# BIOSYNTHESIS OF ENNIATIN B

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## 1. Introduction

The enniatins are cyclic depsihexapeptides with antibiotic properties and are produced by various strains of Fusarium [1,2]. Enniatin B, a lower analogue of valinomycin with a similar ionophoretic specificity for potassium ions [3], consists of three residues each of *N*-methyl-L-valine and 2-D-hydroxyisovaleric acid joined in an alternating fashion (fig.1). Many peptides antibiotics like gramicidin S [4] and tyrocidine [5] are formed from the constituent amino acids in a ribosome- and ribonucleic acid-independent mechanism. This principle seems also to apply to depsipeptides as shown in the case of valinomycin [6]. In this report we describe the enzymatic assembly of enniatin B from its primary precursors, i.e. amino acid and hydroxy acid, in an energy requiring step.

## 2. Materials and methods

### 2.1. *Growth of the organism and identification of enniatin B*

Surface cultures in a lactose medium [7] were prepared by seeding with a spore suspension of Fusarium$_{oxysp.}$ Lamb. et Fautr. ETH 1536/9 and incubating for three weeks at 20–25°C. Illumination with a diurnal rhythm was provided by fluorescent lamps. The wet mycelium was extracted three times with methanol and enniatin B, the main enniatin isomer found in this strain, was purified by thin layer chromatography on silica gel in *n*-hexane/ethyl acetate/methanol/water = 200 : 75 : 17 : 1 [7]. Enniatin B was visualized as a yellow spot by exposure to iodine vapors. Hydrolysis of the cyclic depsipeptide was performed with constant boiling HCl in evacuated tubes at 110°C for 18 h. *N*-methyl-valine and 2-hydroxyisovaleric acid were identified by paperchromatography (*n*-butanol/acetic acid/water = 4 : 1 : 5) and high voltage electrophoresis in a Savant apparatus on Whatman 3 MM paper, at pH 1.9 and pH 6.4, respectively. In addition, the depsipeptide was cleaved by mild alkaline hydrolysis in 2 N ammonia at room temperature for three hours and the resulting hydroxyacyl-amino acid identified by high voltage paper electrophoresis, at pH 6.4, by comparison with the analogue derivative prepared from authentic valinomycin. For in vivo incorporation studies, 0.2 g wet mycelium (8 days old) was washed with 5 mM potassium phosphate, pH 6.7, and incubated in 1 ml of the same buffer with 1 $\mu$Ci of radioactive precursor for 30 min at room temperature under illumination. Labelled enniatin B was extracted with ethyl acetate and characterized as described above.

Radioactive 2-DL-hydroxyisovaleric acid was prepared from DL-[$^{14}$C]valine by reaction with $HNO_2$ [8]. Radioactive compounds were detected with a Berthold Thin Layer Scanner, System BF 210. For quantitation, the radioactive enniatin B was eluted after thin layer chromatography with ethyl acetate and the radioactivity estimated in a Nuclear Chicago Isocap liquid scintillation counter. Autoradiography was performed with Kodak XS-1 film.

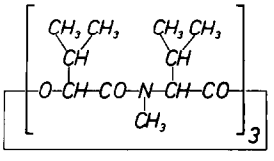


Fig.1. Enniatin B.

2.2. *Enzyme preparation and in vitro synthesis of enniatin B*

Fusarium mycelium (1 g dry weight) was washed with 5 mM potassium phosphate, pH 6.7, and lyophilised. The dry product was homogenized with a 3-fold excess of quartz sand in a mortar and extracted with 50 mM Tris buffer, pH 7.2, containing phenylmethane sulfonyl fluoride (50 mg/l), and 1 mM in dithiothreitol, 5 mM in $MgCl_2$. The extract (10 ml) was centrifuged for 30 min at 20 000 rev/min and desalted by passage through a small Sephadex G-25 column equilibrated with the same buffer. The protein containing fractions (20 ml) were concentrated about 10-fold by ultrafiltration. The reaction mixture contained 250 µl of the enzyme solution and was 1 mM in unlabelled amino acid and hydroxy acid and 4 mM in ATP in a final volume of 270–280 µl. Incubation was performed for 1 h at 37°C.

## 3. Results

3.1. *In vivo incorporation studies*

In the case of valinomycin it is known that L-valine

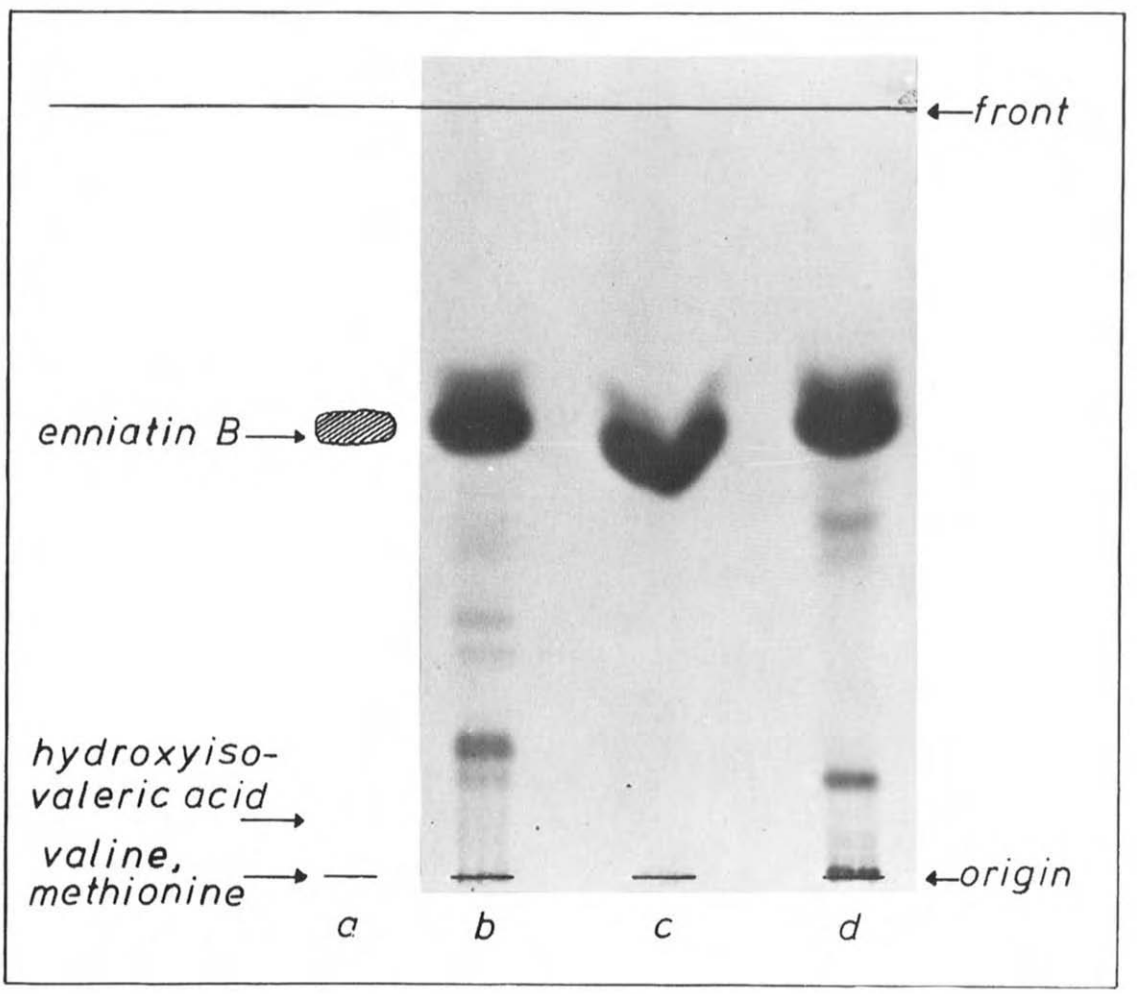



Fig.2. Thin layer chromatography of enniatin B formed in vivo with labelled precursors. (a) unlabelled enniatin B visualized with $I_2$, (b) L-[$^{14}$C]methionine (methyl-$^{14}$C-labelled) incorporation, (c) D,L[$^{14}$C]hydroxyisovaleric acid incorporation, (d) L-[$^{14}$C]valine incorporation. Chromatography on silica gel with ethyl acetate/methanol/water = 100 : 4 : 1 (v/v). Free amino and hydroxy acids are strongly retarded or do not migrate. Exposure of the autoradiogram was 7 days.

forms the precursor of both D- and L-valine, as well as of the D-hydroxyisovaleroyl moiety [9]. Incubation of Fusarium with L-[$^{14}$C]valine resulted in a high incorporation rate into enniatin B (fig.2). Equally, D,L-[$^{14}$C]hydroxyisovaleric acid and L-methionine (*methyl*-$^{14}$C-labelled) were incorporated. An incorporation rate of about 10% of the added precursor was observed for the hydroxy acid within the first 30 min. The rate for valine was about half this value.

Analysis of the product formed from L-[$^{14}$C]valine showed after acid hydrolysis that the radioactivity is evenly distributed between the *N*-methyl-valine and the hydroxy acyl moiety (fig.3). In contrast, D,L-[$^{14}$C] hydroxyisovaleric acid was incorporated exclusively into the hydroxy acyl- and methionine into the valyl-part of the molecule (results not shown). From these experiments it is concluded that the depsipeptide is formed from its primary components and methylation of the amino acid is afforded by methionine as the methyl donor.

### 3.2. *Methylation of valine*

In order to clarify at what stage of the biosynthesis valine is methylated, the influence of unlabelled *N*-methyl-L-valine on the methionine incorporation was investigated by an isotope dilution experiment. As shown in fig.4 the methylation of the [$^{14}$C]valine moiety was independent of any *N*-methyl-valine present, whereas in the control experiment the incorporation of D,L-[$^{14}$C]hydroxyisovaleric acid was drastically depressed by the addition of the unlabelled compound. From these results it is evident, that valine is not methylated prior to peptide bond formation.

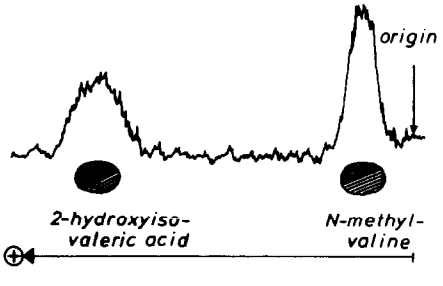



Fig.3. High voltage paper electrophoresis (pH 6.4) of an acid hydrolyzate of enniatin B formed in vivo with L-[$^{14}$C]valine. Scanning of the pherogram for radioactivity. *N*-methyl-valine has been identified, in addition, by its migration at pH 1.9.

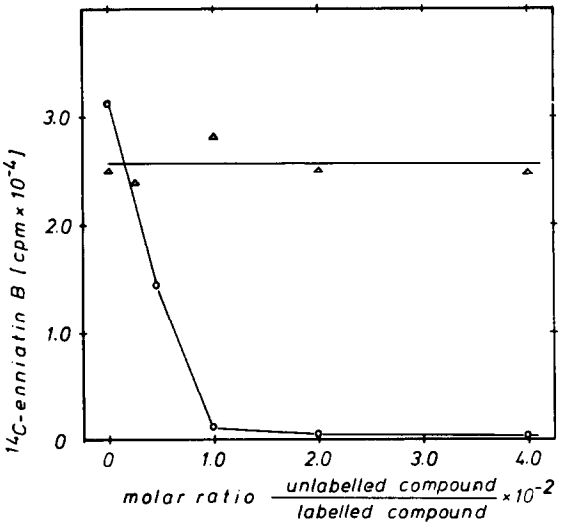



Fig.4. Isotope dilution of precursors of enniatin B during in vivo incorporation. The incorporation rate of labelled precursors in the presence of increasing amounts of unlabelled compounds was determined by isolation and counting of enniatin B formed. (a) L-[$^{14}$C]methionine as precursor versus unlabelled *N*-methyl-L-valine (△——△) (b) D,L[$^{14}$C] hydroxyisovaleric acid versus unlabelled DL-hydroxyisovaleric acid (○——○).

### 3.3. *In vitro synthesis of enniatin B*

In accordance with the results obtained by the in vivo experiments it could be shown that the presumptive precursors of the depsipeptide molecule are also incorporated in an enzymatic in vitro synthesis (fig.5). The formation was energy dependent and was not inhibited by RNAase or streptomycin (results not shown). The enzyme was still functional after removal of ribosomal particles by high speed centrifugation.

Table 1
Double labelling of enniatin B in vitro

| Precursor | Total cpm[a] | pM Incorporated |
|---|---|---|
| L[$^{14}$C]methionine (methyl-$^{14}$C-labelled) | 610 | 5.4 |
| L-[$^{3}$H]valine | 224 500 | 6.5 |

[a]Counts were corrected for counting efficiency with an equimolar standard solution of the labelled amino acids. The specific activities used were 51.2 mCi/mmol for methionine and 15.3 Ci/mmol for valine.

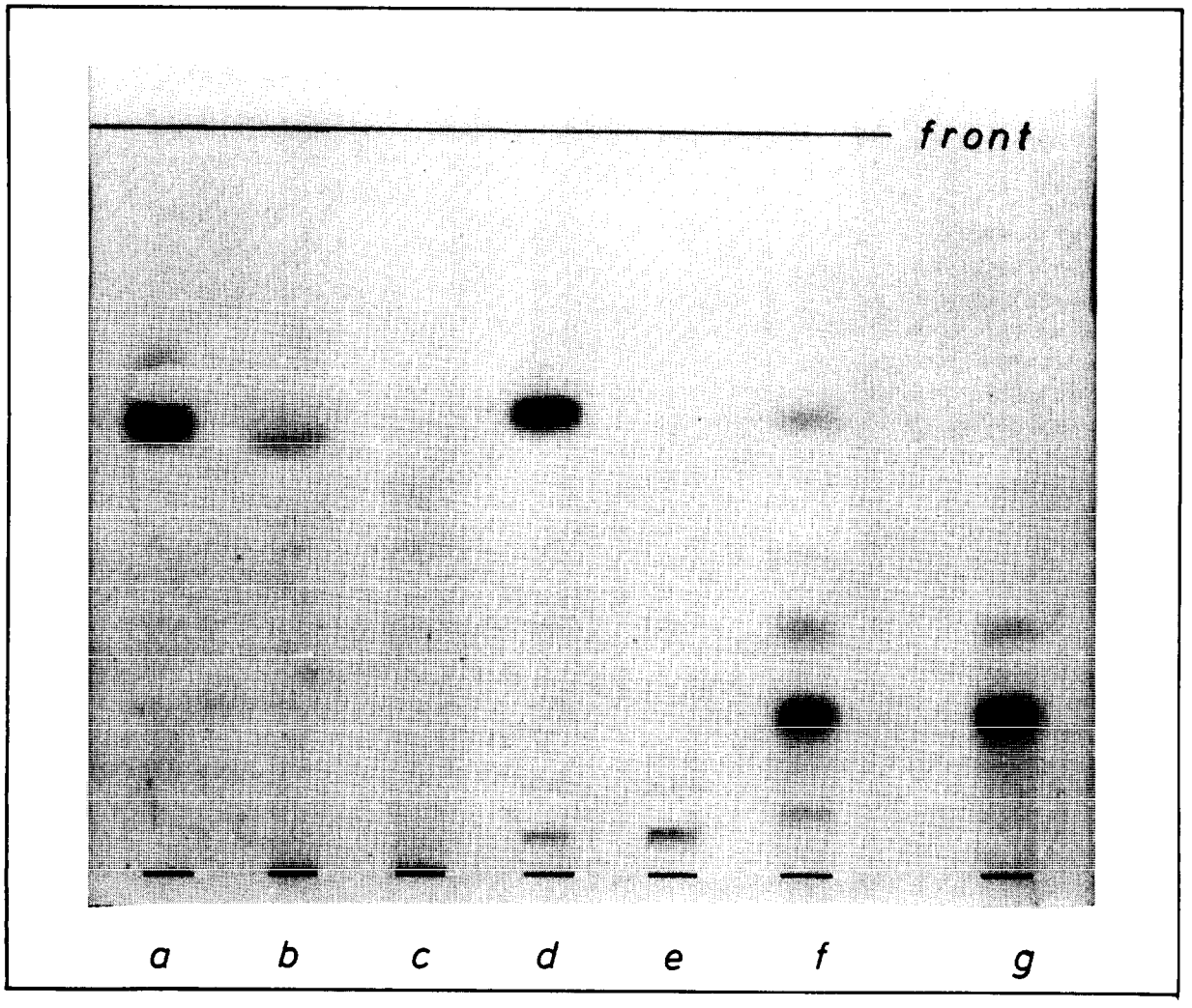
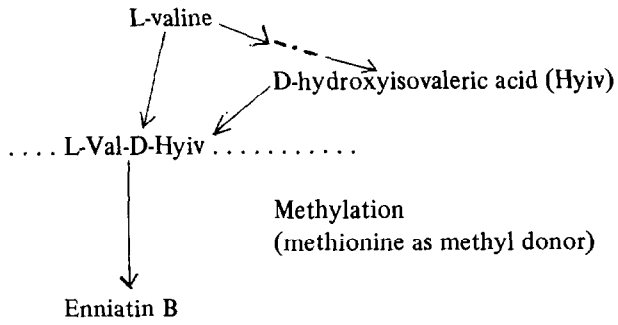



Fig.5. Thin-layer chromatography of enniatin B formed in vitro with labelled precursors. The enzyme assay was complemented by adding the specified precursor in its radioactive form. (a) Enniatin B labelled in vivo as marker, (b) L-[$^{14}$C]valine, (c) L-[$^{14}$C] valine, ATP omitted, (d) DL-[$^{14}$C]hydroxyisovaleric acid, (e) DL-[$^{14}$C]hydroxyisovaleric acid, ATP omitted, (f) *S*-adenosyl-L-[methyl-$^{14}$C]methionine, (g) *S*-adenosylmethionine, ATP omitted. Exposure of the autoradiogram was 7 days.

If valine was substituted by the *N*-methylated derivative, no enniatin B was synthesized demonstrating that *N*-methyl-L-valine was no precursor of the molecule as already indicated by the in vivo studies. Double labelling experiments in vitro with L-[$^{3}$H]valine and methionine (*methyl*-$^{14}$C-labelled) resulted in an incorporation of the methyl group and valine in a ratio of about 1:1 (table 1).

## 4. Discussion

The results described above show that enniatin B is synthesized in a similar way to gramicidin S, tyrocidin and other antibiotics involving a nonribosomal mechanism. From the in vivo and in vitro incorporation studies the following pathway for the biosynthesis is proposed:




Methylation of the valine moiety is probably afforded after peptide bond formation; from the present experiments it is not obvious whether this modification occurs before or after the ring closure. Occurence of small amounts of unmethylated enniatin has, however, been observed [10], indicating that methylation of the molecule represents rather a post-synthetic step.

The possibility of assembling the depsipeptide in a defined in vitro system opens the way for studies which will clarify the methylation step as well as answer questions regarding the activation mechanism of the components and the kinetics of synthesis.

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