Journal of Peptide Science, Vol. 2, 341–350 (1996)

Preparation of Site-Specific Isotopically Labelled Zervamicins, the Antibiotic Peptaibols Produced by *Emericellopsis Salmosynnemata*

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Received 8 January 1996
Accepted 29 February 1996

Abstract: A simple procedure for the preparation of the specifically labelled peptide antibiotic zervamicins IC, IIA and IIB has been developed. The zervamicin molecules are labelled with stable isotopes by culturing the *Emericellopsis salmosynnemata* on a well-defined synthetic medium containing the highly isotopically enriched amino acid. To obtain the peptide with the specifically and highly enriched amino acid residue, precautions have been taken to prevent any *de novo* biosynthesis of the particular amino acid from unlabelled precursors. The enrichment of the labelled peptide is determined by mass spectrometric analysis. Following this method we have incorporated [2′,4′,5′,6′,7′-$^2H_5$]-L-Trp-1, [1′-^{15}N]-L-Trp-1 and [2′,3′,4′,5′,6′-$^2H_5$]-L-Phl-16 into zervamicins IC, IIA and IIB on the preparative scale and without scrambling of the label. Thus, using the procedures described, isotopically labelled zervamicins can be prepared, allowing them to be studied by solid-state NMR.

Keywords: Biosynthesis; deuterium; nitrogen 15; positive ion FAB mass spectrometry; ion channel-forming peptide

INTRODUCTION

The movement of ions through channel-forming membrane proteins is fundamental to a number of physiological processes, including the electrical properties of excitable cells. Channel-forming peptides such as the zervamicins are model ion channels which mimic many of the functional properties of larger and more complex channel proteins. The antibiotic peptaibol [1] zervamicin forms voltage-activated ion channels across phospholipid bilayer membranes [2]. From X-ray studies it is known that the crystal structure of [Leu-1]-zervamicin is highly helical with all the polar residues lying on one face of the helix [3–5]. This distribution of side chains is typical for channel-forming peptides. From a model study it has been reported that channels are formed by a process of self-assembly within the plane of the bilayer which generates bundles of parallel *trans*-membrane helices surrounding a central polar pore [6,7] thus permitting permeation of selected ions. However, experimental data about the structure of these channels are still lacking.

We are interested in the investigation of the structure and function of the zervamicins, their interaction with the membrane and the molecular mechanism of ionic conduction, studied at the

Abbreviations: [$^2H_5$]-L-Trp, [2′,4′,5′,6′,7′-$^2H_5$]-L-tryptophan; [$^2H_5$]-L-Phe, [2′,3′,4′,5′,6′-$^2H_5$]-L-phenylalanine; [$^2H_5$]-L-Phl, 2′,3′,4′,5′,6′-$^2H_5$]-L-phenyl-alaninol; ZIC, ZIIA and ZIIB, respectively, zervamicins IC, IIA and IIB.

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CCC 1075-2617/96/060341-10

atomic level with a multidisciplinary approach of site-directed isotopic labeling and spectroscopic investigation (solid-state NMR and FTIR difference spectroscopy). This approach has been successfully applied for the membrane protein in vision [8], the proton pump bacteriorhodopsin [9] and photosynthetic reaction centres [10,11].

There are two aromatic residues located in the zervamicins IC, IIA and IIB (Figure 1) which are forming the mouth of the ion channel, i.e. tryptophan at position 1 and phenylalaninol at position 16. They may play an important role in the voltage-gated mechanism of opening and closing of the ion channel. Thus, it is necessary to prepare zervamicins labelled at the terminal residues in order to get structural and functional information by means of spectroscopic techniques [8–15].

The method to obtain isotopically labelled zervamicins is to culture *Emericellopsis salmosynnemata* on a medium containing the labelled amino acid. Since only a complex medium for growing the fungi is known so far and this is not suitable for specific-labelling experiments, a well-defined synthetic medium has been developed. Via addition of isotopically labelled amino acids (or their biosynthetic precursors) to the medium, zervamicins have been prepared in isotopically labelled forms.

Isotopic labelling of peptides and proteins often results in dilution or scrambling of the label in the product due to competitive metabolic pathways. In order to find the appropriate conditions for the incorporation of the labelled amino acids into the zervamicins a method for determining the label content of the peptide is needed. FAB-MS is found to be a suitable method for the determination of the amount of incorporation of labelled amino acid in zervamicins IC, IIA and IIB in one single measurement.

Zervamicin-IC:
Ac-Trp*-Ile-**Glu-Iva**-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phl*

Zervamicin-IIA:
Ac-Trp*-Ile-**Gln-Aib**-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phl*

Zervamicin-IIB:
Ac-Trp*-Ile-**Gln-Iva**-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phl*

Figure 1 Primary structure of the isotopically labelled zervamicins IC, IIA and IIB. Iva, D-isovaline; Aib, aminoisobutyric acid; Hyp, hydroxyproline; Phl, phenylalaninol. Labelled positions are indicated by *. Non-conserved amino acids are in bold characters.

In the present paper we describe the preparation of site-specific isotopically labelled zervamicins as well as the analysis of the incorporation of [2′,4′,5′,6′,7′-$^2H_5$]-L-Trp-1, [1′-^{15}N]-L-Trp-1 and [2′,3′,4′,5′,6′-$^2H_5$]-L-Phl-16 into zervamicins IC, IIA and IIB. The spectroscopic studies with the labelled zervamicins will be published in a further paper.

MATERIALS AND METHODS

[$^2H_5$]-L-phenylalanine and [$^2H_5$]-L-tryptophan were synthesized according to the procedure described in the literature [16]. ^{15}N-anthranilic acid was purchased from Icon Services Inc./Summit, New Jersey, USA. Unlabelled amino acids were obtained from Merck (Germany).

Positive ion FAB mass spectra were acquired using a Jeol JMS SX/SX 102A four-sector mass spectrometer, operated at 10 kV accelerating voltage, equipped with a Jeol FAB gun set at 5 mA emission current producing a beam of 6 keV Xe atoms. Full mass spectra were recorded at a speed of 30 s for the m/z 10 to m/z 2400 mass range by averaging five to ten scans and acquired and processed with an HP-9000 data system using Jeol Complement software. Partial profile mass spectra of the molecular mass range, used for the determination of the label incorporation, were obtained by averaging 15–25 spectra scanned in 5 s for the m/z 1800 to m/z 1900 range. The analytes were dissolved in methanol and 1–2 μl of these solutions containing about 10 μl per μl were loaded with a microdroplet of 3-nitrobenzyl alcohol on the FAB probe. Instrumental slit width settings corresponding to a resolution of about 1500 were chosen to obtain good sensitivities. Normal mass spectra were obtained by scanning MS-1, while product-ion spectra of selected precursor ions were acquired by scanning MS-2 in the B/E mode using a collision cell in the third field-free region of the instrument and air as the collision gas. The pressure of the collision gas was adjusted to obtain 50% intensity reduction of the main beam.

Synthetic Medium for Growing *E. salmosynnemata*

For each litre of the synthetic medium 100 ml of A and 20 ml each of stock solutions (*vide infra*) B, C, D and 0.02 ml of E are added. After adding distilled water to a final volume of 1 litre the pH is adjusted to 7.2 with solid malic acid or 1 M ammonia. Cultivation of *E. salmosynnemata* has been performed in 100 ml

of the synthetic medium, to which 22 mg L-Phe, 16 mg of L-Tyr and 4.5 g glucose have been added.

In order to obtain [$^2H_5$-L-Phl-16]-zervamicin 66 mg of [$^2H_5$]-L-Phe is added to 100 ml of the synthetic medium instead of 22 mg of unlabelled L-Phe. For the preparation of [$^2H_5$-L-Trp-1]- or [1′-^{15}N-LTrp-1]-zervamicins either 81 mg of [$^2H_5$]-L-tryptophan or 51 mg of [1′-^{15}N]-anthranilic acid is added. Finally, the medium is autoclaved at 120°C.

Solution A has the following composition (in g/l): 0.373 L-Ala, 0.688 L-Arg.HCl, 0.086 L-Cys.HCl, 2.236 L-Gln, 0.103 Gly, 0.378 L-Ile, 1.376 L-Leu, 1.462 L-Lys, 0.315 L-Met, 0.086 L-Pro, 0.522 L-Ser, 0.430 L-Thr, 0.860 L-Val.

Stock Solution B is prepared by dissolving 134 g malic acid (Sigma) in about 500 ml of distilled water. The pH of this solution is adjusted to 6.9 with concentrated ammonia. Then distilled water is added to a final volume of 1 litre.

Solution C is made by subsequently dissolving 119 g of K_2HPO_4 crystals and 65.0 g of KH_2PO_4 into 1 litre distilled water. The pH of this solution is 6.9.

1 litre of ***solution D*** contains the following nutrients: 10 g of nitrilotriacetic acid, 14.5 g of $MgSO_4$, 3.3 g of $CaCl_2.2H_2O$, 0.25 g of $FeSO_4.7H_2O$, 9.3 mg of $(NH_4)_6Mo_7O_{24}.4H_2O$ and 30 ml of metals '44' stock solution. One litre of metals '44' contains the following solutes: 5.0 g $FeSO_4.7H_2O$, 6.5 g sodium ethylenediamine-tetraacetic acid, 10.893 g of $ZnSO_4.7H_2O$, 1.3 g of $MnCl_2.4H_2O$, 0.392 g of $CuSO_4.5H_2O$, 0.2 g of $CoCl_2.6H_2O$ and 0.114 g of H_3BO_3.

Each litre of ***solution E*** contains 65 mg of nicotinic acid.HCl (Sigma), 50 mg of thiamine.HCl (Sigma) and a trace (1 mg) of biotin (Sigma).

Solutions A, B, C, D and E are kept separately in the refrigerator; the vitamin solution (E) should be used within two weeks of preparation.

Cultures of *E. salmosynnemata*

The microorganism used for the production of antibiotics is *E. salmosynnemata*, strain 336, IMI 58330. A subculture of this microorganism is a kind gift from the Upjon Company, Kalamazoo, Michigan, USA. Stock cultures of *E. salmosynnemata* are kept on the medium containing 1.5% agar and stored in the dark at 4°C. The stock culture is used to inoculate 10 ml of synthetic medium containing the labelled amino acid. This seed culture is grown aerobically by using a rotary shaker operating at 250 rotations per min at 28°C. After 96 h the seed culture is used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of labelled synthetic medium. The cultures have been shaken for 240 h.

Isolation of the Isotopically Labelled Zervamicins IC, IIA and IIB

The fermentation beer is filtrated using a glass filter (G–4). The filter cake is washed with 20 ml of water and the aqueous wash is combined with the clear beer. The filtrated beer is extracted three times with 20 ml portions of 1-butanol. The butanolic extracts are combined and concentrated to dryness. After adding 50 ml of methanol and 20 mg of active charcoal the suspension is heated under reflux for 10 min. The charcoal is removed by filtration and the colourless filtrate is evaporated yielding 190 mg of crude product.

The filter cake is triturated with 50 ml of methanol and the insoluble material is separated by filtration. The methanolic extract, concentrated to dryness *in vacuo*, yields 50 mg of material.

Chromatographic Purification

The methanol as well as the butanol extract, obtained as described above, are purified by means of gel filtration, followed by RP HPLC for further purification. For gel filtration a column (1.5 × 55 cm) packed with Sephadex LH-20 swelled in methanol is used. Methanol is used as an eluant (flow rate 1 ml/min) and 10 ml fractions containing the zervamicins (detected at $\lambda = 280$ nm) are collected. After that, RP HPLC has been used for further separation of zervamicins IC, IIA and IIB.

The analytical and preparative separations of zervamicins are carried out using an HPLC chromatograph, equipped with an Pharmacia LKB HPLC pump, model 2248, a Pharmacia LKB high-pressure mixer, a Pharmacia injector (5 µl or 400 µl sample loop) and a Pharmacia LKB SuperFrac fraction collector. Peaks are monitored using either a FD-300 dual monochromator fluorescence detector ($\lambda_{ex} = 287$ nm, $\lambda_{em} = 348$ nm) or a Pye Unicam LC3 UV-detector ($\lambda = 220$ nm). Peak areas and retention times are determined using the Pharmacia HPLC Manager software. Analytical separations have been carried out using a 250 × 4 mm i.d. SuperPac Spherisorb ODS 5 µm, obtained from Pharmacia (Sweden). For the preparative separation of zervami-

cins a 250 × 20 mm i.d. Europrep 60-10 C18 10 μm column (Knauer, Germany) has been used.

Samples are dissolved in a mixture of $MeOH/CH_3CN/H_2O$ (62.5/22.5/15.0 v/v/v) (the volume of injected sample is 5–400 μl). The mobile phase is composed of: $MeOH/CH_3CN/H_2O$ (62.5/22.5/15.0 v/v/v). The flow rate is 0.4 ml/min for the analytical separation and 7 ml/min for the preparative purification. All separations are performed at room temperature.

Mass Spectrometric Procedure for the Determination of the Isotopic Incorporation into Labelled Zervamicins

The identity of the labelled compounds and their label position is reflected in the m/z values of specific peaks in their mass spectra. Incorporation of $[^2H_5]$-tryptophan in ZIIA and ZIIB shifts all B-ions 5 mass units upwards, while the mass spectra of $[^2H_5]$-phenylalaninol containing zervamicins shown N-terminal sequence ions with identical masses as the unlabelled compound. For both the $[^2H_5]$ labelled Phl and Trp zervamicins the monoisotopic $[M+Na]^+$ peak is found at m/z 1852 (ZIIA) and m/z 1866 (ZIIB), respectively.

The isotopical enrichment was determined using the intensity ratio of the monoisotopic peak of the $[M+Na]^+$ ion of the unlabelled compound and the $[^{15}N]$ or $[^2H_5]$ peak of the labelled compounds, corrected for natural isotopic and background contributions. The correction for the background contribution is needed because in the experiment uncompletely labelled starting materials were used. Mass spectrometric analysis of the labelled amino acids showed a label distribution in L-phenylalanine of 68% $[^2H_5]$, 27% $[^2H_4]$ and 5% $[^2H_3]$, while in L-tryptophan a label content of 67% $[^2H_5]$, 28% $[^2H_4]$ and 5% $[^2H_3]$ was found. This corresponds to a total deuterium labelling of the aromatic part of both amino acids of about 92.5%. The degree of labelling of ^{15}N-anthranilic acid was found to be 97%.

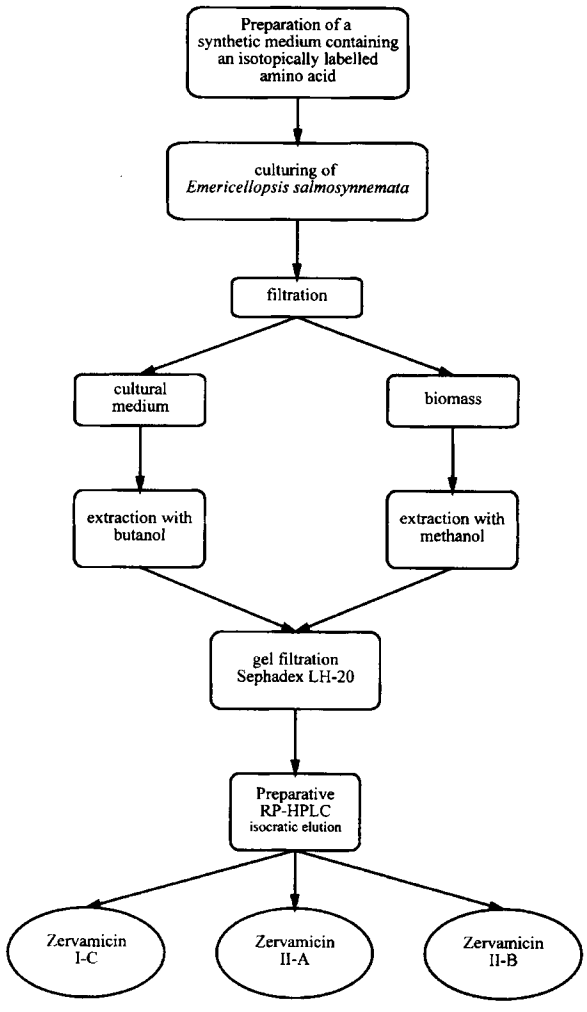
For the unlabelled zervamicin IIB the calculated intensities of the m/z 1861($[M+Na^+]$), m/z 1862, m/z 1863 and m/z 1864 peaks are respectively 92%, 100%, 58% and 23%. For the peak clusters of the unlabelled zervamicins IIA and IC nearly the same intensity patterns are calculated.

In the spectrum of the completely labelled compound the intensity of the m/z 1866 peak in the $[^2H_5]$-Phl or $[^2H_5]$-Trp labeled ZIIB would represent the sum (92.5%) of the contribution of the monoisotopic $[^2H_5]$-compound (0.68 × 92), the combined $^{13}C_1$ and $^{15}N_1$-contributions of the $[^2H_4]$-compound (0.27 × 100) and the $^{13}C_2$-contribution of the $[^2H_3]$-compound (0.05 × 58). Since the intensity of the peak of the unlabelled zervamicin at m/z 1861 (92%) equals approximately the intensity of the peak of the completely labelled peptide at m/z 1866 (92.5%) and taking into account that the accuracy of the label percentage is ±2%, the intensity of the peak of zervamicin IIB (wherein uncompletely labelled starting material has been incorporated) at m/z 1866 can be considered as a reliable representation of the amount of the combined $[^2H_5]$-, $[^2H_4]$- and $[^2H_3]$-compounds: $x \times 0.68 \times 92 + x \times 0.27 \times 100 + x \times 0.05 \times 58$, where x is the degree of incorporation of the (uncompletely) labelled starting material.

RESULTS AND DISCUSSION

A general procedure for the isolation of labelled zervamicins from the cultural medium and biomass is presented in Scheme 1. The method to obtain isotopically labelled zervamicins requires a synthetic medium for culturing *E. salmosynnemata*. In developing a medium the primary goal is to provide a balanced mixture of the required nutrients at concentrations that will lead not only to a good yield of the zervamicins but also to a high level of isotopic enrichment at a specific atomic position of the peptides. The complex medium, described earlier [16,17] for growing *E. salmosynnemata*, contains glucose, starch, molasses, peptone, yeast extract and inorganic nutrients. The peptone and yeast extract are not well-defined amino acid sources and for this reason it is impossible to use the complex medium for the production of isotopically labelled peptides. That is why a well-defined synthetic medium containing a well-defined mixture of chemically pure L-amino acids has been developed. Recently, this procedure was successfully applied to the preparation of the 100 kDa [4′-^{13}C]-Tyr- and [2′-^{15}N]-L-Trp labelled photosynthetic reaction centre protein by using the purple bacterium *Rhodobacter sphaeroides* [18].

After growing the fungi on the synthetic medium the peptaibols have been recovered from the filtered broth and biomass by extraction and further separation using gel filtration and a fast preparative RP HPLC purification step. A simple isocratic elution using water/acetonitrile/methanol allows pure zervamicin IC to be obtained as well as a mixture of zervamicins IIA and IIB. The peak at 15 min corresponds to the charged ZIC whereas the



Scheme 1 General procedure for the preparation of specifically isotopically labelled zervamicins.

peaks between 20 and 25 min are the neutral peptides ZIIA and ZIIB. In this stage of purification the samples have been analysed by means of FAB-MS. The best results have been obtained using 3-nitrobenzylalcohol as a matrix. In the low mass region of the mass spectrum (not shown) of the mixture of zervamicins peaks corresponding to the immonium ions of various amino acids have been found at m/z 58 (Aib), m/z 70 (Pro), m/z 72 (Iva), m/z 86 (Ile and Hyp), m/z 101 (Gln), m/z 120 (Phl) and m/z 201 (acetyl-Trp). From the high mass region of the spectrum shown in Fig. 2 it is clear that monoisotopic peaks of the peptides ZIIA and ZIIB have been found as abundant sodium cationized species at m/z 1847 and m/z 1861, respectively. The spectrum further reveals a number of peaks corresponding to B-type sequence ions [19] at m/z 229.1 (B_1), m/z 342.2 (B_2) and m/z 470.2 (B_3). The observation that from the fourth amino acid onwards couples of B-ions are found with a mass difference of 14 a.m.u. (m/z 555.3 and m/z 569.3 (B_4), m/z 668.4 and m/z 682.4 (B_5, m/z 769.4 and m/z 783.4 (B_6) and m/z 967.6 and m/z 981.6 (B_8)) also reflects the presence of ZIIA and ZIIB in the sample. Cleavage of the Aib-Pro and Aib-Hyp bonds is energetically favourable and results in the formation of relatively abundant B_n-ions, which an be observed at m/z 1052.6 and m/z 1066.6 (B_9), m/z 1378.8 and m/z 1392.8 (B_{12}), and m/z 1576.9 and m/z 1590.9 (B_{14}). Similar fragment ions were observed in the FAB mass spectrum of Z1C ($[M+Na]^+ = m/z$ 1862) [20,21]. The presence of these B-type ions confirms the major part of the amino acid sequence in the peptides. A complete sequence confirmation of each peptide was obtained from the collisionally induced dissocation MS/MS spectra (not shown) of the selected $[M+Na]^+$ ion. The mass difference between two ions of the same type defines the amino acid at this position of the peptide chain. Exceptions are the isobaric amino acids leucine, isoleucine and hydroxyproline. However, these amino acids have been distinguished on the basis of the mass of ions produced by secondary fragmentations. These fragmentations occur at the leucine and isoleucine residues (but not at the hydroxyproline) and involve the cleavages of the C^{β}–C^{γ} bond of the side-chain of these amino acid residues.

To obtain the peptides with the specifically and highly enriched amino acid residue, precautions have been taken to prevent any *de novo* biosynthesis of the particular amino acid from unlabelled precursors. In pilot experiments the concentration of labelled [$^2H_5$]-L-Trp in the synthetic medium has been stepwise increased: 13 mg/100 ml (0.65 mM), 27 mg/100 ml (1.30 mM), 81 mg/100 ml (3.90 mM). In order to find the optimal concentration, the level of incorporation of the labelled Trp-1 residue in the different zervamicins has been determined by means of positive ion FAB-MS. The experiment with 81 mg/100 ml shows that this amino acid has been incorporated into each of the zervamicins to a percentage of $78 \pm 2\%$. It must be noted that when a concentration of 160 mg/100 ml was used, instead of labelled [Trp-1]-zervamicins IC, IIA and IIB, the unlabelled [Leu-1, Aib-4]- and [Leu-1, Iva-4]-zervamicins were produced as the major compounds. This conclusion was extracted from the mass spectrum by the appearance of intense peaks at m/z 1774 and m/z 1787, respectively.

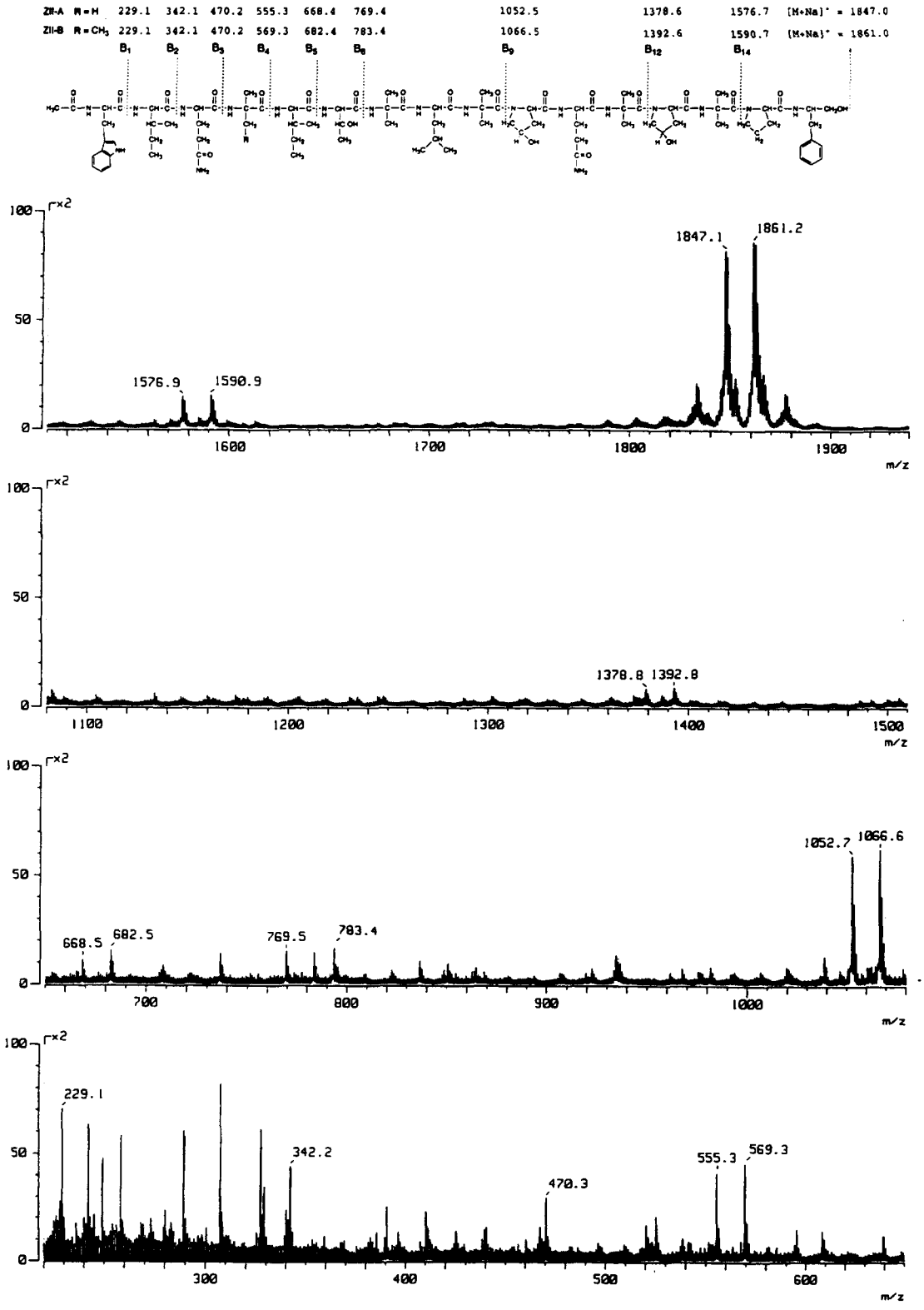


Figure 2 Positive ion FAB mass spectrum of a mixture of zervamicins showing the presence of zervamicin IIA (ZIIA) and zervamicin IIB (ZIIB) as well as characteristic B-type sequence ions. The incorporation of about 17% [$^2H_5$]-L-Phl is, as expected, only visible in the $[M + Na]^+$ ion clusters and not in the B-type ion clusters.

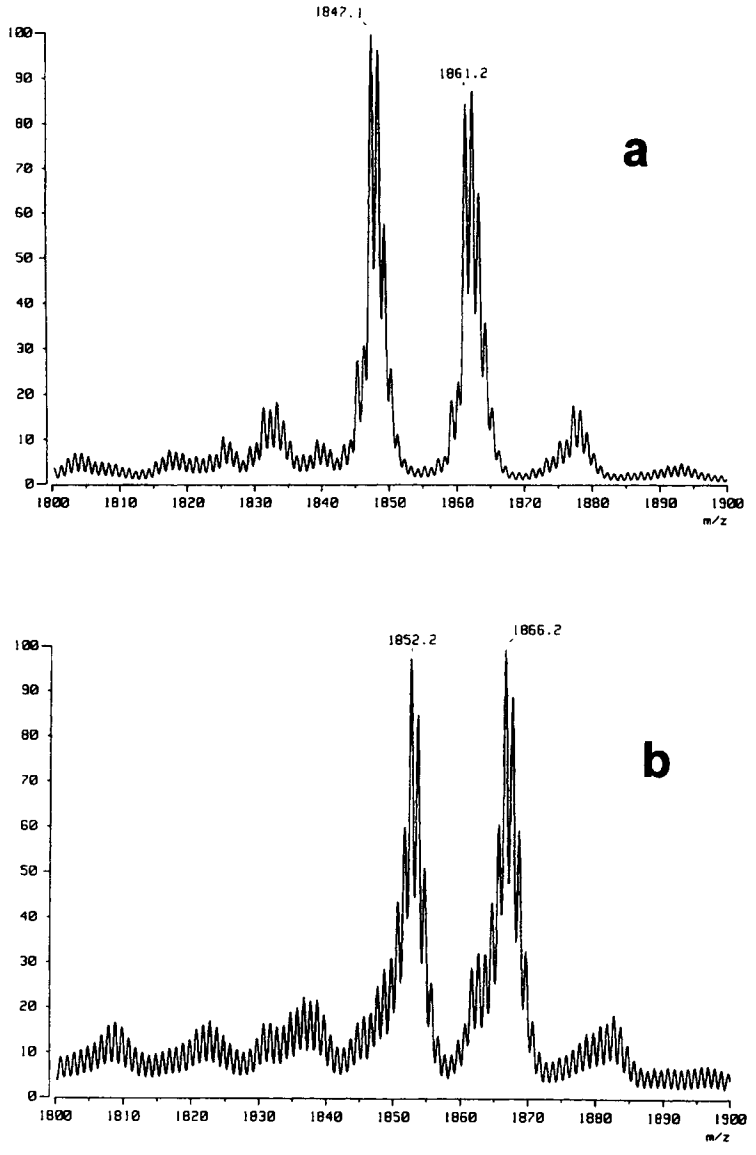


Figure 3 Partial mass spectrum of (a) unlabelled and (b) labelled [$^{2}H_5$-Phl-16]-zervamicin.

Another approach for labelling the peptide at the N-terminal position is adding to the synthetic medium a biosynthetic precursor of tryptophan, i.e. [^{15}N]-labelled anthranilic acid. In order to obtain [^{15}N]-Trp-labelled zervamicin different concentrations of [1'-^{15}N]-anthranilic acid have been used: 9 mg/100 ml (0.65 mM), 18 mg/100 ml (1.31 mM) and 51 mg/100 (3.93 mM) ml of synthetic medium. Fungi grown at a concentration 51 mg [^{15}N]-anthranilic acid per 100 ml medium yield the zervamicins labelled with the [^{15}N]-isotope (55 ± 5%) at the indole ring of the tryptophan residue at position 1. From this result it is clear that *E. salmosynnemata* can synthesize tryptophan from anthranilic acid.

It is of interest to remark that for quantitative label incorporation of tryptophan residues into photosynthetic reaction centres [18] of *R. sphaeroides* R-26 a much lower concentration of either [$^{2}H_5$]-L-Trp (6.8 mg/100 ml) or [^{15}N]-anthranilic acid (4.6 mg/100 ml) was used. For the incorporation of labelled tryptophan into zervamicin the same synthetic medium is used as was reported for labelling of the photosynthetic reaction centres except that in the present study 4.5 g/l glucose has been added. There-

fore, the influence of the amount of glucose on the efficiency of label incorporation has been tested. Decreasing the glucose concentration from 4.5 g/l to 1.5 g/l did not lead to an improved label incorporation, but has a strong effect on the yield of the zervamicin mixture IIA/IIB (decreasing from 240 mg to 80 mg) as well as on the zervamicin ratio IIA : IIB (changing from 1 : 1 to 1 : 2).

Another explanation for the lower efficiency of the labelled tryptophan incorporation that has been considered is a possible higher catabolization rate of the amino acid during the exponential growing phase of the fungi. It is known from the literature that the production of the zervamicins reaches a maximum after 192 h of cultivation of the fungi [16]. For this reason the medium used during the initial period of the cultivation (containing 13 mg of [$^2H_5$]-L-Trp per 100 ml) has been changed after 120 h of cultivation to a fresh medium containing 66 mg of the labelled amino acid per 100 ml. However, it is found that this procedure does not have an effect on the incorporation of the labelled amino acids into the zervamicin.

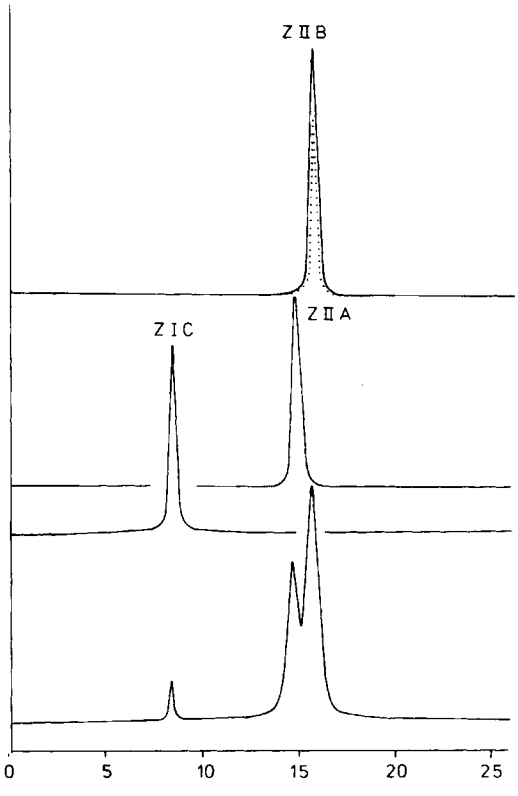


Figure 4 Analytical RP HPLC chromatograms of the isolated zervamicins IC, IIA, IIB and that of an authentical sample of zervamicin IIB (dotted line). Column: SuperPac Spherisorb ODS 5 μm, 250 × 4 mm, mobile phase: MeOH/CH_3CN/H_2O (62.5/22.5/15.0 v/v/v), isocratic elution, flow rate 0.4 ml/min, fluorescence detection $\lambda_{ex} = 287$ nm, $\lambda_{em} = 348$ nm.

In the next series of experiments we used four different concentrations of [$^2H_5$]-L-Phe for labelling of the C-terminal position of the zervamicins, i.e. 11 mg/100 ml (0.65 mM), 22 mg/100 ml (1.30 mM) and 66 mg/100 ml (3.90 mM). After analysing the samples by means of FAB-MS (Figure 3) it has been found that the incorporation of phenylalaninol into zervamicin is higher than 82 ± 2%. Thus, the incorporation of Phl into the zervamicins is more efficient than the incorporation of Trp.

Because pure labelled compounds are needed for the spectroscopic investigation, zervamicins IIA and IIB have been isolated from the mixture by rechromatography on RP HPLC using an isocratic elution procedure (see Materials and Methods). The different peptaibols have been identified by means of tandem FAB-MS. The purity of the isolated zervamicins has been proved by means of analytical RP HPLC (Figure 4).

CONCLUSIONS

A simple procedure for the preparation of specifically labelled zervamicins IC, IIA and IIB in a pure form has been developed. The peptaibols have been labelled with stable isotopes by culturing *E. salmosynnemata* on a well-defined synthetic medium containing the isotopically enriched amino acid. Growing of the fungi on 100 ml of the synthetic medium allows the isolation of 5 mg of pure zervamicin IC, 10 mg of zervamicin IIA and 10 mg of zervamicin IIB. In order to obtain larger amounts of the labelled peptaibols, the production can be easily upscaled.

Positive ion FAB-MS is the method of choice for determining the amount and position of the isotopes. Following the method described we have incorporated [2′,4′,5′,6′,7′-$^2H_5$]-L-Trp-1 (78 ± 2%), [1′-^{15}N]-L-Trp-1 (55 ± 5%) and [2′,3′,4′-5′,6′-$^2H_5$]-L-Phl-16 (82 ± 2%) into three different zervamicins on the preparative scale. From the results obtained it is clear that *E. salmosynnemata* can synthesize tryptophan from anthranilic acid as well as phenylalaninol from phenylalanine.

The procedure described has opened a way to the preparation of zervamicins which are labelled with the ^{13}C-, ^{15}N- and ^2H-isotope at any specific position

or combination of positions of both the N- and C-terminal residues Trp-1 and Phl-16.

Acknowledgements

This work has been supported by the Netherlands Organization for Scientific Research as well as by DSM Research (Geleen, The Netherlands).

We are grateful to the Upjohn Company (Kalamazoo, Michigan, USA) for the kind gift of the subculture of *E. salmosynnemata*. We wish to thank Dr. R. Verhage (Department of Molecular Genetics, Leiden Institute of Chemistry, University of Leiden, The Netherlands) for helpful assistance in the microbiological experiments and Prof. P. Balaram (Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India) for providing samples of unlabelled zervamicins IIA and IIB.

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