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Fungal biosynthesis of non-ribosomal peptide antibiotics and α, α-dialkylated amino acid constituents

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Abstract: Zervamicins (Zrv) IIA and IIB are membrane modifying peptide antibiotics of fungal origin, characterized by a sequence of 15 amino acid residues. The primary structure of Zrv-IIA contains five α-aminoisobutyric acid residues at positions 4, 7, 9, 12 and 14 of the linear peptide. The sequence of Zrv-IIB is similar, but contains a D-isovaline at position 4. When the free amino acid Aib was added to the peptone-glucose culture medium, the fungus *Emericellopsis salmosynnemata* produced Zrv-IIA as the major secondary metabolite, whereas addition of DL-Iva to the culture led to a high production of Zrv-IIB. This observation is rationalized by a lack of selectivity of the non-ribosomal peptide synthetase with respect to the thiolester activated amino acid substrates during step 12 of peptide synthesis. Analysis of the configuration of the Iva residue of Zrv-IIB showed a high enantiomeric purity of the D-enantiomer, indicating a high stereoselectivity of the peptide synthetase for this substrate.

When the culture was supplemented with [^{15}N]DL-Iva, the nitrogen isotope was not only found at the D-Iva residue, but surprisingly also at the Aib residues as well as at the proteinogenic residues of Zrv. The partial catabolism of exogenous [^{15}N]DL-Iva is explained by the assumption of a decarboxylation-dependent transamination reaction, catalysed by 2,2-dimethylglycine decarboxylase. The same enzyme might also be involved in the reversed carboxylation reactions of acetone and 2-butanone, during the anabolic biosynthesis of Aib and Iva, respectively. Zrv might possibly act as a thermodynamic sink to shift these equilibrium reactions towards the reversed side. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: zervamicin; peptide antibiotics

INTRODUCTION

Fungi provide an enormous variety of secondary metabolites, some of which are important pharmaceutics such as immunosuppressants and β-lactam antibiotics. Among the various classes of peptide antibiotics are more than 300 peptaibols with a length varying from 5 to 19 residues [1,2]. Peptaibols [3,4] are linear peptides, generally characterized by a high proportion of α,α-dialkylated amino acids, such as α-aminoisobutyric acid (Aib) and occasionally a D-isovaline (Iva), an acetyl group at the *N*-terminus and an 1,2-amino alcohol at the *C*-terminus [3,4]. The function of the α,α-dialkylated amino acids is to promote the formation of helical structures [1,2,5] and to increase resistance to proteolytic degradation. It is generally believed that peptaibols play an important role in the defensive mechanism of fungi against attack by bacteria [6,7]. Peptaibols have been shown to interact with phospholipid bilayers. Membrane modifying activities, such as leakage of the membrane [8] and ion channel formation [9–12] lead to a loss of the membrane potential, inhibition of cell growth or even cell death.

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Peptaibols are produced, dependent on the strain of the fungus, as mixtures of isoforms, i.e. structurally related products. The non-ribosomal biosynthesis is catalysed by a polyfunctional enzyme that carries a number of modules each of which is involved in (1) selection of a specific amino acid from the pool of available substrates, (2) activation of the carboxy group of the amino acid and (3) coupling this activated amino acid derivative to the *N*-terminus of the growing peptide chain (13–17). The main reason for the generally observed diversity of compounds produced by different strains is certainly due to mutations of the peptaibol synthetase. However, even in the case of single strains, the fungus is still able to produce a mixture of isoforms. This particular contribution to the diversity of metabolic products might be caused by a decreased selectivity at one or more amino acid binding modules of the peptaibol synthetase. Support for this hypothesis has been found recently from an analysis of the set of primary structures of the trichorzin-TVB family and the set of peptaibol synthetase modules that code for the incorporation of amino acids into the peptide chain [17]. Almost identical ‘signal’ regions of the primary structures of the binding modules reflect the 100% conservation found for six of the total seven Aib residues of this peptaibol family. However, either Aib or D-Iva can occupy position 7 of the peptide chain

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of trichorzin, most likely due to a less conserved synthetase module coding for this step of peptide synthesis. Additional evidence for this hypothesis was found in other studies, wherein it was shown that the variation of complex mixtures of isoforms could be manipulated by supplementing the cultures with a specific amino acid [18].

Our laboratories have studied the structure, function and biosynthesis of zervamicin [19–23]. Dependent on the choice of strain the fungus *Emericellopsis salmosynnemata* can produce 12 different isoforms of zervamicin (Zrv) [2,24,25]. Four of them are acidic, with Glu at position-3 of the peptide chain, while eight isoforms are neutral with Gln at the third position and showing different Ile → Val, Leu → Val and Trp → Leu substitutions (Figure 1). It is not clear why the substitutions appear at the *N*-terminal α-helical domain and not at the *C*-terminal 3_{10}-helical domain of the peptide backbone. Nevertheless, it seems plausible that the small structural differences between the side chains (Val vs Ile, etc.) do not alter the secondary structure of the zervamicins significantly. This assumption might not hold for the bioactivity. For example, the lipophilic stationary phase of a C_{18} column can distinguish among a large number of isoforms of peptaibols and it is reasonable that hydrophobic segments of the peptaibol interact similarly with the lipid core region of biomembranes. Thus, members of the natural peptaibol library may exert evolutionary significant, albeit small, membrane modifying actions.

Strain 336 IMI 58330 of *E. Salmosynnemata* does produce, however, only two different isoforms, i.e. Zrv-IIA and Zrv-IIB (Figure 1) [23]. Their primary structures are slightly different with respect to position 4 of the peptide chain with an Aib residue in Zrv-IIA and D-Iva in Zrv-IIB. In the present study, both the regio- and stereoselectivity of the fourth module of zervamicin synthetase were examined. The composition of the peptaibol mixture produced in the [15N]Aib or [15N]Iva supplemented culture was analysed to trace their metabolic pathways to zervamicin. Both Aib and D-Iva were found to be the precursors in this synthesis in spite of the fact that the exogenous DL-Iva was partly catabolized. A 2,2-dialkylglycine decarboxylase (DGD) catalysed carboxylation reaction is suggested for the anabolic pathway to unlabelled α,α-tetrasubstituted amino acids starting from acetyl-CoA and propionyl-CoA. The selectivity of the non-ribosomal peptide synthetase during the peptide coupling of Iva was examined. The experimental results support the hypothesis of a poorly regio-selective but highly stereoselective non-ribosomal peptaibol synthetase.

MATERIALS AND METHODS

Culturing

Highly ^{15}N-enriched (>98% ^{15}N) α-aminoisobutyric acid as well as the racemic mixture of D(*R*)- and L(*S*)-Iva, in the text indicated by DL-Iva, were prepared as described before [26]. The notation D,L-Iva is used when variable amounts of D and L-enantiomers are possible. The strain *E. salmosynnemata* 336 IMI 58330 was kindly donated by Upjohn Co., Kalamazoo, MI, USA. The standard medium (250 ml; pH 7.2) for fungus cultivation contained bacto-peptone (2.5 g), yeast extract (2 g), glucose (2 g) and calcium carbonate (2 g). For incorporation experiments the culture medium was supplemented with 0.5 g of [15N]α-aminoisobutyric acid or 1.0 g of [15N]DL-Iva, respectively. A 96 h seed culture of the fungus was used to inoculate (5% v/v) the fermentation flasks. Under aerobic conditions, the culture was incubated for 8 days at 28 °C on a shaker at 220 rpm. During cultivation white pellets were formed. The increase of biomass was measured as a function

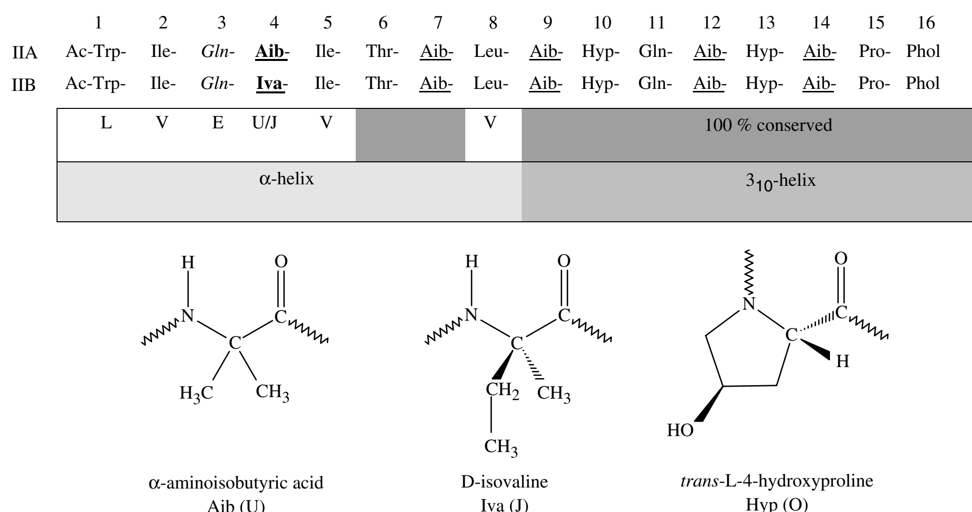

Figure 1 Primary structures of the zervamicins Zrv-IIA and Zrv-IIB, the main isoforms produced by *Emericellopsis salmosynnemata* strain 336 IMI 58330. Formula of the non-protein amino acid constituents of the peptaibols are depicted. The *C*-terminal domain is 100% conserved, whereas some mutations are possible within the *N*-terminal region.

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of time from the volume of the sediment that was formed when shaking was interrupted for a while. At the stationary stage of biomass formation, the fermentation broth was filtered. The filter cake, containing the Zrv isoforms, was extracted with methanol [20]. After evaporation of the solvent, the product was further purified by gel filtration [23]. The protocol ensured that the peptaibol mixture was obtained without resolving the different isoforms. Some experiments were carried out in the presence of an additional amount (2.5 g) of starch. The yield of the product was shown to be directly related to the amount of carbohydrate.

HPLC, GC and MS Analyses

For analytical reversed-phase high-performance liquid chromatography, an Alltima RP C_{18} column (Alltech), diameter 4.6 mm, 150 mm long, 5 μm particle size was used for the isolation of zervamicins. Isocratic elution was achieved, using ethanol/acetonitrile/water (62.5 : 22.5 : 15.0, v/v/v) as the mobile phase, at a flow rate 0.5 ml/min. The peptaibols were detected by the absorbance at 260 and 280 nm (Figure 2) and identified by ESI-MS using an PESciex API165 mass spectrometer.

Chiral amino analysis was performed on a GC-MS model HP 6890 with a mass selective detector model HP 5972 (Agilent, Waldbronn, Germany). The instrument was equipped with a Chirasil-L-Val, i.e. *N*-propanoyl-L-valine-*tert*-butylamide polysiloxane, quartz capillary column, 25 m × 0.25 mm i.d. (Varian-Chrompack, Darmstadt, Germany). EI mass spectra were recorded at an ionization energy of 70 eV. The chirality of Iva was determined after derivatization as *N*-acetyl-Iva-1-propyl ester by GC-MS [27]. The chromatographic conditions were: temperature: 70 °C – 1.0 min –2.5 °C/ min – 100 °C – 2.0 min –3.5 °C/ min – 135 °C – 5.0 °C/ min – 150 °C – 20 °C/ min – 190 °C – 10 min.; pressure: 5 kPa – 1 min –0.2 kPa/ min –7 kPa – 2.0 min –0.3 kPa/ min – 11 kPa – 1.6 kPa/ min –15 kPa – 8.0 min. The split ratio was 30 : 1 at an injection volume of 1.0 μl.

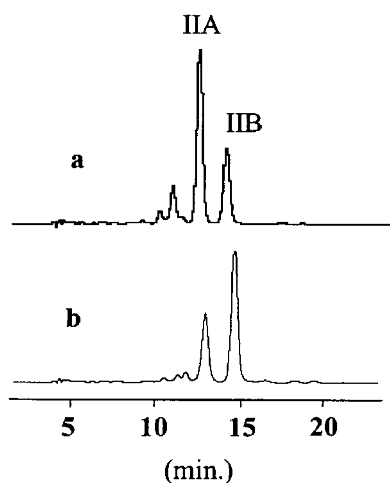

Figure 2 HPLC chromatograms of the products obtained after culturing *E. salmosynnemata* in a complex medium supplemented with (a) Aib and (b) DL-Iva, respectively. The major components Zrv-IIA and Zrv-IIB and minor amounts of the isoforms Zrv-II-1, Zrv-II-2 and Zrv-II-3 are detected at 260 nm.

NMR Analysis

The ^{15}N-labelled zervamicin product was dissolved in 0.5 ml of deuterated methanol (CD_3OH, 99.9% ^{2}H, Cambridge Isotope Laboratories, USA). All NMR experiments were carried out on a Bruker DMX-600 spectrometer. The temperature of the sample was 303 K. The 2D ^{15}N-HSQC spectrum was measured using a Watergate suppression of the $CD_3O\mathbf{H}$ proton signal. The number of scans was 16 in the F1 direction and 400 in the F2 direction. The spectra were processed to 256*2048 complex points. Spectral assignments were achieved by using known ^{1}H,^{15}N-assignments [19] and independently by NOESY experiments. ^{1}H and ^{15}N chemical shifts were given relative to tetramethylsilane and liquid $^{15}NH_3$, respectively, using the internal standard $C\mathbf{H}_3OH$ and the external standard $^{15}\mathbf{N}H_4NO_3$. ^{15}N enrichments were calculated from the splitting pattern of the N$\underline{H}$ in spectrum **A** (top panel of Figure 3), assigned to Aib-14 and in the N$\underline{H}$ of spectrum **B** (bottom panel of Figure 3) assigned to Trp-1, Aib-9 and Gln-11. The other enrichments were obtained from the relative peak integrals.

RESULTS

Incorporation of Aib into the Peptide Chain

The standard complex medium used to grow *E. salmosynnemata* in this study contained glucose and the C,N-sources, peptone and yeast extract. After cultivation until the late stationary stage of biomass formation, the culture was filtered and the peptaibols were isolated by a simple extraction of the mycelium with methanol, followed by gel chromatography. After evaporation of the solvent 25 mg of colourless product was obtained containing 42% Zrv-IIA and 58% Zrv-IIB [23].

In the first experiment Aib was added to the complex medium before inoculation. To investigate the influence of this exogenous substrate on the ratio of Zrv-IIA and Zrv-IIB synthesis, the amino acid was labelled with the stable ^{15}N-isotope. In this way the amino acid, which was added to the culture, could be distinguished from the unlabelled endogenous Aib. The latter is biosynthesized starting from the other C,N-sources present in the culture medium. It was anticipated that addition of exogenous (labelled) Aib would suppress the *de novo* biosynthesis of (unlabelled) Aib by blocking the enzymes that are involved in this process [23].

The culture was supplemented with 0.5 g of [^{15}N]Aib and after 8 days of growth, the peptaibols were recovered from the mycelium as described above. According to HPLC analysis (Figure 2a), the final product contained 64% of Zrv-IIA and 28% of Zrv-IIB as the major components. In addition, minor amounts of other components were formed, which were identified by mass spectrometry. The latter compounds, Zrv-II-1, Zrv-II-2 and Zrv-II-3 (total amount 8%), which were not detected during the cultivation under standard conditions, are isoforms of Zrv-IIA, wherein positions

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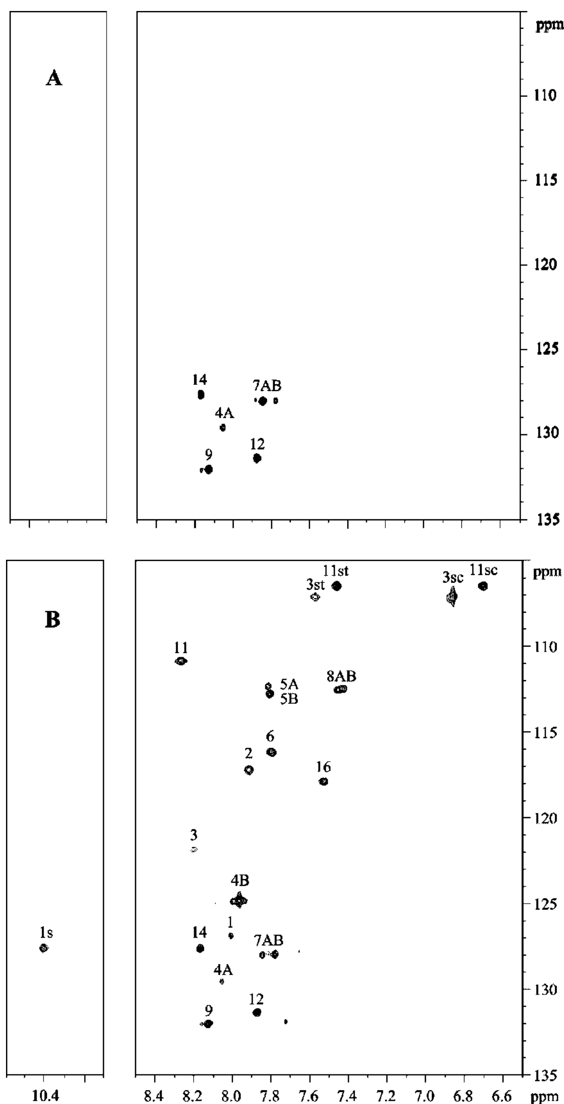

Figure 3 2D-HSQC NMR spectra (600 MHz, 303K) of Zrv-IIA/IIB mixtures in CD_3OH solution. The signals of the ^{15}N-H amide groups (on the right hand side of spectra A and B) and the ^{15}N-H of the indole of Trp (on the left hand side of spectrum B) are numbered according to the sequence positions of the two different isoforms, A (Zrv-IIA) and B (Zerv-IIB). The zervamicin mixtures were produced by culturing under two different conditions using culture media supplemented with [^{15}N]Aib (spectrum A) or [^{15}N]DL-Iva (spectrum B), respectively. s, indole N$\underline{\text{H}}$; sc, *cis* CON$\underline{\text{H}_2}$; st, *trans* CON$\underline{\text{H}_2}$ (see text).

2, 5 and 8 are occupied by a valine residue. Thus, the addition of Aib to the culture medium led to products that were enriched in Aib at position 4 of Zrv-IIA, Zrv-II-1, Zrv-II-2 and Zrv-II-3.

To detect the positions of the labels in the peptide backbone as well as the degree of ^{15}N-content of these residues, a (^{15}N-^{1}H) HSQC-NMR spectrum was recorded of the zervamicin mixture in CD_3OH solution (panel A of Figure 3). The amide region of this spectrum shows five major and two minor ^{15}NH cross peaks. Due to the fact that the structures of Zrv-IIA and Zrv-IIB in methanol solution are very similar, the three cross peaks correspond to amino acid residues at the same positions of the peptide chains, i.e. 9, 12 and 14. Therefore, the assignment of these signals was achieved by a comparison with literature NMR data of Zrv-IIB [19]. The major cross peak at 8.04/129.7 ppm is independently assigned by a NOESY experiment to Aib at position 4 of Zrv-IIA. Note, that the signal 4B that would correspond to Iva-4 of the Zrv-IIB isoform is clearly below the signal to noise ratio. Finally, the strong peak at 7.87/128 ppm and the weak signal at 7.80/128 ppm are assigned to Aib-7 of Zrv-IIA and Zrv-IIB, respectively. The resonances of Aib-7 are split due to the presence of different neighbour residues at position 4 of both helical molecules (Figure 1) [19,20].

The ^{15}N-enrichment of each Aib residue in zervamicin was determined from the ratio between the doublet of the amide proton resonance (due to the ^{1}J coupling with the attached ^{15}N-nitrogens) and the singlet of the residual unlabelled ^{14}NH resonance. The enrichment was found to be ~55%. Thus, in spite of the fact that the culture medium was supplemented with highly enriched [^{15}N]Aib (>99%), 45% of the Aib residues were unlabelled due to *de novo* biosynthesis of the amino acid precursor which was not completely suppressed.

The specific incorporation of [^{15}N]Aib at positions 4, 7, 9, 12 and 14 of Zrv-IIA confirms that this free amino acid is the biosynthetic precursor of the peptide synthesis [28]. In agreement with the postulated lack of selectivity for the corresponding catalytic module of the non-ribosomal peptide synthetase, the experiment shows a significant increase of the Zrv-IIA content, when the total endogenous concentration of Aib (labelled and unlabelled) was increased by supplementing with [^{15}N]Aib.

Incorporation of D-Iva at Position 4 of the Peptide Chain

After culturing *E. salmosynnemata* in the presence of DL-Iva, peptone, yeast extract and glucose the zervamicin mixture was isolated. According to HPLC analysis, the mixture contained 33% of Zrv-IIA and 67% of Zrv-IIB (Figure 2b). The 1 : 2 product ratio was significantly different from that of the control experiment (1 : 1.2), but in striking contrast with the 3 : 1 ratio found in the experiment described above when Aib was added to the culture (Figure 2a). Thus, the effect of the Iva supplemented culture on the yield of Zrv-IIB was much less pronounced. The explanation for this observation is discussed below.

The amide region of the HSQC-NMR spectrum of the ^{15}N-labelled Zrv mixture is depicted on panel B of Figure 3. Since Zrv-IIB contains a single Iva residue, one signal for Iva-4 at 7.97/124.92 ppm would be expected [19]. Surprisingly, the amide region of the ^{15}N-spectrum shows, in addition to this peak, 19 other cross

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peaks (see right panel of spectrum B). Moreover, a peak is detected at 10.40/127.8 ppm (left panel of spectrum B), that is assigned to the ^{15}N-^{1}H of the indole group of Trp-1. The peaks of the amide region of the spectrum are assigned to 16 protons of backbone amide groups and 4 protons of the glutamine side-chain amides (Gln-3 and Gln-11). Three of the backbone amide cross peaks are non-overlapping signals of Ile-5, Aib-7 and Leu-8 of Zrv-IIA/IIB. The Gln side-chain signals are split due to the well-known *cis* and *trans* configurations of the two non-equivalent amide protons. The new weak peak found at 8.04/129.7 ppm is assigned to the NH of Aib-4 (see also panel A).

The isotope content of D-Iva at position 4 of Zrv-IIB is, within experimental error, equal to the ^{15}N-enrichments of the Aib residues (~16%). However, this value is much lower than the label content of Aib found in the former [N^{15}]Aib labelling experiment (~55%). Thus, part of the isotopically labelled D-Iva was not incorporated. It should be noted that, in spite of the fact that racemic [^{15}N]DL-Iva was added to the culture, the initial concentration of [^{15}N]D-isovaline was the same as in the culture, which had been supplemented with [^{15}N]Aib.

The following conclusions can be drawn from the NMR experiments: (1) D,L-[^{15}N]Iva was partly catabolized during the cultivation and this process was not restricted to the L-enantiomer as shown by the observed lower ratio of peptaibol isoforms (1 : 2 instead of 1 : 3) in the produced mixture, (2) D-Iva is the biosynthetic precursor of the corresponding residue at position 4 of Zrv-IIB, (3) the ^{15}N-isotope of the exogenous [^{15}N]Iva was scrambled to all other N positions of the peptaibol molecules.

The question arose as to whether or not Iva would be incorporated in a stereoselective manner. For instance, the phenylalanine binding domain of GrsA peptide synthetase does not show any stereoselectivity for the binding of D and L-Phe [17,29,30]. Since information about the stereoselectivity of the binding modules of zervamicin synthetase is not available, the configurational purity of D-Iva at position 4 of Zrv-IIB was analysed. After isolation of zervamicin from the fungal mycelium, the product was hydrolysed. Analysis of the chiral purity of Iva in the amino acid mixture was achieved by GC/MS using a chiral column. A control (the unlabelled hydrolysate of pure Zrv IIB) contained 100% D-Iva. The labelled hydrolysate, however, contained 97.8% D-Iva (degree of labelling: $^{15}N < ^{14}N$) and 2.2% L-Iva ($^{15}N > ^{14}N$).

Another interesting observation is that the side-chain nitrogen atoms of Trp-1, Gln-3 and Gln-11 of Zrv-IIB are labelled at the same level as the α-nitrogens (see top region of spectrum B). Since it is generally known that ammonia is the only nitrogen source of these amino acid side-chains, it is deduced that [^{15}N]NH_3 is the major N-donating compound in the culture of *E. salmosynnemata*.

DISCUSSION

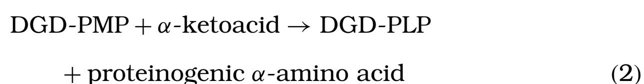
It is established from our experiments that the ratio between the secondary metabolic products of *E. salmosynnemata* can be manipulated by feeding either Aib or Iva. This finding confirms the hypothesis that the diversity of the final peptaibol products is due to a lack of selectivity of the peptide synthetase.

Stable isotope labelling experiments indicate that [^{15}N]Iva is catabolysed during the cultivation process. This is surprising because the well-known transamination reaction of proteinogenic amino acids is not likely to occur. During transamination a proton is removed from the α-carbon of proteinogenic amino acids. Since α,α-dialkylated amino acids do not bear an α-proton, it is evident that a pyridoxal-phosphate dependent transamination reaction cannot take place. Even more surprising, in view of the resemblance of the chemical structures of Aib and Iva, is that the former appears stable during cultivation.

As far as we know, information about the catabolism of C^{α}-tetrasubstituted α-amino acids in fungal cultures has not yet been reported in the literature. The Gram-negative soil bacterium *Burkholderia (Pseudomonas) cepacia*, however, is capable of converting both Iva and Aib to 2-butanone and acetone, respectively [31]. This decarboxylation-dependent transamination reaction is catalysed by 2,2-dialkylglycine decarboxylase (DGD). The bacterial vitamin B6-dependent decarboxylase (EC4.1.1.64) was isolated, cloned and its structure as well as its mechanism of action were solved [32–35]. The enzyme utilizes the cofactor pyridoxal 5′-phosphate (PLP) that is transformed into pyruvate-aldimine (PMP) in two half reactions, 1a-2 and 1b-2:

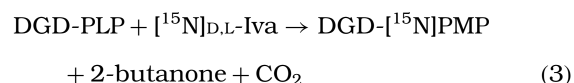
$$\text{DGD-PLP} + \text{Iva} \rightarrow \text{DGD-PMP} + \text{2-butanone} + CO_2 \quad (1a)$$

$$\text{DGD-PLP} + \text{Aib} \rightarrow \text{DGD-PMP} + \text{acetone} + CO_2 \quad (1b)$$

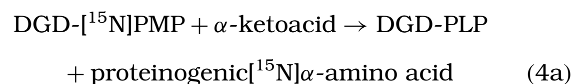


$$\text{DGD-PMP} + \alpha\text{-ketoacid} \rightarrow \text{DGD-PLP} + \text{proteinogenic } \alpha\text{-amino acid} \quad (2)$$

In the fungus *E. salmosynnemata*, the scrambling of the isotope from [^{15}N]D,L-Iva to the nitrogen atoms of the proteinogenic residues of zervamicin might be rationalized in the same manner:



$$\text{DGD-PLP} + [^{15}N]\text{D,L-Iva} \rightarrow \text{DGD-}[^{15}N]\text{PMP} + \text{2-butanone} + CO_2 \quad (3)$$



$$\text{DGD-}[^{15}N]\text{PMP} + \alpha\text{-ketoacid} \rightarrow \text{DGD-PLP} + \text{proteinogenic}[^{15}N]\alpha\text{-amino acid} \quad (4a)$$

The occurrence of these decarboxylation reactions implies that the cells of *E. salmosynnemata* display the same catalytic decarboxylase activity as the bacterial DGD enzyme.

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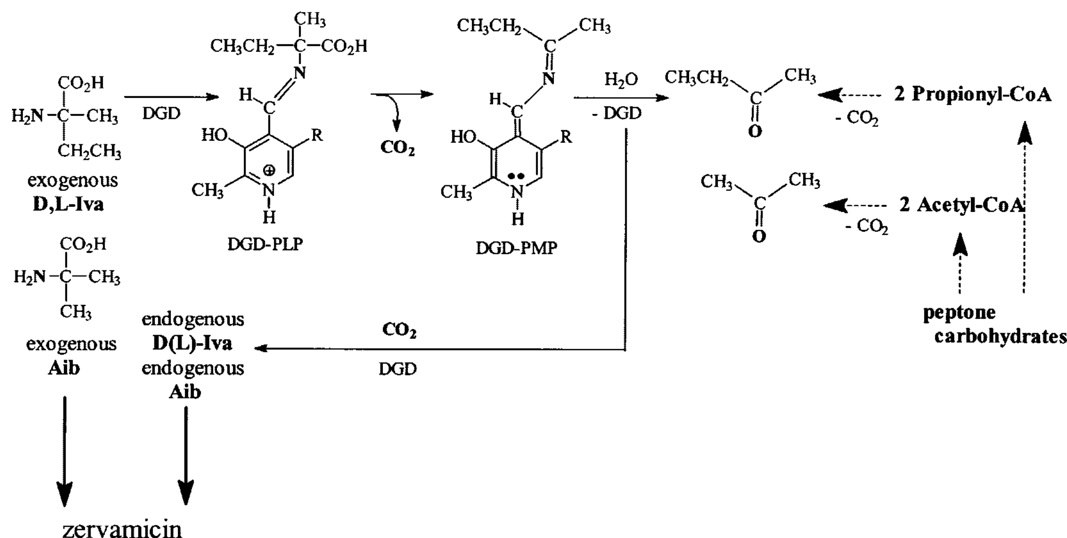
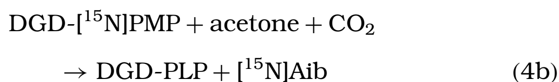
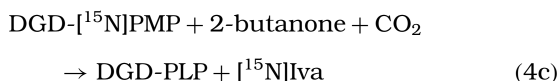


Figure 4 Catabolic and anabolic pathways of the α,α-dialkylated amino acid constituents (Aib and Iva) of zervamicin.

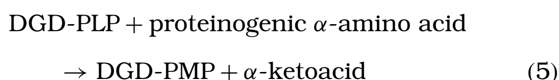
Since we have observed that the ^{15}N-isotope was not only transferred to proteinogenic amino acids but also to the Aib residues of Zrv-IIB, we suggest that reversed carboxylation reactions (4b) and (4c) might have taken place (Figure 4):



$$\text{DGD-}[^{15}\text{N}]\text{PMP} + \text{acetone} + \text{CO}_2 \rightarrow \text{DGD-PLP} + [^{15}\text{N}]\text{Aib} \quad (4b)$$



$$\text{DGD-}[^{15}\text{N}]\text{PMP} + \text{2-butanone} + \text{CO}_2 \rightarrow \text{DGD-PLP} + [^{15}\text{N}]\text{Iva} \quad (4c)$$

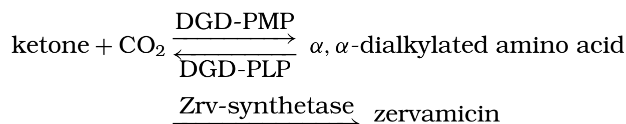


$$\text{DGD-PLP} + \text{proteinogenic } \alpha\text{-amino acid} \rightarrow \text{DGD-PMP} + \alpha\text{-ketoacid} \quad (5)$$

Carboxylation reactions might also occur in the *de novo* biosynthesis of unlabelled Aib and Iva, starting from ketones derived from glucose via acetyl- and propionyl-CoA intermediates (Figure 4) [36]. Previously reported experimental observations support this hypothesis. For example, the yield of zervamicin increases when the pH of the culture broth is kept constant by heavy shaking with solid $CaCO_3$ particles (personal communication from UpJohn Co.). Since solid $CaCO_3$ is in equilibrium with HCO_3^- (aq.) and CO_2 (aq.), it is possible that these C-1 sources are also utilized in carboxylation reactions. Based on ^{14}C- tracer studies, Ooka and Takaeda [37] suggested that L-Val is the precursor of Aib. However, the conversion of this branched C5 amino acid to a C4 amino acid is hard to understand. In our interpretation of the radioactive label study, [^{14}C]Aib is first catabolized to [^{14}C]acetyl-CoA followed by anabolic conversions to [^{14}C]acetone and [^{14}C]Aib [36,37].

The standard medium, used for cultivation of *E. salmosynnemata*, does not contain α,α-dialkylated amino acids. The pathway may start with a Claisen type of condensation of acetyl-CoA and propionyl-CoA, followed by a decarboxylation to acetone and 2-butanone (Figure 4). In this scheme, the latter compounds are further carboxylated to Aib and Iva, respectively.

The question arose as to whether the same DGD enzyme is operative in both extracellular and intracellular compartments of the fungal cell [38]. The implication is that such an enzyme would catalyse a decarboxylation reaction during the transport from the external medium via the cell wall and membranes as well as a carboxylation reaction at the site where the endogenous *de novo* biosynthesis of α,α-dialkylated amino acids takes place. At the intracellular site, the DGD-PLP catalysed carboxylation reaction might be favoured by an irreversible coupling of the α,α-dialkylated amino acids to the peptide chain of the thermodynamically stable peptaibol



$$\text{ketone} + \text{CO}_2 \underset{\text{DGD-PLP}}{\overset{\text{DGD-PMP}}{\rightleftharpoons}} \alpha,\alpha\text{-dialkylated amino acid} \xrightarrow{\text{Zrv-synthetase}} \text{zervamicin}$$

Thus, DGD might be present at two different sites of the fungal cell. In this context it is of interest to note that the expression of DGD cloned in *Escherichia coli* is induced by L-isovaline but not by D-isovaline [35, 39].

It was observed that the non-ribosomal synthetase of *E. salmosynnemata* catalyses the incorporation of Aib and D-Iva equally well into the peptide. However, after supplementing the medium with racemic DL-Iva, analysis of the final product showed a stereochemical purity of 97.8% of D-Iva at position 4 of the peptide chain of Zrv-IIB. In general, the ability of enzymes to distinguish two enantiomeric substrates during the conversion is not surprising. However, it is remarkable that zervamicin synthetase is highly stereoselective for the D and L-enantiomers of Iva, but does not discriminate between Aib and Iva. Figure 5 illustrates schematically the orientations of Aib, D-Iva and L-Iva with regard to their hypothetical complementary

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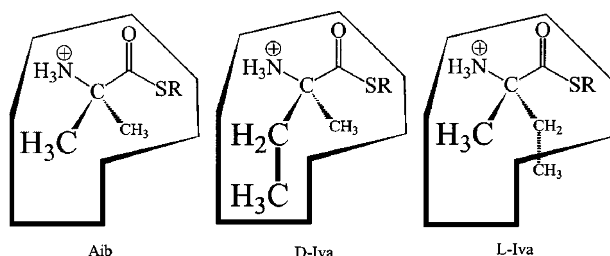

Figure 5 Schematic representation of the binding site of the non-ribosomal zervamicin synthetase for thiolester activated amino acids, indicating why, in contrast to L-Iva, Aib and D-Iva are preferentially incorporated into zervamicin.

binding sites of the enzyme. In this context, it should be noted that part of the incorporated Iva is most likely synthesized from the achiral 2-butanone precursor. Thus, not only zervamicin synthetase, but also the enzyme DGD that is believed to be involved in the synthesis of Iva from 2-butanone, might contribute to the stereochemical purity of Iva in the final peptide product.

It is noteworthy that, compared with the total number of 122 D-Iva containing peptides discovered so far [2], only five peptides were found with one or more L-Iva residues [40–43]. It is not clear yet why Nature has preferred to incorporate D-Iva into peptaibols. From the structural point of view it is plausible that a single D-Iva residue in a sequence of mainly L-proteinogenic amino acids is important for the conformational stability of the secondary structure of the peptide. D-Iva might fit better in a helix of the same screw sense as the proteinogenic amino acids [44,45]. However, the relationship between the structure and biological function of antibiotic peptaibols is not very clear yet. For instance, the influence of the configuration of Iva-12 in emerimicins III and IV on the antimicrobiological activity has been shown not to play an important role [46].

CONCLUSIONS

In summary, it has been demonstrated that Iva as well as Aib are the metabolic precursors of zervamicin. The diversity of the different isoforms produced by *E. salmosynnemata* is most likely the result of a lack of the regiospecific catalytic activity of the non-ribosomal peptide synthetase. The catabolysis of nitrogen-15 labelled D,L-Iva and the subsequent anabolic conversion to labelled Aib, D-Iva and proteinogenic amino acids is explained in terms of an equilibrium reaction catalysed by 2,2-dialkylglycine decarboxylase (DGD).

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