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# ALAMETHICIN BIOSYNTHESIS

# ACETYLATION OF THE AMINO TERMINUS AND ATTACHMENT OF PHENYLALANINOL

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## Summary

Alamethicin synthetase was extracted from the fungus *Trichoderma viride* at the end of its exponential growth phase. It is multienzyme complex with a molecular weight of approx. 480 000. The biosynthesis of alamethicin is initiated on the synthetase by acetylation of thiolester-bound aminoisobutyric acid, which remains enzyme bound. Acetyl-CoA serves as the acetate donor. Of the alamethicin constituents, glycine, alanine and valine are also acetylated when incubated alone. This acetylation is prevented by added aminoisobutyric acid, which indicates that the site on alamethicin synthetase catalyzing the acetylation has a preference for aminoisobutyric acid. Alamethicin formation on the synthetase is terminated by linkage of phenylalaninol to the carboxyl terminus of the peptide. It is unlikely that the amino alcohol is a degradation product of alamethicin or that it had been split off from the synthetase complex. Thus it is probably the reaction product of a separate enzyme system.

## Introduction

We have previously described the isolation and fractionation of a multienzyme complex synthesizing the peptide antibiotic alamethicin in the fungus *Trichoderma viride* [1,2].

We suggest that the molecule is formed in the same manner as gramicidin S or tyrocidine [3,4]. In an ATP-dependent reaction (requiring bivalent cations, e.g. $Mg^{2+}$ or $Mn^{2+}$) the single amino acids are activated as thiolesters at peripheral SH-groups of the synthetase [1]. Peptide formation (which has still to be demonstrated in the case of alamethicin) occurs in a series of transfer steps by which the growing chain is transported from one thiolester-bound amino acid to the next [3]. At first, a cyclic structure of alamethicin was assumed

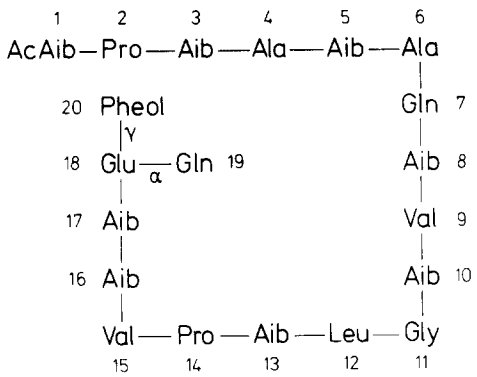


**Fig. 1. Structure of alamethicin according to Martin and Williams [7,8]. Aib, aminoisobutyric acid; AcAib, *N*-acetyl-aminoisobutyric acid; Pheol, phenylalaninol.**

[5,6]. Martin and Williams [7,8] and Jung et al. [9] detected two new structural elements of the molecule, N-acetylated aminoisobutyric acid at the amino terminus and phenylalaninol blocking the γ-carboxyl group of glutamic acid at the carboxyl end. Later observations indicate that the latter may not be correct. Phenylalaninol might bind to a glutamine residue, the C-terminal sequence neighbour of glutamic acid [10,11]. Fig. 1 shows the branched structure proposed by Martin and Williams [7,8] *.

If the biosynthesis of the peptide starts at the amino terminus it will begin with aminoisobutyric acid-1. Our interest was directed to the acetylation of this amino acid and the question whether it might be the initiation reaction that starts peptide formation. Furthermore, we were concerned with the carboxyl terminus of alamethicin: the origin of phenylalaninol and its linkage to the peptide chain.

## Materials and Methods

Radiochemicals were from Amersham-Buchler, Wenden (Germany), acetylcoenzyme A and ATP from Boehringer GmbH, Mannheim (Germany), dithiothreitol from RSA Corp. (Ardsley, N.Y., U.S.A.). All other chemicals were from E. Merck A.G., Darmstadt (Germany). Thin-layer chromatography was performed with thin-layer chromatography plastic sheets F 1500 silica gel from Schleicher and Schüll, Dassel (Germany). The solvents used were ethylacetate/pyridine/water/acetic acid (60 : 20 : 11 : 6, v/v) (solvent 1) and *n*-butanol/acetic acid/water (4 : 1 : 1, v/v) (solvent 2). The chromatograms were autoradiographed on a thin-layer scanner II from E. Berthold, Wildbad (Germany). Radioactivity was counted in an isocap 300 liquid scintillation counter from Nuclear Chicago.

N-acetylated amino[$^{14}$C]isobutyric acid was prepared from amino[$^{14}$C]isobutyric acid and acetic anhydride as described [8]. It was purified by thin-layer chromatography with solvent 1.

[$^{14}$C]Phenylalaninol was synthesized from L-[$^{14}$C]phenylalanine by reduc-

* See note added in proof, p. 385.

tion with $LiAlH_4$. An aqueous solution of 10 μCi L-[$^{14}$C]phenylalanine was evaporated to dryness and the residue suspended in 10 ml dried diethyl ether; 10 mg $LiAlH_4$ were added and the mixture was boiled 4 h under a reflux. Then 2 ml ice-water were added. After $H_2$ formation had terminated, 2 ml 1 M HCl were added to dissolve the precipitate of $Al(OH)_3$. The organic phase was separated and the aqueous phase was extracted twice with 5 ml diethyl ether. The combined ether extracts were evaporated and the residue was dissolved in 2 ml 0.2 M potassium citrate buffer (pH 5.28). The solution was applied to a column of Dowex 50 × 8 ion-exchange resin (size 1 × 2 cm).

L-[$^{14}$C]Phenylalanine was eluted with the citrate buffer. After washing the column with water the labelled phenylalaninol was eluted with 0.5 M ammonia solution. The amino alcohol was further purified by thin-layer chromatography, with solvents I and II.

*In vivo experiments.* The *T. viride* strain was a gift from Dr. F. Reusser, J. Upjohn Co. (Calamazoo, Ind., U.S.A.). It was stored in sterile soil. Stock cultures were grown in 2-l Erlenmeyer flasks containing 300 ml aqueous solution of 2.5% soy peptone and 2.5% malt extract, both from E. Merck AG, Darmstadt (Germany).

The cells were cultured in a New Brunswick incubation shaker (New Brunswick, N.Y., U.S.A.) at 200 rev./min and a temperature of 28°C for 3 days. Fermentation was carried out in a New Brunswick 12 l fermentor, agitation speed 200 per min, supplied with 60 l of air per min. The nutrient broth contained 1.5% soy bean meal, 1.5% fish meal (both gifts from the Schering AG (West-Berlin)), 2% malt extract and 3% dextrin, in tap water. 3 l of this broth were inoculated with the stock culture.

After 40 h, the thick slurry was diluted to 12 l with tap water and fermented for another 20—24 h. Then the mycelia were filtered on a Buchner funnel, washed thoroughly with tap water and stored at −80°C. Yield per fermentation was approx. 250 g wet cells. For labelling experiments, 1 g freshly harvested cells was suspended in 10 ml tap water and incubated with 0.1—1 μCi of the respective radioactive substances. The suspension was shaken for 30 min in a water bath shaker and the cells were then filtered and extracted with 20 ml ethanol. The filtrates were combined and evaporated to dryness. The residue was taken up in 500 μl ethanol and 200 μl of this solution were applied to silica gel plates and chromatographed. Labelled products were detected by radiography on the Berthold scanner, scraped off, extracted with ethanol and counted in toluene scintillation liquid. In other experiments, prior to thin-layer chromatography the ethanolic solution was separated by gel filtration on Sephadex LH 20 (column size 1 × 60 cm). Elution was carried out with ethanol. 2-ml fractions were collected and 50-μl aliquots counted for radioactivity. The peak fractions containing labelled alamethicin were pooled and evaporated. The residue was taken up in 500 μl of ethanol and further purified by thin-layer chromatography. The purified alamethicin was taken up in 0.5 ml 6 M HCl and hydrolyzed in sealed glass tubes at 110°C overnight. The components in a 50 μl aliquot of the hydrolysate were resolved by thin-layer chromatography. The sample was scanned on the thin-layer scanner II from Berthold. For studying the metabolism of radioactive L-phenylalanine in *T. viride* the cells were incubated with L-[$^{14}$C]phenylalanine for 30 min at 28°C, then filtered and

thoroughly washed with water. The cells were lyophilized and extracted with 10 ml acetone. The extract was evaporated and the residue was taken up in 200 µl ethanol and separated by thin-layer chromatography. Radioactive bands were estimated on the Berthold-scanner.

*In vitro studies.* All the work described was done at 0—4° C. 50 g cells were lyophilized in a lyophiliser from Leybold-Heraeus (Köln), pulverized in a mortar and suspended in 200 ml 25 mM sodium phosphate buffer (pH 7.2)/0.5 mM EDTA/1 mM dithiothreitol/10% sucrose (buffer A). To this buffer was added 0.25 M KCl. The suspension was stirred for 45 min and then centrifuged for 15 min at 10 000 × *g* in a Beckman J 21 B centrifuge. The pellet was discarded and to the supernatant were added 3% (v/v) of a 10% (w/v) solution of polyethyleneimine 600 from Serva Feinbiochemica (Heidelberg). After stirring for 30 min, the suspension was centrifuged for 15 min at 10 000 × *g* and the debris, containing cell fragments, organelles and nucleic acids [12], was discarded. The clear yellow supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ and stirred for 60 min. After centrifugation at 10 000 × *g*, the pellet was taken up in a minimum amount of buffer A. The resulting solution was designated 'crude extract' and stored at —80° C. For further purification, 2 ml crude extract were applied to a column (2 × 30 cm) of Ultrogel ACA 34 from LKB (Bromma, Sweden), equilibrated with buffer A. Elution was performed with the same buffer and 2-ml fractions were collected. Alamethicin synthetase activity was determined by measuring the aminoisobutyric acid-dependent ATP-$^{32}PP_i$ exchange reaction [13]. In the assay, about 0.1 µCi $^{32}PP_i$ per sample was used. The fractions containing at least 30% of the enzyme activity in the peak maximum were pooled and used for further studies. Molecular weight of the synthetase was estimated by gel filtration according to Andrews [14], on a column of Ultrogel ACA 23 (1 × 50 cm): 300 µl enzyme solution were applied to the column and eluted with buffer A. 0.75-ml fractions were collected and tested for enzyme activity. The column was calibrated with marker proteins from Boehringer GmbH (Mannheim).

Enzyme-bound substrates: the incubation mixture contained 2 ml enzyme solution, 6 mM ATP, 10 mM $Mg^{2+}$, 0.5 µCi $^{14}C$-labelled (and/or 1 mM unlabelled) amino acid, 0.05 µCi [$^{14}C$]acetyl-CoA (or 20 µg unlabelled acetyl-CoA). Incubations were carried out for 15 min at 32° C. The reaction was terminated by cooling the samples in ice-water; 0.5 ml 30% trichloroacetic acid were added to precipitate the protein. After 30 min in the cold, the precipitate was centrifuged at 3000 × *g*. The pellet was washed by resuspending and recentrifuging, first with a 5% solution of trichloroacetic acid (three times), then twice with ethanol/diethyl ether (2 : 1, v/v) and finally once with diethyl ether, 4-ml portions each time. The pellet was either incubated overnight at 4° C with 200 µl performic acid (95% formic acid, 5% perhydrol) or with 200 µl 50 mM NaOH for 15 min at 60° C. In the first case the acid was removed with a stream of air and the residue extracted with 300 µl 80% ethanol. In the second case, 10 µl 1 M HCl were added to the mixture and the solution was lyophilized. The residue was extracted as described above. The extract was applied to silica gel plates and chromatographed. The chromatograms were assayed by radiography with a Berthold-scanner.

## Results

*Preparation of alamethicin synthetase*

Alamethicin synthetase was partially purified as described in Materials and Methods. By gel filtration on Ultrogel ACA 34, the enzyme complex appears as a single peak in the void volume of the column (Fig. 2); and gel filtration of this peak fraction suggests a tentative molecular weight of 480 000 for the synthetase (Fig. 3). As reported earlier [1,2], the enzyme complex is eluted at about 1.4—1.5 $v_0$ from Sephadex G-200, corresponding to a molecular weight of about 230 000—240 000 [14]. In the present study, the method of enzyme preparation had been modified: instead of disintegrating cells mechanically [1,2], they were lyophilized and then extracted with high ionic strength buffer. This seems to protect the alamethicin synthetase against fragmentation.

*Initiation of alamethicin formation*

*In vivo experiments.* There are three ways in which one can visualize the initiation of the biosynthesis of alamethicin: (1) N-Acetylated aminoisobutyric acid is synthesized by *T. viride*, prior or parallel to the antibiotic production, and is a direct substrate of alamethicin synthetase. Activation of cytoplasmic *N*-acetyl-aminoisobutyric acid followed by its binding to the initiation site of the synthetase would then be the primary reactions in the formation of alamethicin. (2) The complete peptide chain is synthesized and the subsequent acetylation of the $NH_2$-terminus at aminoisobutyric acid is a terminating reaction. (3) Formation of *N*-acetyl-aminoisobutyric acid on the synthetase initiates alamethicin biosynthesis. This would be analogous to the formation of gramicidin A: Kurahashi's group [15] found that it starts with the N-formylation of residue valine-1.

After incubation of *T. viride* cells with amino[$^{14}$C]isobutyric acid the amino acid is utilized only for incorporation into alamethicin. No labelled *N*-acetyl-aminoisobutyric acid is detectable in the cell extract (Fig. 4). Acetyl-amino-

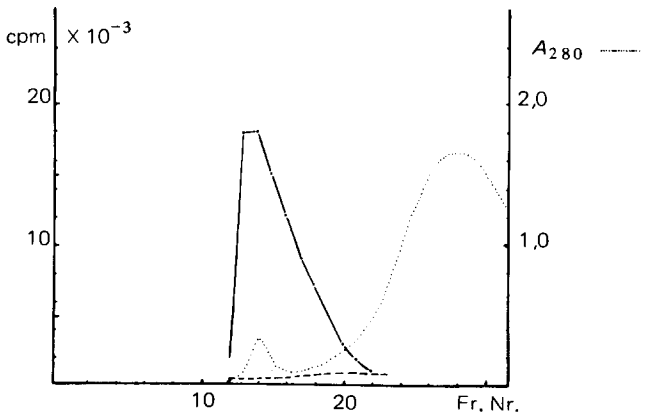


**Fig. 2. Partial purification of alamethicin synthetase by gel filtration. 2 ml crude extract (see Materials and Methods) were applied to a column (2 X 30 cm) of Ultrogel ACA 34. Elution was with buffer A, 2-ml samples were collected. . . . . ., $A_{280nm}$; - - - - - -, amino acid independent ATP-$^{32}PP_i$ exchange; ———, ATP-$^{32}PP_i$ exchange, dependent on aminoisobutyric acid.**

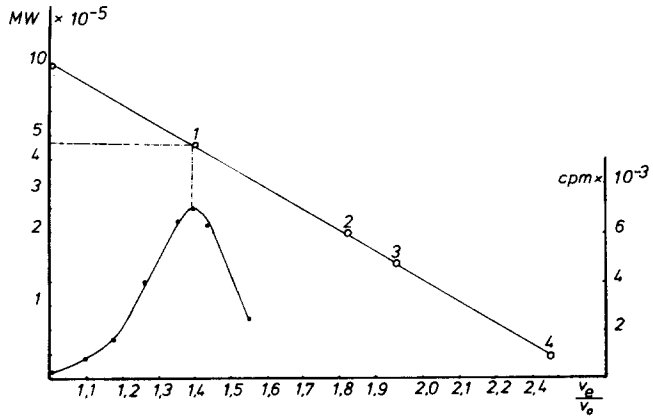


Fig. 3. Estimation of the molecular weight of alamethicin synthetase by gel filtration on Ultrogel ACA 34. 300 µl of the ACA 34 peak fraction (Fig. 2) were applied to the column, size 1 X 50 cm; 0.75-ml fractions were sampled. The synthetase was assayed by the aminoisobutyric acid-dependent ATP-$^{32}PP_i$ exchange (————). Marker proteins: 1, apoferritin, $M_r$ = 480 000; 2, γ-globulin, $M_r$ = 205 000 on gel filtration [15]; 3, aldolase, $M_r$ = 147 000; 4, bovine serum albumin, $M_r$ = 67 000.

[$^{14}$C]isobutyric acid is not accepted by the organism. Under the experimental conditions, it is only hydrolyzed to a small degree (Fig. 5). Therefore, one can conclude that *N*-acetyl-aminobutyric acid itself is neither synthesized by *T. viride* nor is it a direct substrate for alamethicin synthetase.

*In vitro studies.* Alamethicin contains eight aminoisobutyric acid residues,

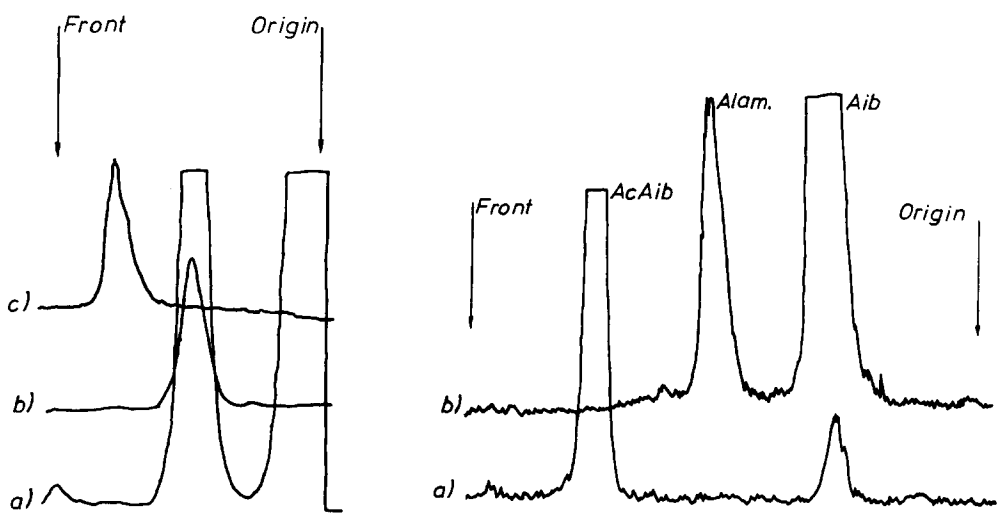


Fig. 4. a, In vivo incorporation of amino[$^{14}$C]isobutyric acid into alamethicin; b, [$^{14}$C]alamethicin marker; c, acetyl-amino[$^{14}$C]isobutyric acid marker. Thin-layer chromatography, scanned with a Berthold-scanner. Solvent: solvent 1.

Fig. 5. Non-acceptance of acetyl-aminoisobutyric acid by alamethicin synthetase. *T. viride* cells were incubated either with acetyl-amino[$^{14}$C]isobutyric acid (a), or with amino[$^{14}$C]isobutyric acid (b). The cell extracts were separated by thin-layer chromatography. Solvents: First solvent 1, then after drying the plate, solvent 2 in the same direction.

hence there might be eight binding sites for aminoisobutyric acid on the synthetase. Presumed the mechanisms of alamethicin formation resembles that of gramicidin S [3] and the peptide chain is formed prior to acetylation of the $NH_2$-terminus, the synthesis starts with a transfer of aminoisobutyric acid-1 from its binding site to proline-2 where the enzyme-bound dipeptide aminoisobutyric acid → proline is formed. The dipeptide is transported to aminoisobutyric acid-3 and the tripeptide aminoisobutyric acid → proline → aminoisobutyric acid is synthesized. The binding sites in positions 1 and 2 simultaneously react with unbound aminoisobutyric acid and proline, respectively, etc. The amount of protein-bound radioactivity should therefore increase by 25% if one incubates alamethicin synthetase together with amino[$^{14}$C]isobutyric acid and L-proline instead of amino[$^{14}$C]isobutyric acid alone. Our experimental data show that the amount of trichloroacetic acid-stable bound amino[$^{14}$C]isobutyric acid is not increasable by the addition of proline or any other of the amino acid components of alamethicin. This means, the peptide is not synthesized in the above discussed manner or in partial sequences, each beginning with aminoisobutyric acid (from the $NH_2$-terminus), which could be linked together to form the complete non-acetylated chain.

Partial sequences of alamethicin could also start with another amino acid than aminoisobutyric acid: with proline-2, alanine-4, alanine-6, glutamine-7, valine-9, glycine-11, leucine-12, proline-14, valine-15 and glutamic acid-18. These amino acids (radioactively labelled) were incubated alone or together

TABLE I

THE INFLUENCE OF C-TERMINAL-DIRECTED SEQUENCE NEIGHBOURS ON THE TRICHLOROACETIC ACID-STABLE BINDING OF ALAMETHICIN CONSTITUENT AMINO ACIDS

Incubation conditions are described in Materials and Methods. Aib, aminoisobutyric acid; AcAib, *N*-acetyl-aminoisobutyric acid.

| $^{14}$C-labelled amino acid | Unlabelled amino acid | Trichloroacetic acid-stable bound (cpm) |
|---|---|---|
| Ala | — | 2257 |
| Ala | Aib | 1481 |
| Ala | Gln | 1656 |
| Gln | — | 1313 |
| Gln | Aib | 1250 |
| Glu | — | 202 |
| Glu | Gln | 230 |
| Gly | — | 727 |
| Gly | Leu | 374 |
| Leu | — | 707 |
| Leu | Aib | 712 |
| Pro | — | 524 |
| Pro | Aib | 460 |
| Pro | Val | 577 |
| Val | — | 586 |
| Val | Aib | 381 |
| AcAib | — | 80 |
| AcAib | Pro | 84 |

with their respective C-terminal sequence neighbours (unlabelled). No increase of protein bound radioactivity was induced by the addition of unlabelled amino acids (Table I). In some cases partial inhibition occurred. This indicated a certain lack of the specificity of substrate binding to the alamethicin synthetase and might be due to the chosen in vitro conditions. *N*-Acetyl-aminoisobutyric acid is not bound to alamethicin synthetase, either if incubated alone or if incubated with the amino acid proline which follows it. This confirms the result of our in vivo studies: *N*-acetyl-aminoisobutyric acid is not a substrate of alamethicin synthetase. The experimental data also demonstrate that no non-acetylated peptide chain or partial sequences of the molecule are synthesized by the enzyme complex. Thus the initiation reaction of alamethicin formation must be the synthesis of enzyme-bound *N*-acetyl-aminoisobutyric acid.

Acetate and acetyl phosphate were tested as possible acetylating reagents but were not utilized by the synthetase (data not shown). In further experiments, alamethicin synthetase was incubated together with labelled acetyl-CoA and unlabelled aminoisobutyric acid or vice versa. Treatment of the trichloroacetic acid precipitate of the incubation mixture liberated radioactivity possessing the

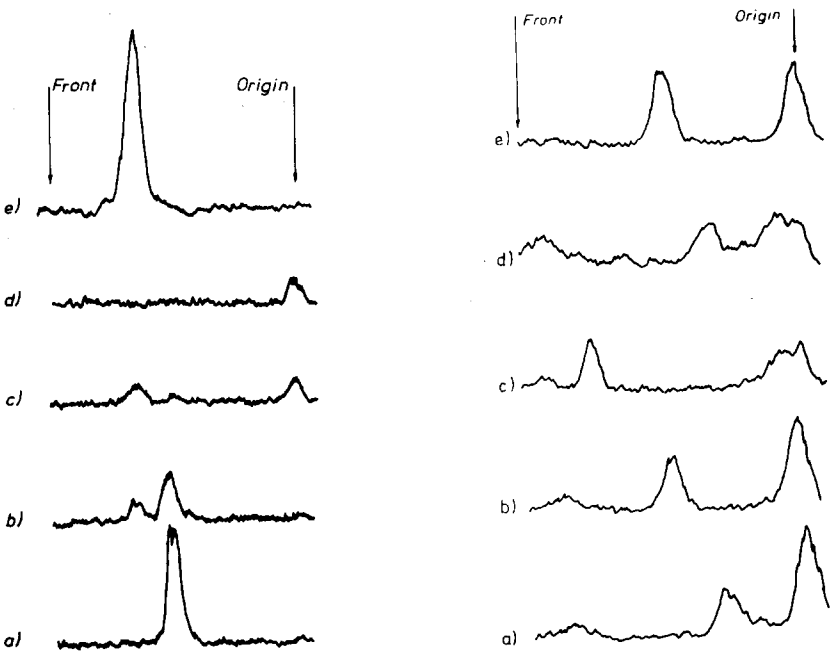


Fig. 6. Formation of enzyme-bound acetyl-aminoisobutyric acid. Thin-layer chromatogram autoradiographed. The incubation mixtures all contained 1 ml of enzyme solution, 6 mM ATP, 10 mM $Mg^{2+}$. Further additions: 1 $\mu$Ci amino[$^{14}$C]isobutyric acid (a); 1 $\mu$Ci amino[$^{14}$C]isobutyric acid plus 20 $\mu$g acetyl-CoA (b); 0.02 $\mu$Ci [$^{14}$C]acetyl-CoA plus 1 $\mu$mol aminoisobutyric acid (c); 0.02 $\mu$Ci [$^{14}$C]acetyl-CoA (d). Marker: *N*-acetyl-amino[$^{14}$C]isobutyric acid (e). Incubation: 15 min at 32°C. Enzyme bound radioactivity was split off with performic acid. Solvent used: solvent 1.

Fig. 7. Lack of specificity of the acetylation reaction catalyzed by alamethicin synthetase and overcoming by the addition of aminoisobutyric acid. Autoradiography of thin-layer chromatograms. The incubation mixture contained enzyme solution, ATP, $Mg^{2+}$, [$^{14}$C]acetyl-CoA (see legend to Fig. 4). Further additions: alanine (a), aminoisobutyric acid (b), valine (c), glycine (d), glycine and aminoisobutyric acid (e) (1 $\mu$mol each). The procedure is described in the legend to Fig. 6. Solvent: solvent 2.

same $R_F$ as *N*-acetyl-aminoisobutyric acid (Fig. 6). In experiments using labelled acetyl-CoA, a further peak of radioactivity appears at the origin of the chromatograms whose nature is unknown. As mentioned above, the presumable *N*-acetyl-aminoisobutyric acid is covalently bound to the synthetase in a linkage which is stable to trichloroacetic acid but can be split by performic acid (and also by diluted NaOH).

These are characteristics of a thiolester bond [16]. Of the alamethicin constituents, alanine, glycine and valine are also acetylated (Fig. 7), glutamine, glutamic acid, leucine and proline are not acetylated. It is noteworthy that the latter four amino acids are structurally more different from aminoisobutyric acid than the former three. Acetylation of these 'wrong' amino acids can be overcome by the addition of equimolar amounts of aminoisobutyric acid, in which case only acetyl-aminoisobutyric acid is synthesized (Fig. 7). Evidently the acetylation of alanine, glycine and valine is of a non-specific nature. If offered alone, they are accepted by the acetylating site of alamethicin synthetase. In vivo, the acetylation reaction seems to be quite specific: no open chain alamethicin has been described possessing another amino terminus than acetyl-aminoisobutyric acid.

*The carboxyl terminus of alamethicin; Origin of phenylalaninol*

Phenylalaninol was identified as a structural element of alamethicin by NMR spectroscopy and gas-liquid chromatography [7—9].

The in vivo incorporation rate of labelled phenylalaninol into alamethicin is similar to that of the other components of the peptide (Table II). L-Phenylalanine is also effectively incorporated, but not the D-form. As Fig. 8 shows, the radioactivity in alamethicin derived from labelled L-phenylalanine is located in the phenylalaninol moiety. Thus no phenylalanine metabolism occurred to shift the final metabolites acetoacetate and fumarate into other amino acids and thereby to label the peptide. The result indicates that L-phenylalanine is the precursor of phenylalaninol.

TABLE II

IN VIVO INCORPORATION OF LABELLED ALAMETHICIN CONSTITUENTS AND NON-CONSTITUENTS INTO THE PEPTIDE

Aib, aminoisobutyric acid; Pheol, phenylalaninol. For experimental details see Materials and Methods.

| $^{14}$C-labelled compound | Incorporation into alamethicin (pmol) | $^{14}$C-labelled compound | Incorporation into alamethicin (pmol) |
|---|---|---|---|
| Aib | 650 | L-Asn | — |
| L-Ala | 220 | L-Asp | 2 |
| L-Gln | 55 | D-Ala | — |
| L-Glu | 14 | L-Ile | 1 |
| Gly | 60 | L-Thr | — |
| L-Leu | 65 | L-Trp | — |
| L-Pro | 90 | L-Ser | 7.5 |
| L-Val | 80 | L-Tyr | 5 |
| L-Phe | 45 | | |
| D-Phe | — | | |
| Pheol | 45 | | |

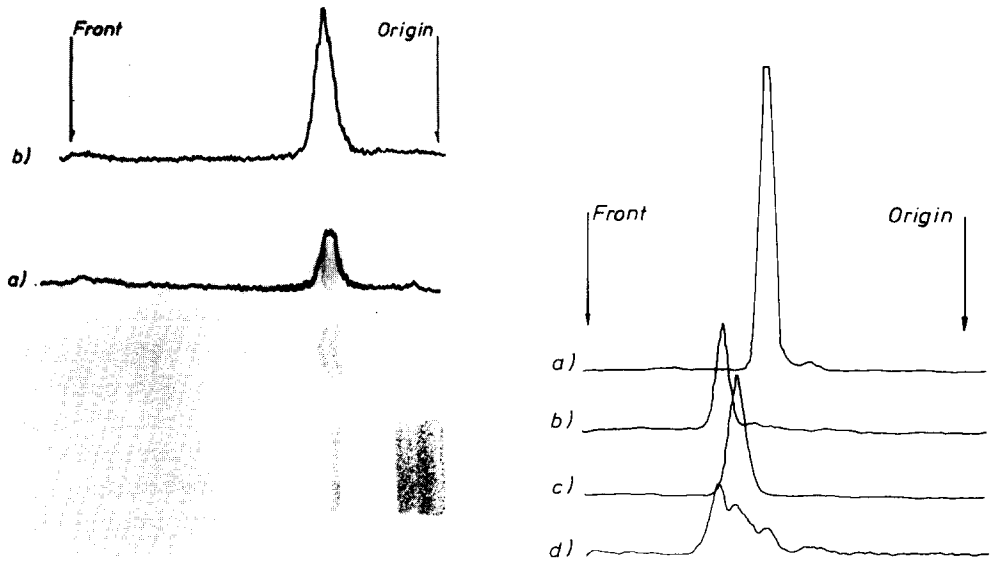


Fig. 8. Incorporation of L-[$^{14}$C]phenylalanine into alamethicin; conversion into phenylalaninol. *T. viride* cells were incubated with L-[$^{14}$C]phenylalanine, the labelled alamethicin isolated and purified and then hydrolyzed. The hydrolysate was separated by thin-layer chromatography and autoradiographed (a). Phenylalaninol marker (b). Bottom: The same chromatogram, stained with ninhydrin. Solvent: solvent 1.

Fig. 9. Unbound pool of phenylalaninol in *T. viride* cytoplasm. Cells were incubated with L-[$^{3}$H]phenylalanine, then washed and lyophilized and extracted with acetone. The extract was concentrated and separated by thin-layer chromatography (d). a, L-[$^{3}$H]phenylalanine; b, [$^{14}$C]phenylalaninol; c, [$^{14}$C]-alamethicin marker. Solvents: First: buffer B, then buffer A, in the same direction.

The next question that had to be answered was, at what stage phenylalanine is converted into phenylalaninol: prior to or after linkage to the carboxyl end of alamethicin. In the latter case, no phenylalaninol should be detectable in the cell sap of *T. viride* unless it is a degradation product of alamethicin. Detailed studies have shown, however, that the antibiotic, due to its complex structure is not degradable by known proteolytic enzymes [6]. If there exists an exopeptidase in *T. viride* capable of splitting phenylalaninol from alamethicin, its action would result in a peptide with two free carboxyl groups instead of one. Such a component must have been detected by the methods used for purification of alamethicin [17,18], but it never was. If phenylalaninol is formed prior to its linkage to alamethicin by an enzyme system which is an integral part of the synthetase complex, one can suppose that it does not accumulate in the cytoplasm, because the synthesis of the peptide chain and phenylalaninol, and the linkage of these two moieties to form alamethicin, are synchronized processes.

A third possibility would be the action of a discrete enzyme system, independent from alamethicin synthetase, leading to the production of phenylalaninol. In this case, the amino alcohol must appear in the cytoplasm of *T. viride*. After incubation of cells with radioactive phenylalanine, two other labelled compounds are detectable in extracts of the organism. Radiography of thin-layer chromatograms shows three peaks (Fig. 9): peak 1 (from the origin)

can be identified as phenylalanine, peak 2 represents labelled alamethicin and peak 3 possesses the same $R_F$ value as the phenylalaninol marker. As discussed above, it is unlikely that the amino alcohol is a degradation product of alamethicin or that it had been split off from the synthetase complex. Thus it is probably the reaction product of a separate enzyme system. In a succeeding communication we will present evidence that phenylalaninol but not phenylalanine is accepted by alamethicin synthetase to be incorporated into the peptide antibiotic.

## Discussions

Lipmann [19] had discussed the similarities in the biosynthesis of fatty acids and of peptide antibiotics. With respect to the group activations and the elongation steps, the enzyme machineries forming the two different classes of polymers resemble each other. The present observations show that, in alamethicin biosynthesis, the initiation step which starts chain growth is also similar to that of fatty acids. In both cases acetyl-CoA serves as the priming agent. In alamethicin biosynthesis, the synthetase catalyzes a transfer of the acetate group to aminoisobutyric acid-1. The exact mechanism of this process has still to be elucidated. The lack of specificity of the acetylation reaction which was observed in our test system was prevented by aminoisobutyric acid and is obviously not of physiological significance.

Our results confirm the finding of Martin and Williams [7,8] and Jung et al. [9], concerning the non-cyclic structure of alamethicin. The synthetase complex is not able to promote the formation of a non-acetylated peptide chain which could finally be cyclisized in the manner of e.g. the tyrocidines [20]. We have further shown that phenylalaninol is an integral part of the alamethicin molecule.

The origin of phenylalaninol could also be clarified: it is derived from L-phenylalanine and probably the reaction product of a separate enzyme system different from alamethicin synthetase. The amino alcohol itself is a substrate of the peptide synthetase. It is not clear whether it possesses any other function in the cell metabolism except to be utilized for the antibiotic production. At times when no alamethicin is synthesized no formation of phenylalaninol is detectable (data unpublished), indicating that the two processes seem to be integrated.

## Acknowledgements

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**Note added in proof** (received July 15th, 1978)

Recently, Pandey et al. [21] assigned a slightly different structure to alamethicin. Their structure differs from that of Martin and Williams [7,8] in so far as phenylalaninol is bound to the C-terminal glutamine on the $\alpha$-carboxyl instead of to the penultimate glutamic acid residue (on the $\gamma_5$-carboxyl group).

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