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Biosynthetic Uniform ^{13}C,^{15}N-Labelling of Zervamicin IIB. Complete ^{13}C and ^{15}N NMR Assignment‡

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Abstract: Zervamicin IIB is a member of the α-aminoisobutyric acid containing peptaibol antibiotics. A new procedure for the biosynthetic preparation of the uniformly ^{13}C- and ^{15}N-enriched peptaibol is described. This compound was isolated from the biomass of the fungus-producer *Emericellopsis salmosynnemata* strain 336 IMI 58330 obtained upon cultivation in the totally ^{13}C,^{15}N-labelled complete medium. To prepare such a medium the autolysed biomass and the exopolysaccharides of the obligate methylotrophic bacterium *Methylobacillus flagellatus* KT were used. This microorganism was grown in totally ^{13}C,^{15}N-labelled minimal medium containing ^{13}C-methanol and ^{15}N-ammonium chloride as the only carbon and nitrogen sources. Preliminary NMR spectroscopic analysis indicated a high extent of isotope incorporation (>90%) and led to the complete ^{13}C- and ^{15}N-NMR assignment including the stereospecific assignment of Aib residues methyl groups. The observed pattern of the structurally important secondary chemical shifts of $^{1}H^{\alpha}$, $^{13}C{=}O$ and $^{13}C^{\alpha}$ agrees well with the previously determined structure of zervamicin IIB in methanol solution.

Keywords: antibiotic; peptaibol; zervamicin; ^{13}C; ^{15}N; isotope labelling; NMR

INTRODUCTION

State of the art NMR techniques can provide a wealth of information about the structure and dynamics at atomic resolution of self-assembling, amphipathic and ion-channel forming peptaibol molecules. These techniques allow investigations of biomolecules that are functionally active in phospholipid membranes, either in a membrane mimetic solution or in a membrane bound state. In any case, the technique of isotope labelling combined with multidimensional NMR techniques gives better resolved spectra and permits additional information to be obtained. In particular, uniform labelling facilitates the assignment procedure of NMR studies and avoids the laborious preparation of dozens of specific-labelled samples [1–4].

Zervamicins are peptide antibiotics synthesized by the mycelial fungus *Emericellopsis salmosynnemata* strain 336 IMI 58330 [5]. These antibiotics belong to peptaibol family, namely to the class of linear polypeptide containing Aib residues and a

Abbreviations: Zrv-IIB, zervamicin IIB; Aib, α-aminoisobutyric acid; Iva, D-isovaline; Hyp, hydroxyproline; Phl, phenylalaninol; EPS, exopolysaccharides; CSI, chemical shift index.

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Ac-Trp[1]-Ile[2]-Glu[3]-Iva[4]-Ile[5]-Thr[6]-Aib[7]-Leu[8]-Aib[9]-Hyp[10]-Gln[11]-Aib[12]-Hyp[13]-Aib[14]-Pro[15]-Phl[16]

Zervamicin IC

Ac-Trp[1]-Ile[2]-Gln[3]-Aib[4]-Ile[5]-Thr[6]-Aib[7]-Leu[8]-Aib[9]-Hyp[10]-Gln[11]-Aib[12]-Hyp[13]-Aib[14]-Pro[15]-Phl[16]

Zervamicin IIA

Ac-Trp[1]-Ile[2]-Gln[3]-Iva[4]-Ile[5]-Thr[6]-Aib[7]-Leu[8]-Aib[9]-Hyp[10]-Gln[11]-Aib[12]-Hyp[13]-Aib[14]-Pro[15]-Phl[16]

Zervamicin IIB

Figure 1 Primary structures of the zervamicins IC, IIA and IIB.

C-terminal amino alcohol Phl (Figure 1). Zervamicins are more active against Gram-positive than Gram-negative bacteria [6]. The peptaibols adopt an active completely helical conformation in the presence of lipid membrane or suitable membrane mimetic [7,8]. The mode of zervamicin action implies the formation of voltage-dependent multilevel ion channels formed from amphipathic helical bundles in phospholipid bilayer membranes [9,10].

Zervamicin IIB is of special interest for studying the detailed ion channel mechanism because of the regular distribution of polar and apolar residues in the peptide chain. Of more interest are the studies on the molecular interaction of the peptaibol with lipid membranes as well as on the determination of the mechanism and dynamics of zervamicin-induced pore formation. A very efficient approach to the elucidation of structure–function relationships for peptaibols is based upon solid- or solution-state NMR spectroscopy, which can provide valuable information about the structure, dynamics and orientation of peptaibol molecules in bilayer membranes and membrane mimetics as well [8,11,12]. However, in order to exploit the full power of NMR techniques, stable isotope enriched samples of the peptaibols are necessary. In particular, the availability of uniformly ^{13}C,^{15}N-labelled samples will be important in providing detailed information at the atomic level on peptaibol function, such as the detailed structure of the molecule including the pattern of intramolecular hydrogen bonds, the mode of interaction with solvent molecules, the dynamics and modes of peptide aggregation in membrane mimicking media. Moreover, it permits determination of the precise structure of the zervamicin channel in a membrane by solid state NMR technique.

A biosynthetic approach based upon cultivating the corresponding fungus-producer on a medium containing stable isotopes, which should be incorporated into target molecules, proved to be appropriate for deriving uniformly labelled biologically active compounds [13]. However, application of this technique to obtain uniformly ^{13}C,^{15}N-double-labelled peptaibols met with some difficulties. An attempt to prepare the uniformly ^{13}C,^{15}N-labelled peptaibol alamethicin by growing the fungus *Trichoderma viride* in a medium containing [U-^{13}C]-glucose and K $^{15}NO_3$ showed that ^{15}N was incorporated to a level of about 98% but ^{13}C to a relatively low level of about 50% [14].

Our previous studies demonstrated the efficient use of methylotrophic bacteria as producers of stable isotope labelled amino acids [15,16]. The autolysed 'methylotrophic' biomass was utilized as the basis for preparing the totally labelled complete growth medium to cultivate the heterotrophic producers of uniformly deuterated inosine [17] and delta-endotoxin [18].

Here the biosynthetic approach employed to obtain the preparation of uniformly ^{13}C,^{15}N enriched Zrv-IIB is described. Preliminary spectroscopic characterization of the obtained compound indicated a high purity and high extent of isotope incorporation (>90%). The product was further characterized by mass spectrometry, and the full assignment of ^{13}C and ^{15}N NMR signals of Zrv-IIB was made in methanol solution. The observations ensure that highly isotopically enriched Zrv-IIB was obtained. The chemical shifts of all the $^{1}H^{\alpha}$, $^{13}C^{\alpha}$ and $^{13}C{=}O$ nuclei were in excellent agreement with the previously determined structure of Zrv-IIB [7]. The stereospecific assignment of both the pro-*R* and pro-S methyl groups for each of the four different Aib residues was obtained from the $^{13}C^{\beta}$ chemical shifts, and they agree with the assignment that has been reported elsewhere [7].

MATERIALS AND METHODS

The following materials were used: methanol, isopropanol, ethyl acetate, starch, salts (Reachim, Russia); acetonitrile (Lecbiopharm, Russia); glucose (Merck, Germany); bacto peptone, yeast extract, agar (Difco, USA). The labelled compounds, $^{15}NH_4Cl$ (^{15}N, 98%), $^{13}CH_3OH$ (^{13}C, 99%) and [U-^{13}C]-glucose (^{13}C, 99%) were purchased from Cambridge Isotope Laboratory, USA.

Culture Medium and Cultivation Conditions for *Methylobacillus flagellatus*

To produce a totally labelled biomass and exopolysaccharides, the obligate methylotroph *M. flagellatus* KT [19] obtained from the Laboratory of Genetics of Methylotrophic Bacteria, State Research Institute for Genetics and Selection of Industrial Microorganisms (Moscow, Russia) was used. Methylotrophic bacteria were grown at 42 °C in standard minimal M9 medium containing per litre: Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), $MgSO_4{\cdot}7H_2O$ (370 mg), $CaCl_2$ (11 mg), $FeSO_4{\cdot}7H_2O$ (7.4 mg), $ZnSO_4{\cdot}7H_2O$ (1 mg), $MnCl_2{\cdot}4H_2O$ (0.01 mg), $CoCl_2{\cdot}H_2O$ (0.74 mg), $CuCl_2$ (0.25 mg), $NiCl{\cdot}H_2O$ (0.07 mg), Na_2MoO_4 (0.07 mg), H_3BO_3 (0.64 mg), EDTA (10 mg), NH_4Cl (1 g) and CH_3OH was added gradually (no more than 10 g/l) as the only carbon source, the pH was maintained at 7.0–7.2. To obtain the uniformly $^{13}C,^{15}N$-labelled biomass, minimal medium of the same composition was used, but prepared with $^{13}CH_3OH$ and $^{15}NH_4Cl$. The seeding culture of *M. flagellatus* was grown in 50 ml of the $^{13}C,^{15}N$-labelled medium. Cultivation was carried out in a laboratory-scale fermenter Bioflo C30 (New Brunswick Scientific Co., USA) with a 500 ml working volume. The cells were grown in 300 ml of the $^{13}CH_3OH$ and $^{15}NH_4Cl$ containing medium and harvested by centrifugation at 10 000 **g** for 15 min. The optical density of bacteria cultures was measured at 590 nm.

Isolation of ^{13}C-labelled Exopolysaccharides (EPS) from *M. flagellatus* Culture

Exopolysaccharides were precipitated from the *M. flagellatus* culture broth by the addition of 2 volumes of cold ethanol (4 °C) during intensive stirring. The mixture was stored at 0 °C for 2 h. The obtained white flakes of EPS were separated by centrifugation (15 000 rpm, 30 min).

Preparation of the Autolysed $^{13}C,^{15}N$-labelled Biomass of Methylotrophic Bacteria

To use the components of the bacterial biomass as growth substrates for the next fungi cultivation, soft autolysis of the cells was performed according to [20] under conditions to exclude reverse isotope exchange. The $^{13}C,^{15}N$-labelled biomass was resuspended in 0.3 M HCl, autolysed for 24 h at 60 °C, dissolved with water up to a 1% solution of free labelled amino acids, neutralized with 2 M NaOH, and finally sterilized by means of an autoclave. The autolysed $^{13}C,^{15}N$-labelled biomass was used instead of the unlabelled bacto peptone and yeast extract as a $^{13}C,^{15}N$-source for preparing the complete growth medium for fungi cultivation.

Culture Medium and Cultivation Conditions for *E. salmosynnemata*

The *E. salmosynnemata* strain 336 IMI 58330, producing the zervamicins [7], was a kind gift of Upjohn Co. (Kalamazoo, MI, USA). The standard medium for fungus cultivation contained per litre: glucose (10 g), starch (10 g), bacto peptone (10 g), yeast extract (2.5 g) and calcium carbonate (8 g), pH 7.2. The seeding culture of the fungus was cultivated in tubes (5 ml of the medium) for 96 h at 28 °C. The seeding culture (10 ml) was used to inoculate 200 ml of the fermentation medium. The culture was incubated for 7 days at 28 °C on a shaker at 220 rpm. To obtain [U-$^{13}C,^{15}N$]-zervamicin the fungus was grown in medium consisting of the autolysed $^{13}C,^{15}N$-enriched 'methylotrophic' biomass (7 g/l), instead of the unlabelled bacto peptone and yeast extract and [U-^{13}C]-EPS instead of glucose and starch.

Comparison of Labelled and Unlabelled Media for Cultivation of *E. salmosynnemata*

To compare the efficiency of the complete totally $^{13}C,^{15}N$-labelled medium with the standard one, compositions of the autolysates were analysed. Two parameters of the nutrients value of each media were determined, i.e. concentrations of glucose and free amino acids. The glucose concentration was assayed by a colorimetric approach [21], and the contents of free amino acids were detected by thin layer chromatography using the eluent isopropanol/ethyl acetate/25% ammonia/water (40/40/16/32, by volume).

Isolation of the Uniformly $^{13}C,^{15}N$-labelled Zervamicins from the Fungal Biomass

After the fermentation was completed the broth was filtered on a glass filter and the obtained filter cake of the fungus mycelium was triturated with methanol (5 ml/g). The methanol extract was concentrated to a small volume and then dried *in vacuo* on a Speed Vac concentrator. The resulting solid was redissolved in 1.5 ml of methanol and the insoluble residue was removed by centrifugation. The peptaibols were extracted from the filtered fermentation broth with ethyl acetate. Reversed-phase high-performance liquid chromatography on the Vydac column 208TP54 C8, 5 μm particle size, 300E pore size, 4.6 mm ID, 250 mm long (Vydac, USA) was used for isolation of the uniformly $^{13}C,^{15}N$-labelled zervamicins from the methanol and ethyl acetate extracts. Separation of zervamicins IIA and IIB was achieved using the eluent methanol/acetonitrile/water (67/14/19, by volume) at a flow rate 0.5 ml/min. The peptaibols were detected by A_{214}.

Determination of the Molecular Mass and Isotope Enrichment of the $^{13}C,^{15}N$-labelled Zervamicin IIB

The molecular mass of the isolated peptaibol was determined by MALDI-TOF mass spectrometry (MALDI-MS/TOF/VISION 2000, ThermoBioAnalysis, UK). The content of isotope incorporation was determined by 1D heteronuclear NMR spectroscopy. The samples were shown to be pure by 2D heteronuclear NMR spectroscopy.

NMR Spectroscopy and Spectral Assignments

The purified $^{13}C,^{15}N$-labelled Zrv-IIB was dissolved in 0.6 ml of deuterated methanol (CD_3OH, 99.5% deuterium, Stohler Isotope Chemicals, USA) to a concentration of about 4 mM without adjustment of the pH (the measured apparent pH was 6.2). This sample was used for NMR experiments. All NMR experiments were carried out on a Bruker DRX-500 spectrometer equipped with a 5 mm TXI-gradient probe. The temperature of the sample was 305 K.

Spectral assignment of proton bearing ^{13}C and ^{15}N nuclei was achieved by means of ^{13}C- or ^{15}N-HSQC spectra, using known proton assignments [7]. The ^{15}N-HSQC spectrum [22] was measured with spectral widths of 2 kHz × 5 kHz (128 × 1024 complex points). Two ^{13}C-HSQC spectra were acquired with the ^{13}C carrier placed in the centre of the aliphatic (40 ppm) or aromatic (110 ppm) region with spectral widths of 12.5 kHz × 5 kHz (256 × 1024 complex points). All HSQC spectra were processed to 512 × 2048 complex points. Spectral assignments of the carbonyl ^{13}C nuclei were achieved by means of 2D variants of HNCO [23] and HN(CA)CO [24] experiments measured with spectral widths of 1.25 kHz × 5 kHz (128 × 1024 complex points). The spectra were processed to 256 × 2048 complex points.

Chemical shifts were measured relative to the residual ^1H signal of the methyl group of methanol, assuming its chemical shift value of 3.3 ppm. Chemical shifts of ^{13}C and ^{15}N were referenced indirectly using ratios from [25].

BioMagnetic Resonance Bank Accession Code

The chemical shifts of Zrv-IIB in methanol solution were deposited into the BioMagnetic Resonance-Bank (www.bmrb.wisc.edu accession code 4601).

RESULTS AND DISCUSSION

Cultivation of the Methylotrophic Bacteria *M. flagellatus* to Obtain Totally $^{13}C,^{15}N$-labelled Biomass and Exopolysaccharides

Methanol and formaldehyde as its product are toxic for methylotrophic bacteria at a concentration in the medium exceeding several volume per cent. However, a high yield of biomass and EPS can be obtained by bacteria grown in the culture medium with a very low concentration of methanol. Cultivations of methylotrophic bacteria were performed in a laboratory-scale fermenter at a constant oxygen level: methanol was automatically injected in accordance with changes of the partial oxygen pressure (pO_2) of the culture. Thus methanol was added to the culture as the only carbon source in amounts that permitted both biomass and EPS to be obtained in a high yield.

In the present work, the techniques were applied for growing the obligate methylotrophic bacterium *M. flagellatus* in the medium containing $^{13}CH_3OH$ and $^{15}NH_4Cl$ to produce a totally $^{13}C,^{15}N$-enriched complete culture medium for fungi cultivation instead of commercially available $^{13}C,^{15}N$-labelled media. This methylotroph is an ideal microorganism for obtaining a high isotope incorporation into biomass and carbohydrates owing to their ability to use only methanol as the carbon and energy

source and inability to use nitrogen from the atmosphere. Besides, ^{13}C-methanol and ^{15}N-ammonium chloride are relatively inexpensive sources of stable isotopes, rendering large-scale uniformly stable isotope labelling economical. The presence of ^{13}C-methanol and ^{15}N-ammonium chloride in the medium did not much affect the growth of the bacteria and for this reason no preliminary adaptation of the cells was required (Figure 2). Fermentation was performed in media of different isotope compositions (Table 1). The methylotrophic bacteria displayed high growth capacities in three test cultures of varying compositions. Besides, the ability of *M. flagellatus* to produce EPS on stable isotope-labelled media (indicated earlier for highly deuterated medium [26]) is of significant importance for the preparation of complete isotope-labelled media with additional carbon sources. The preparations of $^{13}C,^{15}N$-labelled biomass and ^{13}C-EPS were used to compose highly isotope enriched complete culture media for *E. salmosynnemata* cultivation to produce $^{13}C,^{15}N$-labelled zervamicin.

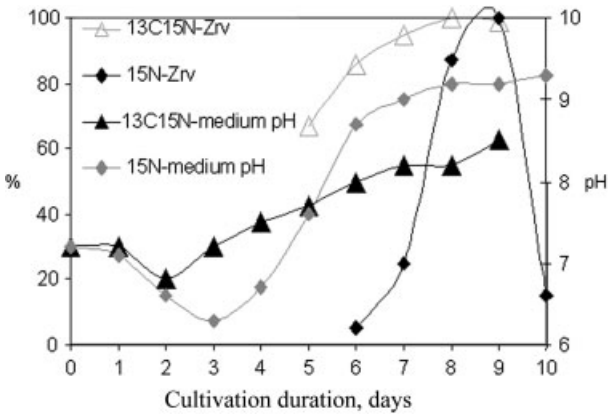

Figure 2 Relative yield of the isotope labelled Zrv-IIB in the biomass of *E. salmosynnemata* compared with the native peptaibol, and pH dynamics of the isotope-containing culture media.

Composition of the Totally $^{13}C,^{15}N$-labelled Complete Culture Medium for *E. salmosynnemata* Cultivation

A procedure to prepare site-specific labelled zervamicin has been described earlier [27].

In our previous studies [11], a procedure for uniform ^{15}N-labelling of zervamicin was developed. The procedure was based upon the cultivation of *E. salmosynnemata* on the media containing ^{15}N-labelled autolysed methylotrophic biomass. As described, the maximum yield of peptaibol can be achieved upon cultivation of the fungus on a complete medium with a high content of glucose and starch. It was observed that the fungus culture grown in the media with lower concentrations of the carbon and nitrogen sources exhibited a marked reduction in zervamicin accumulation. In order to optimize the production of the uniformly $^{13}C,^{15}N$-labelled Zrv-IIB, the culture media prepared from the $^{13}C,^{15}N$-labelled biomass and ^{13}C-EPS was used with about the same concentrations as used in the standard medium for *E. salmosynnemata* cultivation.

To prepare a complete culture medium from the $^{13}C,^{15}N$-labelled biomass, fresh cells of the methylotroph were autolysed. The $^{13}C,^{15}N$-labelled autolysed methylotrophic biomass and ^{13}C-EPS was dissolved with water up to the same concentrations of free amino acids and glucose as in the standard complete culture media containing bacto peptone, yeast extract and starch (Table 2). The EPS of *M. flagellatus* mainly consists of polyfructan [26] which is significantly different to the carbohydrate constituents (glucose and starch) of the standard

Table 1 Biosynthesis Parameters for the Strain *M. flagellatus* KT Culture grown on different Culture Media

Parameter	Standard (unlabelled) medium	^{15}N-labelled medium	$^{13}C,^{15}N$-labelled medium
Biomass yield[a] (g/l)	3.8	3.8	3.6
EPS yield[a] (g/l)	1.4	1.3	1.1
Conversion of ^{13}C-methanol into biomass[a] (%)	11.1	10.6	9.2
Conversion of ^{13}C-methanol into EPS[a] (%)	2.6	2.5	2.1

[a] Calculated per dry weight.

Table 2 Composition of Complete Culture Media used for *E. salmosynnemata* Culturing and Obtaining of Isotope-labelled Zrv-IIB

Complete media	Carbon and nitrogen source	Additional carbon source
Standard (unlabelled) medium [5]	Bacto peptone and yeast extract	Starch and glucose
^{15}N-labelled medium [11]	Autolysed ^{15}N-labelled methylotroph biomass	Starch and glucose
^{13}C,^{15}N-labelled medium	Autolysed ^{13}C,^{15}N-labelled methylotroph biomass	^{13}C-EPS, [U-^{13}C]-glucose[a]

[a] In some preliminary experiments.

culture medium used for *E. salmosynnemata* cultivation. That is why in our preliminary labelling experiments both [U-^{13}C]-glucose and ^{13}C-EPS were added to the culture medium containing the ^{13}C,^{15}N-labelled autolysed biomass of the methylotrophic bacteria. However, the addition of ^{13}C-labelled glucose did not result in a substantially increased yield of the target product, so in further experiments the ^{13}C-EPS was used as the only additional carbon source.

It was demonstrated that the methylotrophic bacteria *M. flagellatus* KT is unable to fix atmospheric nitrogen [19] and therefore can not dilute the ^{15}N-label during the growth of *E. salmosynnemata* in the $^{15}NH_4Cl$-containing culture medium. But addition of $CaCO_3$ as a pH stabilizer of the culture medium can substantially dilute the ^{13}C-label. Also it was tested whether the addition of $CaCO_3$ was critical for ensuring a high zervamicin yield during the growth of *E. salmosynnemata.* Cultivation of the fungus in the ^{13}C,^{15}N-labelled medium without $CaCO_3$ revealed a much lower pH shift compared with that in the standard unlabelled or ^{15}N-labelled growth media (Figure 2). At the same time the zervamicin yields did not increase dramatically in the presence of $CaCO_3$. To exclude a possible ^{13}C-label dilution, $CaCO_3$ was not added to the culture medium in our further experiments. Finally 140 ml of ^{13}C,^{15}N-labelled medium was prepared at a concentration based on the dry weights of the autolysed biomass and EPS.

Cultivation of the Fungus *E. salmosynnemata* on Totally ^{13}C,^{15}N-labelled Complete Culture Medium

To establish the optimum conditions, the time dependent zervamicin production was investigated (Figure 2). Every day, starting with day 5 of cultivation, 1.5 ml samples of the fungus culture were tested for the pH value and zervamicin concentration in the mycelium. In the course of the study it became apparent that the maximum yield of zervamicin was achieved on day 8 of cultivation, which corresponds well with the product accumulation in the standard glucose and starch containing medium [28]. In order to obtain uniformly ^{13}C,^{15}N-labelled zervamicin preparation, the fungus seeding culture was grown on the ^{13}C,^{15}N-labelled medium in order to exclude any isotope label dilution and then cultivation of the fungus was performed in two 350 ml flasks, each containing 70 ml of the complete ^{13}C,^{15}N-labelled medium. The wet weight of the collected fungus mycelium was 12 g (dry weight: 1 g).

Isolation of the ^{13}C,^{15}N-labelled Zervamicin IIB from the Fungus Culture

The obtained ^{13}C,^{15}N-labelled mycelium was triturated with methanol as described in the Materials and Methods section. The peptaibols were extracted from the fermentation broth with ethyl acetate (Figure 3). Reversed-phase high-performance liquid chromatography was used for the ^{13}C,^{15}N-labelled zervamicin isolation from methanol and ethyl acetate extracts and for the separation of the two zervamicin analogues IIA and IIB (Figure 4). The total amount of the purified uniformly ^{13}C,^{15}N-labelled Zrv-IIB was 14 mg. The molecular mass of the isolated peptaibol was determined by TOF-MALDI mass spectrometry. The m/z 1970 corresponds to the calculated mass of the sodium adduct $[M + Na]^+$ of the uniformly ^{13}C,^{15}N-labelled Zrv-IIB.

NMR Spectroscopic Characterization and Assignment of ^{13}C and ^{15}N Resonances

The complete assignment of the proton bearing ^{13}C and ^{15}N nuclei was made in a straightforward

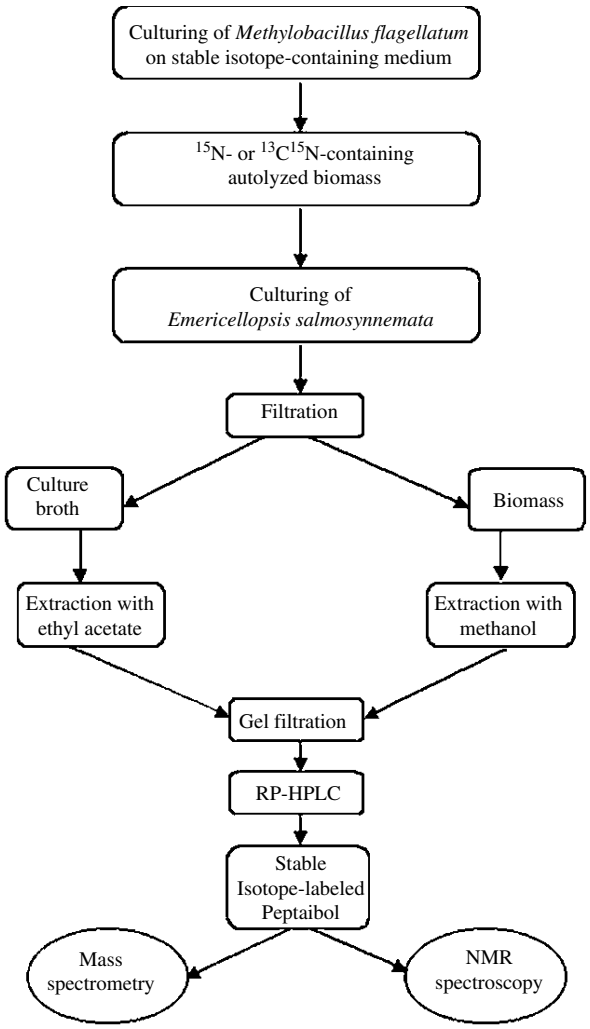

Figure 3 General scheme of procedure for the preparation of isotopically labelled zervamicins.

manner from ^{13}C- or ^{15}N-HSQC spectra, using the known proton assignment [7]. There were only few ambiguities caused by overlap in proton resonances. The assignment of the carbonyl ^{13}C resonances in residues followed by a non-proline residue was made in the 2D HNCO spectrum. Then this assignment was verified and extended to carbonyl signals of the proline preceding residues in the 2D HN(CA)CO spectrum, which gives complementary information. The absence of surplus resonances in these spectra and the correct mass of the obtained peptide revealed by mass-spectrometry, made us confident of the chemical purity and its correct sequence of residues.

The level of isotope incorporation was determined using 1D heteronuclear NMR spectroscopy. Each

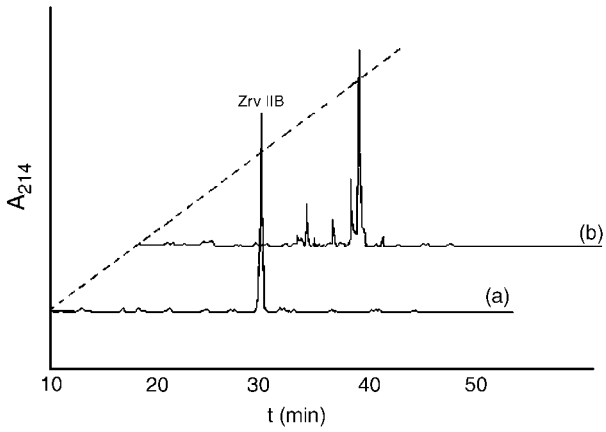

Figure 4 HPLC purification of Zrv-IIB (a) isolated from methanol extracts of *E. salmosynnemata* (b) biomass. Vydac column 208TP54 C8, 300E, 4.6 mm ID, 250 mm, 5 μm (Vydac, USA), a linear gradient of the tertiary mixtures methanol/acetonitrile/water A (60/5/35, by volume) and B (75/22.5/2.5, by volume) from 0 to 95% B for 45 min at the flow rate 0.5 ml/min.

peak in the amide region of the 1D-^{1}H spectrum, acquired without ^{15}N decoupling, represents a triplet resonance. The middle component arises from protons connected with the ^{14}N nucleus, and the two side bands (split by approximately 90 Hz) arise from protons connected with the ^{15}N nucleus. The intensity ratio of these peaks indicated that the level of ^{15}N incorporation was about 95%.

In a similar manner, the extent of ^{13}C incorporation was deduced from the carbonyl region of the 1D-^{13}C spectrum measured with ^{1}H and ^{15}N decoupling. The intensity ratio of the carbonyl signals connected with $^{13}C^{\alpha}$ and $^{12}C^{\alpha}$ implies that the extent of ^{13}C incorporation in the C^{α} positions was about 90%.

Application of the Chemical Shift Index Procedure to the N-terminal Fragment of Zrv-IIB and Stereochemical Assignment of the Aib Methyl Groups

It has been shown that the chemical shifts could give invaluable information about the assignment of secondary structure elements in proteins and peptides [29,30]. In order to deduce some information about structure elements the so-called Chemical Shift Index (CSI) method utilizes the secondary shifts of $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C{=}O$ resonances [29]. In helices, analysis of the ^{1}HN chemical shift is also very useful as it can identify the distortion of the

helix, helix-end effects and solvation effects. Certainly, such analysis should be quite cautious in the case of peptaibols containing many unusual residues. There is no experimental or statistical study of the random coil chemical shifts in these residues or of the behaviour of their chemical shifts in different types of secondary structures. However, in some cases, the arguments for helical conformation can be gained from other chemical shift sources in unusual residues, for example from the large difference in the chemical shifts of the two $^{13}C^{\beta}$ atoms in Aib residues [31]. Therefore, the CSI protocol was applied only to the common amino acids in the primary sequence of Zrv-IIB (Figure 5). The observed picture of CSI agrees well with the calculated structures [7], the *N*-terminus of the peptide is completely helical. At Leu^{8}, the residue that terminates the α-helical part of the molecule, the 'helical'-CSI values turn to 'coil'-CSI indicating the start of a helix bend. Analysis of the secondary HN chemical shifts $\Delta\delta^1$HN (δ^1HN$-\delta^1$HN$_{\text{random-coil}}$) also confirms this idea. All amide groups show negative secondary shifts that are typical for helices (data not shown).

There is another matter in which chemical shift analysis is an indispensable tool for peptaibol structure determination, namely the stereospecific assignment of the Aib methyl groups. Each Aib residue has two stereochemically different β-methyl groups. The signals of these groups are the only side chain resonances in the residue, and these groups are involved in the majority of the

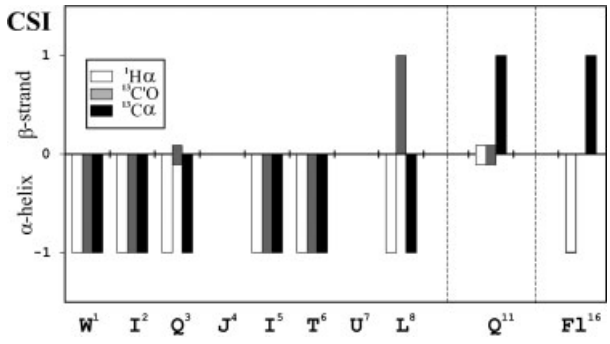

Figure 5 CSI of common amino-acid residues in Zrv-IIB. Black, grey and white boxes denote the indices for $^{1}H^{\alpha}$, $^{13}C{=}O$ and $^{13}C^{\alpha}$ nuclei, respectively. The signs of $^{13}C{=}O$ and $^{13}C^{\alpha}$ indices are reversed in order to give a more clear picture. The negative indices indicate helicity. CSI protocol was applied as in [29] and random coil $^{1}H^{\alpha}$, $^{13}C{=}O$ and $^{13}C^{\alpha}$ chemical shifts were taken from [30]. The amino acid sequence is given in the one letter code, where Aib, Iva and Phl are abbreviated as U, J and Fl, respectively, and other residues as usual.

structurally important NOE contacts. Therefore, the stereospecific assignment of the methyl groups plays a crucial role in the peptaibol structure determination, and in previous peptaibol studies the lack of such assignments leads to an absence of structural convergence [31,32]. One of the Aib residues methyl groups occupies the position of the H^{α} proton (pro-*S* methyl, $C^{\beta 2}$) and the other corresponds to C^{β} side chain group (pro-*R* methyl, $C^{\beta 1}$). In the helical conformation, the methyl groups are spatially non-equivalent and take different positions relative to intraresidual and sequential HN protons (that result in different NOE intensities between the methyl groups and the HN protons) and to the intraresidual carbonyl (that lead to different shielding of $^{13}C^{\beta}$ nuclei). This spatial non-equivalence underlies two strategies of the methyl assignment [31,33]. One should note that the results of these assignment approaches are completely dependent on helix handedness and should be reversed in the left-handed helix.

The group of L.R. Brown [34,35] have proposed another assignment strategy that utilizes the exhaustive grid-search before structure calculation. This method permits a simultaneous determination of the Aib methyl assignments and the helix handedness. In the course of the structural investigation of chrysospermin C, they additionally have proposed a simple rule for the methyl assignment. Namely, in the right-handed helix the $C^{\beta 1}$ and $C^{\beta 2}$ methyl groups have different ^{13}C chemical shifts. The $^{13}C^{\beta 1}$ group resonates in the down-field region (27–29 ppm) and the $^{13}C^{\beta 2}$ group resonates in the up-field region (24–26 ppm) of the ^{13}C spectrum, with the exception of Aib-Pro dipeptide fragments, in this case the $^{13}C^{\beta 1}$ resonates up-field relative to $^{13}C^{\beta\ 2}$.

Approximately the same computational algorithm for the Aib methyl assignment was utilized in our previous works, the stereospecific assignment was made simultaneously with the structure calculation [8]. Using the results of heteronuclear assignments, the hypothesis of L.R. Brown and co-workers can be verified. Zrv-IIB has four Aib residues and three of them precede a proline or hydroxyproline residue. All eight ^{13}C-methyl signals resonate in two distinct spectral regions (Figure 6). $C^{\beta 1}$ groups of Aib residues that precede Pro/Hyp and $C^{\beta 2}$ group of Aib^{7} (that precede Leu) resonate in the up-field (24–26 ppm) region of ^{13}C spectrum, while other methyl groups resonate in the down-field region (27–29 ppm). In this way, our results confirm the above-mentioned hypothesis: assignment of the Aib

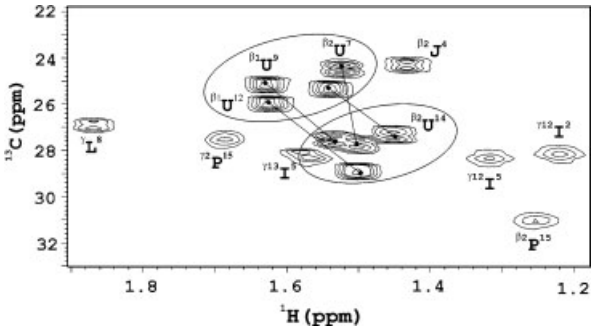

Figure 6 Aib methyl region of ^{13}C-HSQC spectrum of Zrv-IIB. Assignments for all resonances are shown; amino acids are abbreviated as in Figure 1. Methyl resonances of the same Aib residue are connected by straight line. It is clearly seen that Aib methyl signals resonate in two different spectral regions (circled), see text for details.

methyl groups in peptaibols can be fulfilled based on $^{13}C^{\beta}$ chemical shifts in case other data (for example NOE and J coupling constants for common residues) unambiguously indicate a right-handed helix.

CONCLUSIONS

A new biosynthetic approach to obtain uniformly ^{13}C,^{15}N-labelled peptaibol zervamicin IIB is described. The method is based upon cultivation of the antibiotic-producing fungus *Emericellopsis salmosynnemata* on the totally ^{13}C,^{15}N-labelled complete medium prepared from the autolysed biomass and exopolysaccharides of the obligate methylotrophic bacteria *Methylobacillus flagellatus*. The methylotroph cells were grown on ^{13}C,^{15}N-labelled medium containing ^{13}C-labelled methanol as the only carbon source and ^{15}N-labelled ammonium chloride. Preliminary spectroscopic characterization of ^{13}C,^{15}N-zervamicin IIB indicates a high purity and a high extent of isotope-label incorporation (above 90%) as well. Taking into account the obtained quantitative data, it can be concluded that the methylotrophic bacteria can be used for efficient uniform stable isotope labelling of peptide and protein preparations, which will be beneficial for NMR and other isotope sensitive spectroscopic techniques.

The measured chemical shift values argue for a high helicity of Zrv-IIB at the *N*-terminus in methanol solution (in excellent agreement with the previously determined structure [7]).

Moreover, the $^{13}C^{\beta}$ chemical shifts pattern in Aib residues points to a simple and convenient rule for the stereospecific assignment of the two different prochiral methyl groups of Aib, that might plays a key role in the future for other peptaibol structure determinations by NMR spectroscopy.

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REFERENCES

1. Henry G, Sykes B. Methods to study membrane protein structure in solution. *Methods Enzymol.* 1994; **239**: 515–535.
2. Damberg P, Jarvet J, Graslund A. Micellar systems as solvents in peptide and protein structure determination. *Methods Enzymol.* 2001; **339**: 271–285.
3. Opella S, Ma C, Marassi F. Nuclear magnetic resonance of membrane-associated peptides and proteins. *Methods Enzymol.* 2001; **339**: 285–313.
4. Fu R, Cotten M, Cross T. Inter- and intramolecular distance measurements by solid-state MAS NMR: determination of gramicidin A channel dimer structure in hydrated phospholipid bilayers. *J. Biomol. NMR* 2000; **16**: 261–268.
5. Argoudelis A, Dietz A, Johnson L. Antibiotics zervamicins I and II and process for preparing the same. 1975; *Patent USA* 3.907.990.
6. Jen W, Jones G, Brewer D, Parkinson V, Taylor A. The antibacterial activity of alamethicins and zervamicins. *J. Appl. Bacteriol.* 1987; **63**: 293–298.
7. Balashova T, Shenkarev Z, Tagaev A, Ovchinnikova T, Raap J, Arseniev A. NMR structure of the channel-former zervamicin IIB in isotropic solvents. *FEBS Lett.* 2000; **466**: 333–336.

8. Shenkarev Z, Balashova T, Efremov R, Yakimenko Z, Ovchinnikova T, Raap J, Arseniev A. Spatial structure of zervamicin IIB bound to DPC micelles: implications for voltage-gating. *Biophys. J.* 2002; **82**: 762–771.
9. Sansom M, Balaram P, Karle I. Ion channel formation by zervamicin-IIB. A molecular modelling study. *Eur. Biophys. J.* 1993; **21**: 369–383.
10. Agarwalla S, Mellor I, Sansom M, Karle I, Flippen-Anderson J, Uma K, Krishna K, Sukumar M, Balaram P. Zervamicins, a structurally characterized peptide models for membrane ion channels. *Biochem. Biophys. Res. Commun.* 1992; **186**: 8–15.
11. Bechinger B, Skladnev D, Ogrel A, Li X, Rogozhkina E, Ovchinnikova T, O'Neil J, Raap J. ^{15}N- and ^{31}P-solid-state NMR investigations on the orientation of zervamicin II and alamethicin in phosphatidylcholine membranes. *Biochemistry* 2001; **40**: 9428–9437.
12. Korzhnev D, Bocharov E, Zhuravlyova A, Orekhov V, Ovchinnikova T, Billeter M, Arseniev A. Backbone dynamics of the channel-forming antibiotic zervamicin IIB studied by ^{15}N NMR relaxation. *FEBS Lett.* 2001; **495**: 52–55.
13. Muchmore D, McIntosh L, Russell C, Anderson D, Dahlquist F. Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. *Methods Enzymol.* 1989; **177**: 44–73.
14. Yee A, Marat K, O'Neil J. The interactions with solvent, heat stability, and ^{13}C-labelling of alamethicin, an ion-channel-forming peptide. *Eur. J. Biochem.* 1997; **243**: 283–291.
15. Karnaukhova E, Reshetova O, Semenov S, Skladnev D, Tsygankov Y. ^{2}H- and ^{13}C-labelled amino acids generated by obligate methylotrophs. Biosynthesis and MS monitoring. *Amino Acids* 1994; **6**: 165–176.
16. Mosin O, Skladnev D, Shvets V. Biosynthesis of ^{2}H-labelled phenylalanine by a new mutant of RuMP facultative methylotroph *Brevibacterium methylicum*. *Biosci. Biotechnol. Bioengin.* 1998; **62**: 225–229.
17. Kazarinova L, Korol'kova N, Mironov A, Mosin O, Skladnev D, Yurkevich A. Method for obtaining of high deuterated nucleosides and nucleotides. 1995; *Patent* RF 95118778.
18. Skladnev D, Smirnova T, Nikolaenko M, Azizbekian R, Lapshina M, Strahova E. Obtaining of high deuterated crystal delta-endotoxin of *Bacillus thuringiensis* on complete growth medium, prepared from autolysed high deuterated biomass of methylotrophic bacteria. *Biotechnology (Moscow)* 2000; **1**: 23–29.
19. Kletsova L, Chibisova E, Tsygankov Y. Mutants of the obligate methylotroph *Methylobacillus flagellatum* KT defective in genes of the ribulose monophosphate cycle of formaldehyde fixation. *Arch. Microbiol.* 1988; **149**: 441–446.
20. Krylov I, Krasnoshtanova A, Manakov M. Acid hydrolysis of biomass proteins of industrial microorganisms. *Biotechnology (Moscow)* 1996; **3**: 43–55.
21. Kochetkov N. *Methods in Carbohydrate Chemistry (in Russian)*. Mir: Moscow, 1967; 116.
22. Kay L, Keifer P, Saarinen T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity *J. Am. Chem. Soc.* 1992; **114**: 10 663–10 665.
23. Grzesiek S, Bax A. Improved 3D triple-resonance NMR techniques applied to a 31-kDa protein. *J. Magn. Reson.* 1992; **96**: 432–440.
24. Clubb R, Thanabal V, Wagner G. A constant-time 3-dimensional triple-resonance pulse scheme to correlate intraresidue ^{1}H^{N}, ^{15}N, and ^{13}C' chemical-shifts in ^{15}N-^{13}C'-labeled proteins. *J. Magn. Reson.* 1992; **97**: 213–217.
25. Wishart D, Bigam C, Yao J, Abildgaard F, Dyson H, Oldfield E, Markley J, Sykes B. ^{1}H, ^{13}C and ^{15}N chemical-shift referencing in biomolecular NMR. *J. Biomol. NMR* 1995; **6**: 135–140.
26. Ponomareva E, Antonova S, Skladnev D. Total stable labelled carbohydrates synthesized by methylotrophic bacteria. *Glycoconjugate J.* 2001; **18**: 122.
27. Egorova-Zachernuk T, Shvets V, Verslius K, Heerma W, Lugtenburg J, Raap J. Preparation of site-specific isotopically-labelled zervamicin, the peptaibols, produced by *Emericellopsis salmosynnemata*. *J. Pept. Sci.* 1996; **12**: 956–968.
28. Rogozhkina E, Lapshina M, Eremin S, Shvets V, Skladnev D. Investigation of the peptide antibiotic zervamicin IIB mycelial fungus-producer *Emericellopsis salmosynnemata* cultivation processes. Development of the HPLC express-method. *Chem. Pharm. J. (Moscow)* 2000; **34**: 50–53.
29. Wishart D, Sykes B. Chemical shifts as a tool for structure determination. *Methods Enzymol.* 1994; **239**: 363–392.
30. Wishart D, Case D. Use of chemical shifts in macromolecular structure determination. *Methods Enzymol.* 2001; **338**: 3–34.
31. Yee A, Babiuk R, O'Neil J. The conformation of alamethicin in methanol by multinuclear NMR spectroscopy and distance geometry simulated annealing. *Biopolymers* 1995; **36**: 781–792.
32. Franklin J, Ellena J, Jayasinghe S, Kelsh L, Cafiso D. Structure of micelle-associated alamethicin from ^{1}H NMR. Evidence for conformational heterogeneity in a voltage-gated peptide. *Biochemistry* 1994; **33**: 4036–4045.
33. Esposito G, Carver J, Boyd J, Campbell I. High-resolution ^{1}H NMR study of the solution structure of alamethicin. *Biochemistry* 1987; **26**: 1043–1050.
34. Anders R, Wenschuh H, Soskic V, Fischer-Fruhholz S, Ohlenschlager O, Dornberger K, Brown L. A solution NMR study of the selectively ^{13}C, ^{15}N-labeled peptaibol chrysospermin C in methanol. *J. Pept. Res.* 1998; **52**: 34–44.
35. Anders R, Ohlenschlager O, Soskic V, Wenschuh H, Heise B, Brown L. The NMR solution structure of the ion channel peptaibol chrysospermin C bound to dodecylphosphocholine micelles. *Eur. J. Biochem.* 2000; **267**: 1784–1794.

