ala	MeLeu	**Leu**	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	MeAOAAbu	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	Gly	MeLeu	Val	MeLeu	Ala

occurence of amino acid selection sites of low stringency, such as position 6. Obviously variations in this position define the major constituents of the natural peptide family. Not more than one additional alteration is tolerated, although many are known. Especially lack of N-methylation as a modification preceding amino acid incorporation is known to reduce the rate of synthesis. Upon inhibition of the respective *N*-methyl-transferase by, e.g., sinefungin, Billich & Zocher (1987, 1990) have produced demethyl derivatives of enniatins and shown a reduction of the rate of synthesis by 90%. If each elongation step utilizing a methylated aminoacyl residue were similarly reduced, the expected rate for two unmethylated sites in a peptide would be 1%. Such a reduction could explain why, despite the presence of seven N-methyl functions, no natural peptides exceeding the didemethyl state have been found.

Likewise, in the case of the antifungal cyclononapeptidolactone aureobasidin (Table 3) with four N-methyl residues only single demethyl analogs have been identified, among a total of 29 isolated (Awazu *et al.*, 1995; Kleinkauf & von Döhren, 1997). These observations suggest that the overall rate of peptide biosynthesis by the complex pathway is determined by a variety of individual steps. Reduced rates of single steps will be additive, perhaps in a complex manner, and thus specific steps of elongation may be slowed down so that intermediates are rather lost by hydrolysis than allowed to proceed on the biosynthetic pathway.

However, also the opposite may be found, as has been demonstrated in the gramicidin S system. Dipeptides containing imino acids or N-methylated residues easily form piperazinediones, a well-known side reaction in solid-phase peptide synthesis. Substitu-

Table 3. Aureobasidins produced by *Aureobasidium pullulans* in different nutrient broths (Awazu *et al.*, 1995)

Peptide	1	2	3	4	5	6	7	8	9
A	(2R,3R)Hmp	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
T1	(2R,3S)Hmp	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
T2	(2R,3S)Hmp	MeVal	Phe	MePhe	Pro	aIle	MeLeu	Leu	ßhMeVal
T3	DHiv	MeVal	Phe	ßhMePhe	Pro	aIle	MeLeu	Leu	MeVal
T4	DHiv	MeVal	Phe	ßhMePhe	Pro	aIle	MeaIle	Leu	MeVal
U1	DHiv	Val	Phe	ßhMePhe	Pro	aIle	MeaIle	Leu	MeVal
U2	DHiv	Val	Phe	2hMePhe	Pro	Val	MeaIle	Leu	MeVal
S1	Hmp	MeVal	Phe	MePhe	Pro	Met(O)	MeVal	Leu	ßhMeVal
S2a	Hmp	MeVal	Phe	MeTyr	Pro	aIle	MeVal	Leu	ßhMeVal
S2b	2,5h3mP	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
S3	2,3h3mP	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
S4	2,4h3mP	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
B	Hiv	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
C	Hmp	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
D	Hmp	MeVal	Phe	MePhe	Pro	Val	MeVal	Leu	ßhMeVal
E	Hmp	MeVal	Phe	ßhMePhe	Pro	Val	MeVal	Leu	ßhMeVal
F	Hmp	MeVal	Phe	MePhe	Pro	Val	Val	Leu	ßhMeVal
G	Hmp	MeVal	Phe	MePhe	Pro	Val	MeVal	Leu	MeVal
H	Hmp	MeVal	Phe	MePhe	Pro	Val	MeVal	Leu	Val
I	Hmp	MeVal	Phe	MePhe	Pro	Leu	MeVal	Leu	ßhMeVal
J	Hmp	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	NßMeAsp
K	Hiv	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	MeVal
L	Hmp	MeVal	Phe	MePhe	Pro	Val	MeVal	Leu	MeVal
M	Hmp	MeVal	Phe	Phe	Pro	aIle	MeVal	Leu	MeVal
N	Hmp	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	MeVal
O	Hmp	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	ßhMeVal
P	Hmp	MeVal	Phe	MePhe	Pro	aIle	Val	Leu	MeVal
Q	Hmp	MeVal	Phe	MePhe	Pro	aIle	MeVal	Leu	MePhe
R	Hmp	MeVal	Phe	ßhMePhe	Pro	aIle	MeVal	Leu	MeVal
a1	Hmp	MeVal	oFPhe	ßhMeoFPhe	Pro	aIle	MeVal	Leu	MeVal
a2	Hmp	MeVal	mFPhe	ßhMemFPhe	Pro	aIle	MeVal	Leu	MeVal
a3	Hmp	MeVal	Phe	ßhMePhe	Hyp	aIle	MeVal	Leu	MeVal
a4	Hmp	MeVal	Phe	ßhMePhe	SPro	aIle	MeVal	Leu	MeVal
a5	Hmp	MeVal	Phe	ßhMePhe	Pro	Nle	MeVal	Leu	MeVal
a6	Hmp	MeVal	Phe	ßhMePhe	Pro	Met	MeVal	Leu	MeVal
a7	Hmp	MeVal	Phe	ßhMePhe	Pro	aIle	MeVal	aIle	MeVal
a8	Hmp	MeVal	Phe	ßhMePhe	Pro	aIle	MeVal	Nva	MeVal

tion of proline in a gramicidin S biosynthetic system by 3,4-dehydro-proline enhanced piperazinedione formation by a factor of five, and thus decreased the overall yield of the cyclodecapeptide (Kleinkauf & von Döhren, 1981). Likewise, ornithyl-bound intermediates tend to abort by piperidone formation, a restriction not observed if lysine is substituted with its sterically unfavorable length (Vater *et al.*, 1987). Thus side reactions also known from solid phase procedures may lead to additional products in enzymatic reactions.

CONCLUSIONS FOR LIBRARY CONSTRUCTIONS USING PEPTIDE SYNTHETASES

As has been shown for cyclosporins, the preparative synthesis of a variety of cyclic peptide analogues is feasible (Fig. 5). However, not all combinations of alterations will be accessible to the system. To extend the repertoire of each synthetase, defined structural alterations are required. Synthesis with the use of a synthetase obviously does not match the performance of chemical methods with regard to structural analog utilization, since additional constraints are involved including substrate selection and protein stability. However, peptide synthetases combine a number of advantages including stereoselectivity and efficient cyclization. In addition valuable information on peptide-target interactions may be obtained in some cases, since strictly controlled positions appear to be essential for the biological role of these peptides.

Since the variation of specificity as well as complete exchange of activation domains has

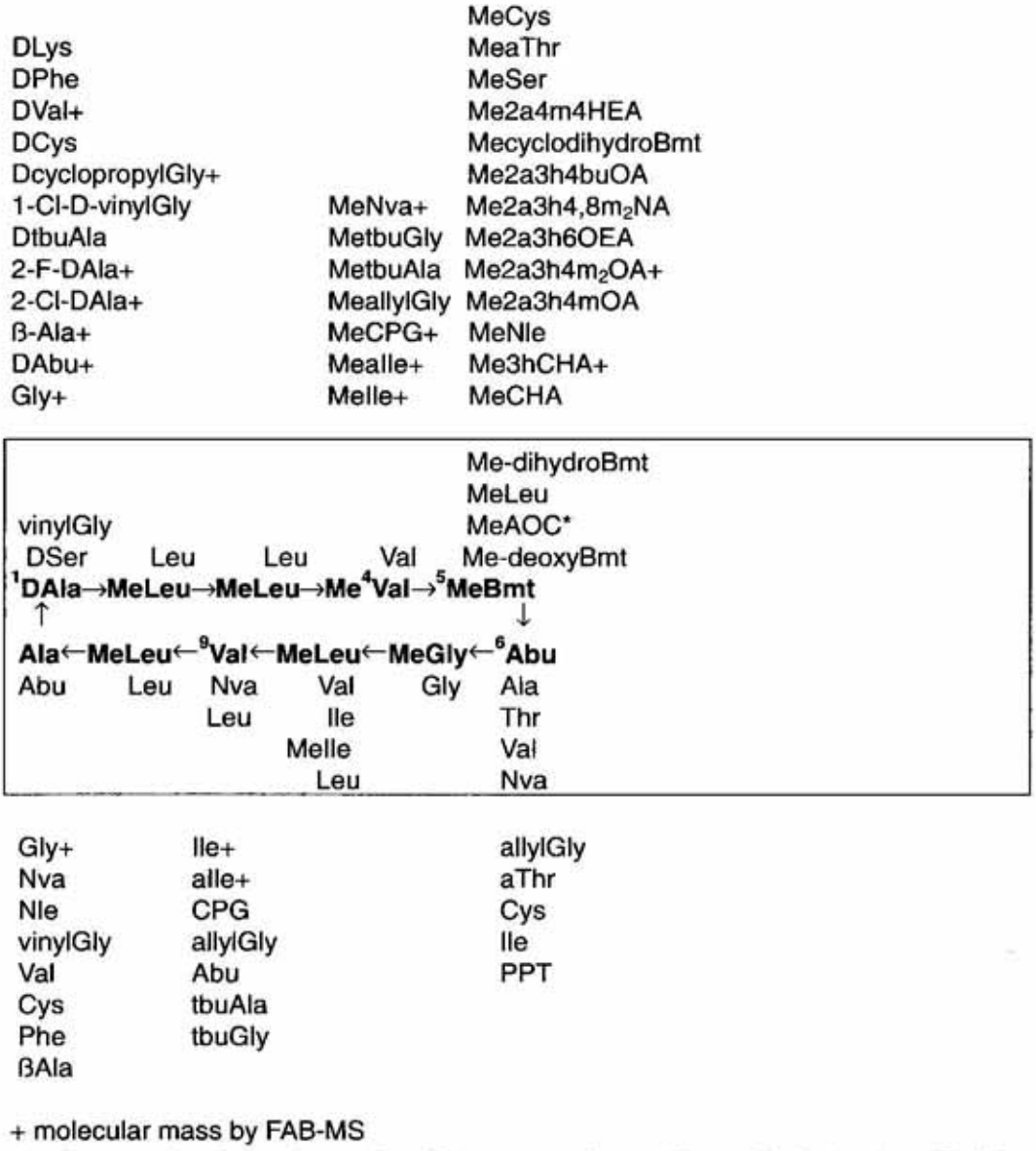

Figure 5. Analogues of cyclosporin produced by enzymatic peptide synthesis *in vitro* (Kleinkauf & von Döhren, 1997).

Except for positions 4 and 9, compounds with single exchanges were produced. Boxed substitutions are compounds isolated from fermentation broths.

been demonstrated at the molecular level, exciting prospects for peptide chemistry have been opened.

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