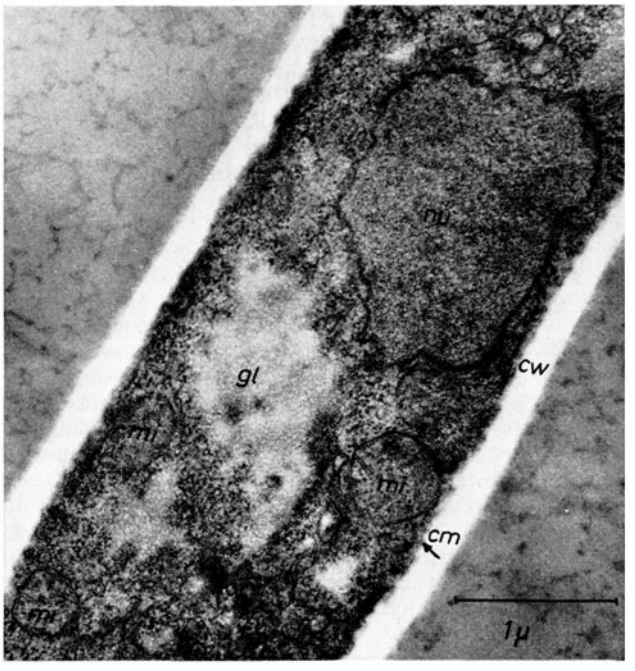

Fig. 5. Ultrathin section of *C. lipolytica* cultivated on glucose medium.

The principal differences in the ultrastructure of yeast cells grown on glucose or on hydrocarbons can be summarized into 8 features, shown schematically in Figure 1. (1) The surface of the yeast cell wall after growth on hydrocarbons is covered with a thin layer of hydrocarbons which penetrate through the cell wall to the cell membrane. The accumulation of hydrocarbons is especially marked in yeast cells grown on gas oil which never disappears completely from the growth medium (Figure 3). The mechanism of penetration could not be analyzed in full detail but it appears that ultrafine pores are involved. Hydrocarbons accumulate on the surface of the cytoplasmic membrane. (2) The cytoplasmic membrane of cells grown on hydrocarbons is always thicker and clearly visible and contains deep invaginations and digital projections which represented an increase of the surface of the cytoplasmic membrane. Pinocytotic vesicles were frequently observed at the ends of deep invaginations, suggesting the possibility of an active translocation of hydrocarbons into the cytoplasm (Figures 2 and 3). (3) Yeast cells grown on hydrocarbons contain more abundant endoplasmic reticulum (Figure 2). (4) Cells grown in media with hydrocarbons contain more fat vacuoles than do cells grown in a glucose-containing medium (Figures 2 and 5). (5) Yeast cells grown on hydrocarbons have more mitochondria which contain frequently an intramitochondrial vacuole (Figure 4). (6) The cell wall of these yeasts is thinner than in cells grown on glucose (Figures 3 and 5). (7) The cytoplasm of cells grown on hydrocarbons is more electron-dense and contains more ribosomes. (8) Cells grown on glucose contain numerous glycogen granules (Figure 5) whereas the hydrocarbon grown cells contain less polysaccharide and more fat vacuoles.

Our observations support the view that hydrocarbons penetrate through the cell wall of *C. lipolytica*, are concentrated at the surface of the cytoplasmic membrane and bring about numerous morphological changes of the cell. The cytoplasmic membrane seems to play an important role in the metabolism of hydrocarbons and in their transport into the cell. The question remains whether the hydrocarbons are oxidized at the cytoplasmic membrane or whether they penetrate by pinocytosis into the cytoplasm to be oxidized there by enzymes associated with the membraneous system of the cytoplasm.

Zusammenfassung. Durch Lösung von 0,1% Vanadium- oder Nickel-Naphthenat in Kohlenwasserstoffen kann deren Durchtritt in die Zelle der Hefe *Candida lipolytica* elektronenmikroskopisch verfolgt werden. Die Kohlenwasserstoffe durchdringen die Zellwand, reichern sich an der Zytoplasmamembran an und verursachen im Zellinnern reiche Veränderungen. Diese beweisen meistenteils die Schlüsselaufgabe der Zytoplasmamembran, den direkten Kontakt der Kohlenwasserstoffe mit den Oxydationsenzymen zu vermitteln.

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DISPUTANDUM

Peptide Antibiotic Biosynthesis: A New Approach

It is now well established that the biosynthesis of peptide antibiotics is independent of the ribosomal RNA-requiring processes operating in protein synthesis. A wealth of evidence from studies with tyrocidines[1], gramicidin S[2-4], polymyxins[5], bacitracin[2-6], actinomycins[7-8], and U-22324[9], together with a retraction[10] of earlier contra-indicatory findings for gramicidin S, leaves no doubt that a purely enzymatic process is involved. A recent cell-free

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synthesis of gramicidin S[11] affords an elegant demonstration of these facts. Nevertheless, much remains to be revealed concerning, for example, the precise origins of the D-amino acid residues and the cyclic structures found in most peptide antibiotics.

A process of peptide elongation by stepwise, enzyme-controlled addition of amino acids, long known for glutathione[12], has been demonstrated for the peptides of bacterial uridine nucleotides[13], into which D-amino acids are directly incorporated. However, for most peptide antibiotics, there is strong evidence against such a process. Thus L-valine, not D-valine, is the precursor of the D-penicillamine moiety of penicillin[14–16] and of the D-valine residues in actinomycin[17] and valinomycin[18]. Similarly, the D-ornithine[19,20] and D-phenylalanine[6,20] in bacitracin, and the D-leucine in polymyxin D[21], are derived from the corresponding L-isomers; in some cases the free D-amino acids are inhibitory[15,17,20]. It has been shown that inversion of the L-amino acid does not involve a deamination step, for example in penicillin[22] and actinomycin[23]. Inversion of configuration in those amino acids which possess 2 asymmetric centers occurs only at the α-carbon; hence, the occurrence of D-allo-hydroxyproline[24] in etamycin[25] and of D-allo-isoleucine in certain actinomycins[26]. In the latter case L-isoleucine is demonstrably the precusor[27]. For gramicidin S the evidence is ambiguous, but recent findings[11] indicate that L-phenylalanine is a more efficient precursor than the D-form.

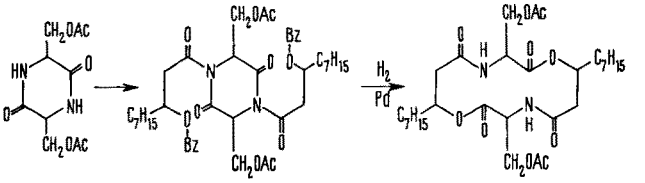
The probable explanation for these observations is that inversion of an L-amino acid is preceded by its incorporation into a biosynthetic intermediate. In considering possible structures for such intermediates it seems pertinent that they are generally destined for incorporation into peptides which are cyclic. Possible modes of construction of such cyclopeptides include ring-closure of a completed acyclic peptide or a process of ring-expansion via insertion reactions. The former scheme presupposes that antibiotic-producing organisms can constrain peptide chains in the required conformation for ring-closure. This seems unlikely in view of the wide variations in ring-size encountered in these compounds.

A plausible alternative is provided by the following hypothesis: (a) D-amino acids in antibiotics are formed from L-amino acids after incorporation of the latter into stereochemically labile intermediates. (b) Such intermediates are cyclic dipeptides, i.e. diketopiperazines (DKP's). (c) DKP intermediates undergo ring-expansion via condensation with other DKP's and/or incorporation of amino acid or hydroxy acid residues according to the 'insertion principle'[28,29].

The following key stages in the chemical synthesis of serratamolide[30] illustrate the insertion principle (Diagram 1). SHEMYAKIN and ANTONOV have suggested that related reactions may play a role in the biosynthesis of depsipeptides[29]. The successful insertion of amino acids[31] into peptides suggests wider application of the insertion idea to cyclopeptides in general. As a further extension of the postulate, it is here proposed that insertion of diketopiperazines into peptides may be involved in cyclopeptide biogenesis, though such reactions have as yet no synthetic parallel.

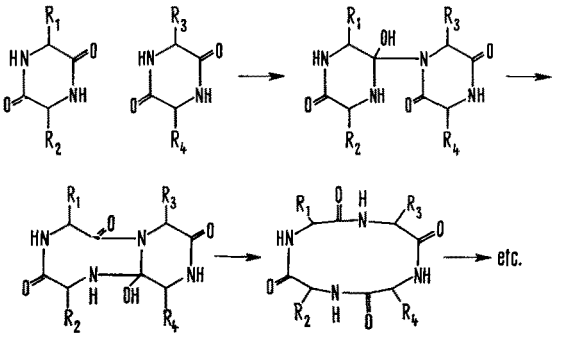
Condensation of two DKP's, as illustrated, would require prior activation, e.g. by acylation by an enzyme, and can be conceived as a trans-peptidation proceeding via ortho-amide intermediates of the kind discussed by WRINCH[32] (Diagram 2). Intervention of such small-ring structures may account for the presence of D-amino acid residues in antibiotics, since DKP's have long been known to epimerize readily[33]. In particular, it is energetically favorable for a *cis*-disubstituted DKP (derived from 2

Diagram 1



Diacetyl serratamolide

Diagram 2



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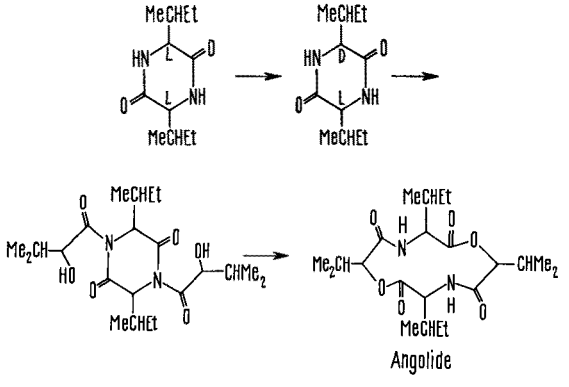
L-amino acids) to isomerize to the thermodynamically more stable trans (L-D)-form. For example, base treatment of *cyclo*-[L-phenylalanyl-L-prolyl] affords *cyclo*-[D-phenylalanyl-L-prolyl][34]. Formation of the latter DKP[35] upon incubation of L-phenylalanine and L-proline with a cell-free extract of *B. brevis* Nagano (which produces gramicidin S containing the D-phe-L-pro sequence) shows that similar transformations can be effected by enzymes. During biosynthesis, specific amino acid residues could undergo inversion at the DKP stage or in more complex intermediates still containing DKP-like structural features. This conclusion receives support from some experimental observations and from the distribution pattern of D-amino acids in antibiotics (see below).

Occurrence of DKP's in nature, particularly in fungi and in *Streptomyces* strains[36], is widespread. For example, a variant of *S. noursei* produces a number of DKP's, including *cyclo*-[L-phenylalanyl-L-leucyl][37], and phalamycin, an antibiotic peptide containing these 2 amino acids[38]. However, involvement of DKP's in peptide antibiotic biosynthesis has not been postulated hitherto.

The following examples illustrate the above concepts.

Cyclodepsipeptides. The possible role of hydroxy acid insertion reactions in the biosynthesis of depsipeptides[39] has been pointed out by SHEMYAKIN and ANTONOV[29]. The simple case of angolide[40] is illustrative of the principles outlined above. Epimerization of L-isoleucine DKP, followed by hydroxy acid insertion is a probable biosynthetic route (Diagram 3).

Diagram 3



Valinomycin, *cyclo-Tris* (L-lactyl-L-valyl-D-α-hydroxy-valeryl-D-valyl)[41], is a more complex example of alternating L- and D-amino acid residues interspersed with hydroxy acid residues. Such a structure could be formed by trimerization of *cyclo*-[D-valyl-L-valyl] and subsequent hydroxy acid insertion, or by some other sequence of these reactions. Striking support for such a scheme (Diagram 4) is the observation[18] that L-valine-C^{14} is incorporated exactly equally into the D- and L-valine residues of the antibiotic – a direct consequence of the involvement of a symmetrical precursor such as L-valine DKP. Likewise, sporidesmolide I[42] can be envisaged as a condensation product of 2 epimerized DKP's and 2 hydroxy acid residues; L-valine is known[43] to be the precursor of both the L- and D-valine residues in the antibiotic. The *N*-methyl amino acids which occur in this and several other antibiotics never possess the D-configuration; hence the enniatins[39] contain no D-amino acids.

Peptide lactones. This group of antibiotics includes the actinomycins, etamycin and related 3-hydroxy-picolinoyl peptides, and the quinoxaline antibiotics such as echinomycin. The hydroxy acid component is a serine or threonine residue which could be incorporated by insertion into DKP-derived precursors. From studies by KATZ and WEISSBACH, the biosynthesis of actinomycins[44] appears to involve oxidative dimerization[45] of a 3-hydroxy-4-methyl-anthraniloyl-pentapeptide lactone. This intermediate could be constructed from 3 fragments as illustrated (Diagram 5). This scheme is supported by the

Diagram 4

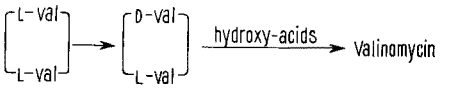
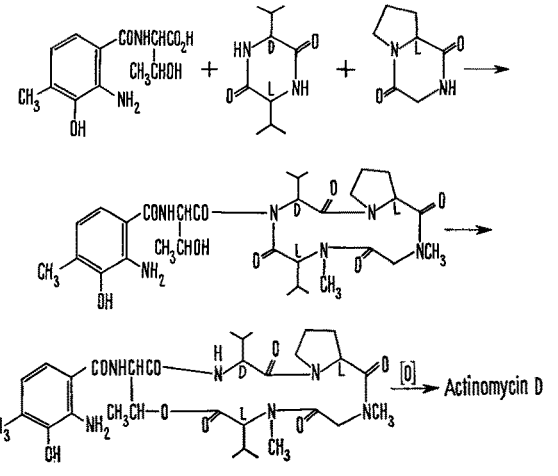


Diagram 5



The above scheme is not intended to represent the precise sequence of the various stages, e.g. *N*-methylation, or to imply that 4-methyl-3-hydroxyanthranilic acid, which has been implicated as a precursor[46], is necessarily present in the form shown throughout. An alternative pathway involving pro-val and meval-sar DKP's is excluded because sarcosine is known to replace proline in certain actinomycins[47] and the inversion of L-valine would not then be explained.

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exactly equal incorporation of L-valine-C^{14} into the D-valine and L-*N*-methylvaline residues[7], recalling the case of valinomycin.

It is assumed that DKP's containing sarcosine do not epimerize, since there is no drive to relieve steric strain of the type present in DKP's possessing 2 (*cis*) side-chains.

The case of etamycin[25] is strikingly analogous, although here 3 precursor DKP's are involved (Diagram 6).

The insertion scheme[29] requires that the initial acylation takes place at the nitrogen atom of a primary amino acid. It is therefore noteworthy that despite the prevalence of secondary amino acids in the peptide lactone antibiotics, the serine or threonine residue is invariably located adjacent to a primary amino acid.

The examples discussed thus far illustrate the principle that, with the exception of those amino acids (e.g. threonine) which are incorporated singly, equal numbers of D- and L-amino acids are found, provided sarcosine and/or glycine are considered as 'D'-amino acids.

A related explanation may account for the presence of D-serine residues in the quinoxaline antibiotics such as echinomycin[48], the quinomycins[49], and the triostins[50]. Although serine lies outside the DKP-derived portion of the molecule, it is at one stage of the insertion reaction part of a 6-membered ring wherein its inversion might be favored for steric reasons (Diagram 7).

In relating the hexapeptide lactone antibiotics (staphylomycin S[51], ostreogrycin B[52], and the vernamycins[53]) to the scheme shown for actinomycin and etamycin, the presence of an additional L-amino acid is seen, which must be singly incorporated in such a scheme. The presence of L-4-ketopipecolic acid exclusively in this group suggests that this may be the 'extra' amino acid. Conceivably this is a case of hydroxy acid insertion (and subsequent oxidation to the keto compound after O → N-acyl shift). It appears that those amino acids possess additional functional groups (OH or NH_2) are best accommodated on the basis of single insertion, in contrast to the simpler amino acids incorporated via DKP's. Such functional groups could facilitate insertion reactions and rearrangements based upon acyl shift.

Antibiotics containing α,γ-diaminobutyric acid. Extension of the above concepts to those antibiotics which contain α,γ-diaminobutyric acid (dab) is feasible. Polymyxin B_1[54] exemplifies this group of substances (Diagram 8), in which the cyclic portion of the peptide appears to derive from a DKP 'core' by insertion of L-dab and L-threonine residues. Polymyxins B_2, E_1 and E_2, and circulins A and B all possess analogous structures[55]. The DKP 'core' provides 1 D- and an adjacent L-amino acid in the final structure, the remaining amino acids all consisting of L-dab and L-threonine. In view of the ready N,N'-acyl migrations observed in this series, the branch-point may be mobile during biosynthesis, providing another type of ring-expansion process. The presence of D-dab in polymyxins A_1 and A_2 and of D-serine in the corresponding (3) position of polymyxins D_1 and D_2 requires explanation.

Gramicidin S and the tyrocidines. This group of cyclopeptide antibiotics contain L-ornithine, a higher homolog of L-dab, so that it is logical to seek in their structures possible DKP-derived moieties which could combine with ornithine via insertion reactions (Diagram 9). Gramicidin S at first appears to present an obvious case – 4 DKP's (2 epimerized and 2 not) and 2 singly-inserted ornithine residues could combine in various ways to form the cyclic decapeptide. One possibility, as shown, involves leucylvaline DKP and D-phenylalanylproline DKP as precursors; the latter substance has actually been isolated from cell-free extracts of *B. brevis* nagano incubated with L-phenylalanine and L-proline[35].

Diagram 6

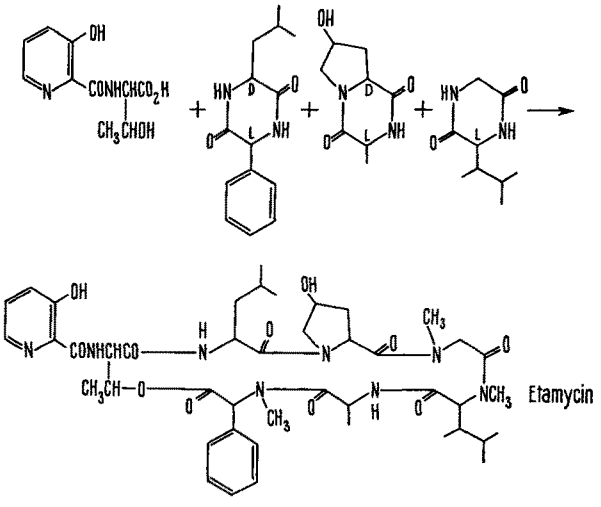

Diagram 7

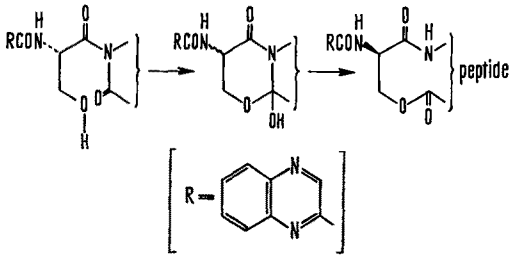

Diagram 8

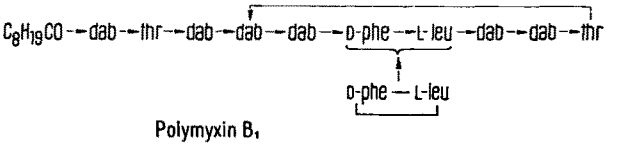

Diagram 9

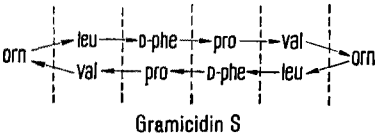

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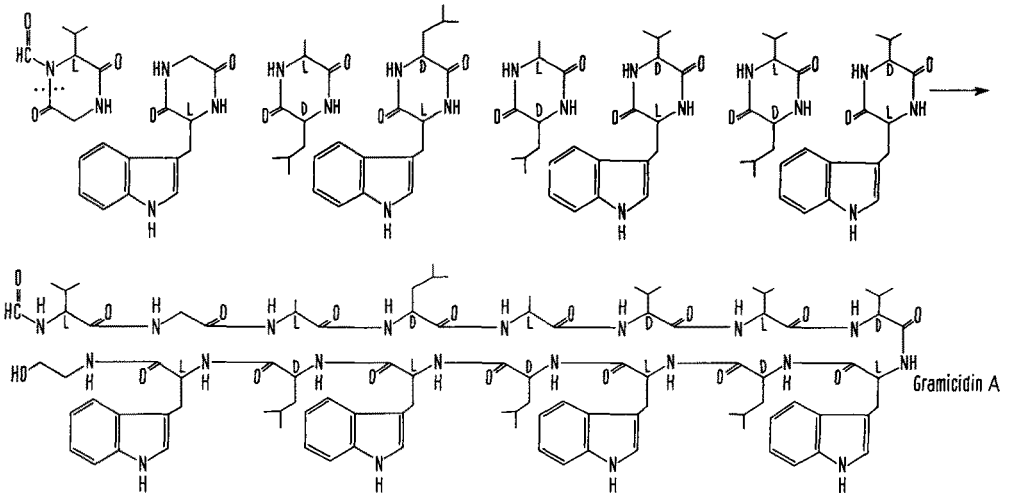
Diagram 10



Nevertheless, it is necessary to interpret the biosynthesis of this group of antibiotics with caution, for several reasons. There exists a close relationship between gramicidin S and the tyrocidines; all contain within the cyclodecapeptide structure the sequence val-orn-leu-D-phe-pro, suggesting that this pentapeptide, possibly in a bound form, is itself a precursor. Conceivably there is combination of 2 cyclic pentapeptides, one of which in each case possesses the above sequence and is in turn derived from L-ornithine and the 2 above-mentioned DKP's. However, experimental studies on these antibiotics do not entirely support the above concepts. For example, cell-free synthesis of D-phenylalanyl-L-prolyl-L-valine[56] and of a related (bound) tetrapeptide containing ornithine[57] have been described, though there is no proof that these peptides are actual precursors. The ambiguities observed in the genesis of the D-phenylalanine residue contrast strangely with the evidence obtained for other antibiotics. However, in view of the conflicting data[10] reported for this group of compounds it seems premature to preclude the possibility of DKP involvement in their biosynthesis.

Examples of D- *and* L-*amino acid alternation.* It is apparent that in those biosyntheses where intervention by hydroxy acids or diamino acids is absent, the intermediacy of epimerized DKP's could lead to structures possessing an alternating sequence of D- and L-amino acids. Fungisporin[58], *cyclo-bis*-[D-phenylalanyl-L-phenylalanyl-D-valyl-L-valyl], is such a case, and can be regarded as a copolymer of 2 epimerized DKP's.

Further examples are gramicidins A–C[59], a closely related series of acyclic peptides which present a remarkable unbroken pattern of alternating D- and L-amino acid residues, with the earlier stated proviso that glycine is regarded as 'D'. Thus gramicidin A is Formyl-L-val-gly-L-ala-D-leu-L-ala-D-val-L-val-D-val-L-try-D-leu-L-try-D-leu-L-try-D-leu-L-try-$NHCH_2CH_2OH$; the other members differ only in the replacement of an L-try by L-phe or L-tyr and/or of *N*-terminal L-val by L-ileu. Assuming that the C-terminal ethanolamine is derived reductively from glycine, the biosynthetic process could be based upon an 'assembly line' of epimerized DKP's utilizing a concerted or consecutive series of transpeptidation reactions. It is of interest to consider whether, in such a chain of events, the formylation of the terminal DKP plays an initiating or terminating role, or both (Diagram 10). The intermediacy of DKP's in this manner explains why glycine occupies a 'D' position in the alternating sequence.

In the foregoing examples, D-amino acid distribution has been correlated with the possible role of epimerized DKP's as biosynthetic precursors. The aim has been to illustrate general principles rather than precise pathways in the absence of more positive experimental evidence. Furthermore, it is not intended to exclude related concepts in which discrete DKP's would not be involved. For example, amino acids could enter an enzyme-controlled assembly system and pass through intermediate stages in which DKP rings are always bound to the enzyme or are part of larger intermediates in the transpeptidation process. Mechanisms similar to those already discussed could then account for the D-amino acid residues and macrocyclic structures observed in the peptide antibiotics. It is intended to investigate experimentally some of the possibilities discussed here[60].

Zusammenfassung. Eine verallgemeinernde biosynthetische Theorie über die Entstehung von D-Aminosäuren in Peptid-Antibiotika wird dargestellt und zur Bildung von makrozyklischen Strukturen aus kleineren Ringsystemen in Beziehung gesetzt.

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