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Total Synthesis of Zervamicin IIB and its Deuterium-labelled Analogues

ALEXEI OGREL, WIM BLOEMHOFF, JOHAN LUGTENBURG and JAN RAAP

Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands

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Abstract: For the first time the total synthesis of the peptaibol antibiotic zervamicin IIB is described. Synthesis of this peptaibol was achieved by the Fmoc/*tert*-butyl strategy in solution using a fragment condensation approach. Three fragments of zervamicin IIB were obtained by stepwise elongation with Fmoc amino acids using BOP as a coupling reagent. For the introduction of the highly sterically hindered α-aminoisobutyric acid residues BOP/DMAP activation was applied. The Fmoc group was removed by reaction with 0.1 M NaOH in dioxane/methanol/water (30/9/1, v/v/v). Peptide fragments were coupled by means of a new coupling reagent, CF_3-PyBOP. Using the strategy developed, zervamicin IIB and two analogues specifically deuterium-labelled at different positions of the glutamine-11 residue have been synthesized in 40% overall yield based on the isotopically labelled amino acid and with 98 ± 2% of isotope enrichment. FAB mass spectroscopy, 600 MHz ^{1}H-NMR spectroscopy and high-performance liquid chromatography provided convincing evidence that the synthetic products, zervamicin IIB and its deuterium-labelled analogues, fully correspond to the naturally occurring zervamicin IIB. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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(No. of Figures: 6. No. of Tables: 1. Number of Refs: 40.)

Keywords: peptaibol; antibiotic; peptide synthesis; NMR spectroscopy; mass spectrometry; isotope labelling

INTRODUCTION

Zervamicins are members of a family of 16-residue peptaibols (peptides containing both Aib residues and a C-terminal amino alcohol [1]) which are isolated from the fungus *Emericellopsis salmosynnemata* [2]. Peptaibols have common structural elements. All of them contain α,α-dialkylated amino acid residues (e.g. Aib and Iva), an acylated N-terminal amino acid residue and a 1,2-amino alcohol residue at the C-terminus [3]. These peptides belong to the class of antibiotics that form voltage-gated ion channels in phospholipid membranes [4,5]. It is of interest that their toxicity appears to be due to their membrane interactions [4, 6, 7].

A rational design of novel peptaibol antibiotics is only possible if a profound knowledge of the structure–function relationship of the wild-type molecules is available. In order to obtain structural and functional information at the atomic level of the zervamicin bound in the membrane, we apply a method developed in our group in collaboration with others [8–15] which consists of three steps: (1). synthesis of amino acids specifically labelled at predesigned positions with stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{17}O and ^{18}O); (2). incorporation of these labelled

Address for correspondence: Dr J. Raap, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands. Tel. (31)71-5274419; fax (31)71-5274537.

Abbreviations: Aib, α-aminoisobutyric acid; BOP, (benzotriazol-*l*-yloxy)-tris(dimethylamino)-phosphonium hexafluorophosphate; CF_3-PyBOP(6-trifluoromethyl)-benzotriazole-*l*-yl-oxy)-tris(pyrrolidino)-phosphonium hexafluoro phosphate; DIPEA, diisopropylethylamine; DMAP, *4*-dimethylaminopyridine; Fmoc-OSu, *9*-fluorenylmethyl *N*-succinimidyl carbonate; Hyp, *4-trans*-hydroxyproline; Iva, isovaline; Phl, phenylalaninol; TDM, (*N,N,N′,N′-tetramethyl-4,4′*)-diaminodiphenylmethane; TMS, tetramethylsilane; Xxx, arbitrary amino acid.

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amino acids into the peptide or protein of interest; (3). studying of the site-specific isotopically labelled peptide or protein with non-invasive, non-destructive, isotope-sensitive techniques, such as solid-state NMR, FT-IR and resonance Raman spectroscopy. Using this strategy, information can be obtained that is otherwise impossible or very difficult to obtain. The two big advantages of this strategy are that, despite the large size of the systems, atomic resolution can be achieved and that undisturbed, functionally intact systems can be studied.

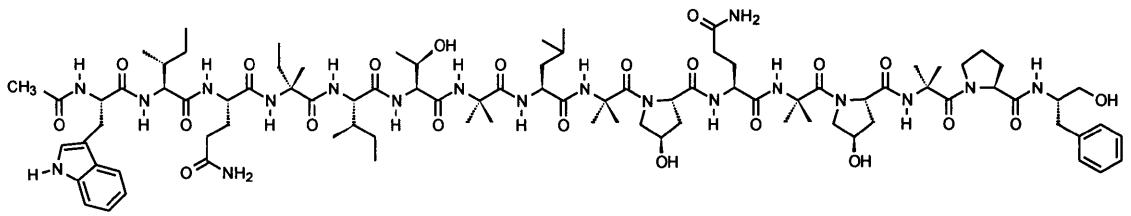
Up to now, our group has elaborated synthetic schemes for preparation of isotopically labelled L-Tyr [16], L-Lys [17], L-Trp [18], L-Pro [19], L-His [20], L-Ser [21], L-Thr [21], L-Glu [22, 23] and L-Gln [24].

L-Amino acids specifically labelled with stable isotopes can be incorporated into a peptaibol either biochemically or by means of total chemical synthesis. The biosynthetic approach, which is based on growing microorganisms on media supplemented with isotopically enriched amino acids, can only be used effectively to incorporate those labelled amino acid residues that are end products of the primary biosynthetic pathway and the biosynthesis of which is regulated by an efficient end-product inhibition mechanism [25]. However, for specific isotope labelling of amino acids that have a central role in the metabolic pool (e.g. Gln) this approach is not suitable. Therefore, only total chemical synthesis allows one to label any position or any combination of positions of the peptide. In the present work we describe the first synthesis of zervamicin IIB **1**$^{\mathbf{I}}$ and of its isotopically labelled analogues [2-^{2}H-Gln^{11}]-zervamicin IIB **1**$^{\mathbf{II}}$ and [4,4-$^{2}H_2$-Gln^{11}]-zervamicin IIB **1**$^{\mathbf{III}}$. The labelling of the Gln-11 residue in the zervamicin molecule is of primary interest because in the X-ray structure its side chain is folded in an unusual fashion [6]. It is supposed that such folding has a direct relationship to the gating mechanism for the cation transport through the ion channel [4, 6, 7]. Solid-state deuterium NMR (^{2}H-NMR) spectroscopy can provide valuable information about the zervamicin molecules in phospholipid membranes. Furthermore, the orientation of the site-directed ^{2}H-labelled zervamicin IIB in the membrane can be determined by ^{2}H-NMR [26].

Strategy

Zervamicin IIB is a linear, amphiphatic peptide which contains a high proportion of α,α-dialkylated amino acids, such as Aib and Iva [3]. The molecule contains no charged amino acids; the N-terminus is acetylated, whereas the C-terminus is occupied by an 1,2-amino alcohol, L-Phl (Figure 1). The peptaibol contains three acid-labile peptide bonds, Aib^{9}-Hyp^{10}, Aib^{12}-Hyp^{13} and Aib^{14}-Pro^{15}. Consequently, synthesis of zervamicin IIB should be performed using Fmoc strategy rather than Boc strategy which requires frequent use of acid for repetitive N^{α}-Boc deprotections. In contrast, the N^{α}-Fmoc protective group is removed under basic conditions and acid treatment is required only once during the final removal of acid-labile side-chain protective groups (e.g. Bu^{t}, Boc and Trt). Nevertheless, possible peptide bond cleavages during the final acidolytic removal of protective groups requires careful control of this step.

Furthermore, zervamicin IIB contains five highly sterically hindered α,α-dialkylated amino acids, incorporation of which into peptides using the well-established coupling reagents was found to be extremely difficult [27–29]. Therefore, new more powerful reagents have to be considered for their incorporation.



Ac Trp^{1} Ile Gln Iva Ile Thr^{6} Aib Leu Aib Hyp Gln^{11} Aib Hyp Aib Pro Phl^{16}

Figure 1 The primary structure of zervamicin IIB.

For the synthesis of zervamicin IIB $\mathbf{1}^{I}$ and its isotopically labelled analogues $\mathbf{1}^{II}$ and $\mathbf{1}^{III}$ a solution synthesis using a fragment condensation approach proved to give the best results. The strategy for the synthesis is based on condensation between the fragments **2a** and $\mathbf{3a}^{I-III}$ (Scheme 1). Compound **2a** was the product of a condensation reaction of fragments **4a** and **5a**. The heptapeptaibols $\mathbf{3a}^{I-III}$ have been prepared after couplings of unprotected L-Phl onto the hexapeptides $\mathbf{6a}^{I-III}$, respectively, followed by Fmoc-group deprotection (Scheme 2).

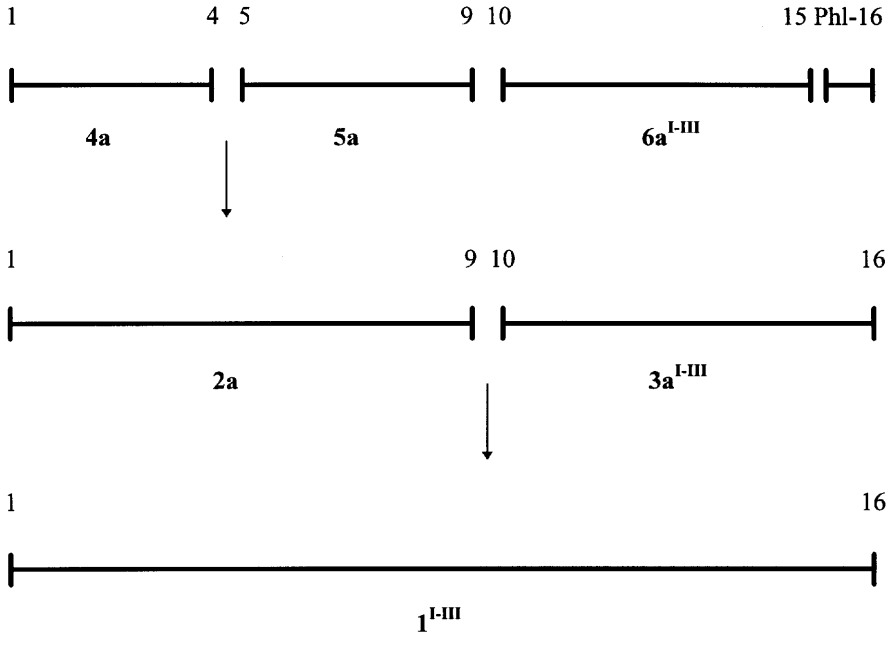
In order to avoid any risk of racemization, for all segment condensations either fragments with the α,α-dialkylated amino acids, Iva (fragment **4a**) and Aib (fragment **2a**), or with Pro (fragments $\mathbf{6a}^{I-III}$) as the C-terminal amino acid were used. It should be also noted that in our synthetic strategy the introduction of the most expensive amino acids, the specifically isotopically labelled L-Gln, is postponed until the later stages of the synthesis.

The protected peptide fragments, tetrapeptide **4**, pentapeptide **5** and hexapeptides $\mathbf{6}^{I-III}$ were synthesized by stepwise elongation from the C-terminus with Fmoc amino acids.

MATERIALS AND METHODS

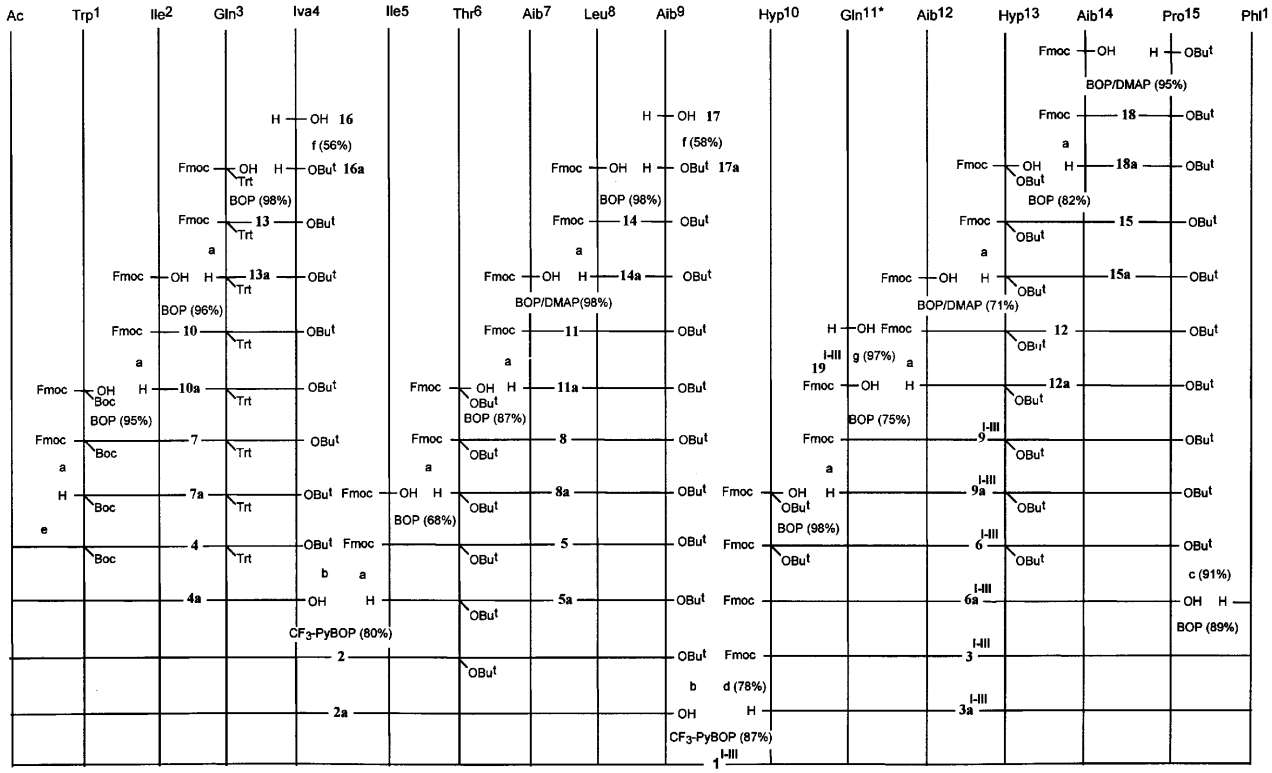
Fmoc-amino acids were purchased from NovaBiochem (Switzerland). R-Iva was obtained as a gift from DSM (The Netherlands). Native, HPLC pure, zervamicin IIB was obtained as a gift from Indian Institute of Science, Bangalore, India. BOP reagent was purchased from Richelieu Biotechnologies (Canada). CF_3-PyBOP was obtained according to [27]. DMAP was obtained from Sigma. DMF was analytical grade and stored over molecular sieves (4 Å). DIPEA was distilled from KOH. AcOBu^t and Bu^{t}OH were distilled from $MgSO_4$.

Reactions were run at room temperature. The pH's were measured using wet pH-indicator strips. TLC analysis was performed on DC-Alufolien, Kieselgel 60 F_{254} (Merck) employing the following solvent systems: A (CH_2Cl_2/MeOH, 9/1, v/v), B (CH_2Cl_2/MeOH/AcOH, 20/5/1, v/v/v), C (CH_2Cl_2/diethyl ether, 10/1, v/v), D (diethyl ether), E (CH_2Cl_2/MeOH, 20/1, v/v), F (CH_2Cl_2/MeOH, 6/1, v/v) and G (CH_2Cl_2/MeOH, 2/1, v/v). Compounds were visualized by UV (254 nm), ninhydrin (0.2% in ethanol) and TDM reagent [30]. Solvents were



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]

Scheme 1 General strategy for the synthesis of zervamicin IIB: top peptides containing unlabelled Gln^{11}; middle, peptides containing [2-^{2}H]-Gln^{11}; bottom, peptides containing [4,4-$^2H_2$]-Gln^{11}.



Scheme 2 Synthetic scheme of zervamicin IIB: (a) $AcOBu^t/Bu^tOH/H_2SO_4$ (15/15/2, v/v/v); (b) 0.1 M NaOH in dioxane/MeOH/H_2O (30/9/1, v/v/v); (c) Ac_2O/CH_2Cl_2 (1/10, v/v); (d) TFA/H_2O/triisopropylsilane (90/5/5, v/v/v), (g) Fmoc-OSu/Et_3N. (I) peptides containing unlabelled Gln^{11}; (II) peptides containing [2-^{2}H]-Gln^{11}; (III) peptides containing [4,4-$^2H_2$]-Gln^{11}.

evaporated under reduced pressure at 40°C. Silica gel column chromatography was performed on Kieselgel 60, 230–400 mesh, ASTM, Merck. Gel filtration was performed using an column (Sephadex LH-20, 800 × 20 mm), a UV detector operating at 226 nm and a fraction collector. Elution was effected using MeOH (flow rate 3 ml/min). Analytical and semi-preparative HPLC was performed using a Jasco HPLC apparatus consisting of an analytical reversed-phase (C_{18}) column (Spherisorb ODS-2, 250 × 4 mm, 5 μm), and UV-detector operating at 214 nm. Elution was effected using an appropriate gradient from 0.1% TFA/H_2O to 0.1% TFA/CH_3CN or employing a gradient of 50% MeOH to 100% MeOH over 40 min (flow rate 1 ml/min).

Melting points were measured on a Büchi melting-point apparatus and are uncorrected. Optical rotations were determined with a PROPOL automatic polarimeter at 20°C in a 0.5 dm cell.

Electrospray mass spectra (ES-MS) were recorded on a Finnigan MAT SSQ-710 equipped with an electrospray interface. The ES-MS spectra were recorded from the neat peptides, without derivatization. Fast atom bombardment mass spectrometry (FAB-MS) was performed as described in [25].

^{1}H-NMR spectra were recorded on a Jeol JNM-FX 200 and a Bruker DMX-600 spectrometer. Chemical shifts (δ) are given in p.p.m. relative to the signal of tetramethylsilane (TMS: δ 0.00) as an internal standard. ^{13}C-NMR spectra were recorded using a Jeol JNM-FX 200 at 50.1 MHz, or a Bruker DMX-600 at 150.9 MHz. Chemical shifts (δ) are given in p.p.m. relative to the signal for internal chloroform (δ 77.0 p.p.m.) or methanol (δ 49.0 p.p.m.). ^{13}C and ^{1}H-NMR spectra have been recorded for the various compounds, confirming their structure and homogeneity.

Zervamicin IIB 1^I

The nonapeptide **2a** (1.86 mg, 1.7 μmol) was coupled with heptapeptaibol **3a**^{I} (2.38 mg, 3.07 μmol) by means of CF_3-PyBOP (1.5 mg, 2.5 μmol) in DMF (0.02 ml). The pH was adjusted

to 9–10 by the addition of DIPEA. After standing overnight, 2 ml of water was added to the reaction mixture followed by evaporation to dryness. The residue was resuspended in water and the solvent removed again. The product was isolated by gel filtration on Sephadex LH-20 (eluent: MeOH) and purified by semi-preparative RP-HPLC to result pure **1^{I}**. Yield 2.7 mg (87%). FAB-MS (m/z): 1861.076 $(M+Na)^+$, (calculated 1861.034).

[2-^{2}H-Gln^{11}]-Zervamicin IIB 1^{II}

1^{II} was obtained and purified according to the procedure described for the preparation of **1^{I}** using the nonapeptide **2a** (6.1 mg, 5.63 μmol), heptapeptaibol **3a^{II}** (21 mg, 27.2 μmol) and, CF_3-PyBOP (16 mg, 27.2 μmol). Yield 9.0 mg (86%). FAB-MS (m/z): 1862.036 $(M+Na)^+$, (calculated 1862.041).

[4,4-^{2}H$_2$-Gln^{11}]-Zervamicin IIB 1^{III}

1^{III} was obtained and purified according to the procedure described for the preparation of **1^{I}** using the nonapeptide **2a** (1.86 mg, 1.7 μmol), heptapeptaibol **3a^{III}** (2.38 mg, 3.07 μmol), CF_3-PyBOP (1.5 mg, 2.5 μmol) in DMF (0.02 ml). Yield 2.7 mg (87%). FAB-MS (m/z): 1863.128 $(M+Na)^+$, (calculated 1863.047).

Ac-Trp-Ile-Gln-D-Iva-Ile-Thr(Bu^t)-Aib-Leu-Aib-OBu^t (2)

The tetrapeptide **4a** (26 mg, 0.044 mmol) was coupled with pentapeptide **5a** (29 mg, 0.044 mmol) by means of CF_3-PyBOP (37 mg, 0.062 mmol) in DMF (0.50 ml). The pH was adjusted to 9–10 by addition of DIPEA. After standing overnight, 2 ml of water was added to the reaction mixture followed by evaporation to dryness. The residue was resuspended in water and the solvent removed again. The product **2** was isolated by gel filtration on Sephadex LH-20 (eluent: MeOH) and purified by semi-preparative RP-HPLC. Yield 35 mg (80%). R_F 0.53 (system F); $[\alpha]_D^{22} = -15.0$ ($c = 0.04$, MeOH); ES-MS (m/z): 1197 $(M+H)^+$, (calculated 1197), 1219 $(M+Na)^+$, (calculated 1219), 1235 $(M+K)^+$, (calculated 1235).

Ac-Trp-Ile-Gln-D-Iva-Ile-Thr-Aib-Leu-Aib-OH (2a)

Some 0.40 ml of a mixture consisting of trifluoroacetic acid, triisopropylsilane and water, (90/5/5, v/v/v) was added to the nonapeptide Ac-Trp-Ile-Gln-D-Iva-Thr(Bu^t)-Aib-Leu-Aib-OBu^t **2** (12 mg, 0.010 mmol). After stirring for 30 min, the solution was evaporated to dryness under reduce pressure. The product **2a** was isolated by gel filtration on Sephadex LH-20 (eluent: MeOH) and purified by semi-preparative RP-HPLC. Yield: 8 mg (74%). M.p. = 140°C; R_F 0.59 (system G); $[\alpha]_D^{22} = -15.0$ ($c = 0.08$, MeOH); ES-MS (m/z): 1085 $(M+H)^+$, (calculated 1085), 1107 $(M-H+Na)^+$, (calculated 1107), 1129 $(M-2H+2Na)^+$, (calculated 1129), 1084 $(M)^-$, (calculated 1084).

Fmoc-Hyp-Gln-Aib-Hyp-Aib-Pro-Phl (3^{I})

To **6a^{I}** (45 mg, 0.052 mmol) dissolved in DMF (1.5 ml), Phl (7.8 mg, 0.052 mmol) and BOP (23 mg, 0.052 mmol) were added. The pH was adjusted to 9–10 by addition of DIPEA. After stirring for 30 min the reaction mixture was diluted with methanol and the product **3^{I}** was purified by means of gel filtration on Sephadex LH-20 (eluent: MeOH). Yield: 46 mg (89%). M.p. = 128°C; R_F 0.59 (system F); $[\alpha]_D^{22} = -45.2$ ($c = 1$, CH_2Cl_2).

Fmoc-HYp-[2-^{2}H-Gln]-Aib-Hyp-Aib-Pro-Phl (3^{II})

Compound **3^{II}** was prepared according to the same procedure as described for the synthesis of heptapeptaibol **3^{I}** using **6a^{II}** (25 mg, 0.029 mmol), Phl (13 mg, 0.087 mmol) and BOP (13 mg, 0.029 mmol). Yield: 25 mg (85%). M.p., R_F and $[\alpha]_D^{22}$ parameters are identical to those for **3^{I}**.

Fmoc-Hyp-[4,4-^{2}H$_2$-Gln]-Aib-Hyp-Aib-Pro-Phl (3^{III})

Compound **3^{III}** was prepared according to the same procedure as described for the synthesis of heptapeptaibol **3^{I}** using **6a^{III}** (45 mg, 0.052 mmol), Phl (7.8 mg, 0.052 mmol) and BOP (23 mg, 0.052 mmol). Yield: 46 mg (89%). M.p., R_F and $[\alpha]_D^{22}$ parameters are identical to those for **3^{I}**.

H-Hyp-Gln-Aib-Hyp-Aib-Pro-Phl (3a^{I})

The heptapeptaibol **3^{I}** (46 mg, 0.05 mmol) was deprotected by means of 40% diethylamine in dimethylformamide (10 ml) for 10 min). The reaction mixture was concentrated and the residue obtained was purified by gel filtration on Sephadex LH-20 (eluent: MeOH) followed by RP-HPLC to afford pure **3a^{I}**. Yield: 28 mg (78%). M.p. = 155°C; R_F 0.73 (system G); $[\alpha]_D^{22} = -51.2$ ($c = 0.06$, MeOH); FT-1R:

1649 cm^{-1} (amide I), 1544 cm^{-1} (amide II); ES-MS, m/z: 775.0 [M + H] (calculated 774.9).

H-Hyp-[2-^{2}H-Gln]-Aib-Hyp-Aib-Pro-Phl (3a^{II})

The heptapeptaibol **3**^{II} (25 mg, 0.025 mmol) was deprotected by 40% diethylamine in dimethylformamide (10 ml) for 10 min according to the procedure described for the preparation of heptapeptaibol **3a**^{I}. Yield: 18 mg (98%). M.p., R_F and $[\alpha]_D^{22}$, FT-IR parameters are identical to those for **3a**^{I}. ES-MS, m/z: 775.9 [M + H] (calculated 775.9).

H-Hyp-[4,4-$^2H_2$-Gln]-Aib-Hyp-Aib-Pro-Phl (3a^{III})

The heptapeptaibol **3**^{III} (46 mg, 0.05 mmol) was deprotected by means of 40% diethylamine in dimethylformamide (10 ml) for 10 min according to the procedure described for the preparation of heptapeptaibol **3a**^{I}. Yield: 28 mg (78%). M.p., R_F, $[\alpha]_D^{22}$ and FT-IR parameters are identical to those for **3a**^{I}. ES-MS, m/z: 777.1 [M + H] (calculated 776.9).

Ac-Trp(Boc)-Ile-Gln(Trt)-D-Iva-OBu^t (4)

Fmoc group from **7** (0.38 g, 0.32 mmol) was removed by reaction with 0.1 M NaOH in dioxane/methanol/water (30/9/1, v/v/v). After 2 min, the reaction mixture was neutralized with 1 M HCl to pH 6–7 and evaporated to dryness. To the residue, containing **7a**, acetic anhydride (1 ml, 10.6 mmol) in dichloromethane (10 ml) was added. The pH of the reaction mixture was adjusted and maintained at 9–10 by addition of DIPEA. After stirring for 15 min, the mixture was diluted with ethyl acetate, washed with 10% $NaHCO_3$, 5% NaH_2PO_4 (pH 4.5), saturated NaCl solution and dried ($MgSO_4$). After evaporation of the solvent, the product was purified on a silica gel column (eluent: dichloromethane/methanol, 30/1, v/v) to afford 0.30 g (94%) of pure **4**. R_F 0.30 (system E).

Ac-Trp-Ile-Gln-D-Iva-OH (4a)

Some 4 ml of a mixture consisting of trifluoroacetic acid, triisopropylsilane and water (90/5/5, v/v/v) was added to **4** (65 mg, 0.066 mmol). After stirring for 30 min the solution was evaporated to dryness under reduced pressure. The product **4a** was isolated by gel filtration on Sephadex LH-20 (eluent: MeOH) and purified by semi-preparative RP-HPLC. Yield: 32 mg (83%). M.p. = 178°C; R_F 0.85 (system B); $[\alpha]_D^{22} = -40.0$ ($c = 0.2$, MeOH); ES-MS (m/z): 585.3 $(M - H)^-$, (calculated 585.3); 621.5 $(M + Cl)^-$, (calculated 621.8).

Fmoc-Ile-Thr(Bu^t)-Aib-Leu-Aib-OBu^t (5)

Removal of the Fmoc group from **8** (0.40 g, 0.54 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. To the suspension of the residue containing **8a** in 6 ml DMF, were added Fmoc-Ile-OH (0.23 g, 0.65 mmol), BOP (0.29 g, 0.65 mmol) and DIPEA (0.31 ml, 1.84 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate, washed with 10% $NaHCO_3$, 5% NaH_2PO_4 (pH 4.5) and, saturated NaCl solution, and was dried ($MgSO_4$). After evaporation of the solvent, the product was purified by gel filtration on Sephadex LH-20 (eluent: MeOH) afforded HPLC pure **5**. Yield: 0.33 g (68%). M.p. = 162°C; R_F 0.40 (system E); $[\alpha]_D^{22} = -17.0$ ($c = 2$, CH_2Cl_2).

HCl · H-Ile-Thr(Bu^t)-Aib-Leu-Aib-OBu^t (5a)

Removal of the Fmoc group from **5** (77 mg, 0.091 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. Purification by gel filtration on Sephadex LH-20 (eluent: MeOH) afforded HPLC pure **5a**. Yield: 64 mg (94%). M.p. = 168°C; R_F 0.50 (system F); $[\alpha]_D^{22} = -3.5$ ($c = 0.4$, MeOH); ES-MS (m/z): 628.0 $(M + H)^+$, (calculated 627.9).

Fmoc-Hyp(Bu^t)-Gln-aib-Hyp(Bu^t)-Aib-Pro-OBu^t (6^I)

Removal of the Fmoc group from **9**^{I} (0.22 g, 0.24 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. To the suspension of the residue containing **9a**^{I} in 3 ml DMF, were added Fmoc-Hyp(Bu^t)-OH (0.34 g, 0.72 mmol), BOP (0.32 g, 0.72 mmol) and DIPEA (0.28 ml, 1.68 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate, washed with 10% $NaHCO_3$, 5% NaH_2PO_4 (pH 4.5), saturated NaCl solution and dried ($MgSO_4$). After evaporation of the solvent, the product was purified on a silica gel column (eluent: dichloromethane/methanol, 20/1, v/v) followed by gel filtration on Sephadex LH-20 (eluent: MeOH). Yield: 0.23 g (98%). M.p. = 161°C; R_F 0.43 (system A); $[\alpha]_D^{22} = -38.2$ ($c = 1$, CH_2Cl_2).

Fmoc-Hyp(Bu^t)-[2-^{2}H-Gln]-Aib-Hyp(Bu^t)-Aib-Pro-OBu^t (6^{II})

The hexapeptide **6^{II}** was prepared according to the same procedure as described for the synthesis of hexapeptide **6^I** using **9^{II}** (53 mg, 0.052 mmol). The coupling was performed with Fmoc-Hyp(Bu^t)-OH (64 mg, 0.156 mmol) by means of BOP (69 mg, 0.156 mmol) and DIPEA (0.062 ml, 0.364 mmol). Purification on a silica gel column (eluent: dichloromethane/methanol, 30/3, v/v) yielded 51 mg (98%) of pure **6^{II}**. M.p., R_F and $[\alpha]_D^{22}$ parameters are identical to those for **6^I**.

Fmoc-Hyp(Bu^t)-[4,4-^{2}H$_2$-Gln]-Aib-Hyp(Bu^t)-Aib-Pro-OBu^t (6^{III})

The hexapeptide **6^{III}** was prepared according to the same procedure as described for the synthesis of hexapeptide **6^I** using **9^{III}** (0.11 g, 0.12 mmol). The coupling was performed with Fmoc-Hyp(Bu^t)-OH (0.17 g, 0.36 mmol) by means of BOP (0.16 g, 0.36 mmol) and DIPEA (0.14 ml, 0.84 mmol). Yield: 0.12 g (98%). M.p., R_F and $[\alpha]_D^{22}$ parameters are identical to those for **6^I**.

Fmoc-Hyp-Gln-Aib-Hyp-Aib-Pro-OH (6a^I)

The hexapeptide **6^I** (72 mg, 0.07 mmol) was deprotected by means of 50% TFA in dichloromethane (6 ml) for 30 min. After the addition of heptane (5 ml) the reaction mixture was evaporated to dryness. The residue was resuspended in heptane and the solvents were evaporated. The product **6a^I** was isolated and purified by gel-filtration on Sephadex LH-20 (eluent: MeOH). Yield: 54 mg (91%). M.p. = 205°C; R_F 0.20 (system B); $[\alpha]_D^{22} = -55.3$ ($c = 0.38$, MeOH).

Fmoc-Hyp-[2-^{2}H-Gln]-Aib-Hyp-Aib-Pro-OH (6a^{II})

The hexapeptide **6^{II}** (33 mg, 0.03 mmol) was deprotected by means of 50% TFA in dichloromethane (6 ml) for 30 min according to the procedure described for the preparation of hexapeptide **6a^I**. Yield: 25 mg (91%). M.p., R_F and $[\alpha]_D^{22}$ parameters are identical to those for **6a^I**.

Fmoc-Hyp-[4,4-^{2}H$_2$-Gln]-Aib-Hyp-Aib-Pro-OH (6a^{III})

The hexapeptide **6^{III}** (72 mg, 0.07 mmol) was deprotected by means of 50% CF_3COOH in dichloromethane (6 ml) for 30 min according to the procedure described for the preparation of hexapeptide **6a^I**. Yield: 54 mg (91%). M.p., R_F and $[\alpha]_D^{22}$ parameters are identical to those for **6a^I**.

Fmoc-Trp(Boc)-Ile-Gln(Trt)-D-Iva-OBu^t (7)

Removal of the Fmoc group from **10** (0.95 g, 1.08 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. To the suspension of the residue containing **10a** in 7 ml DMF were added Fmoc-Trp(Boc)-OH (0.68 g, 1.29 mmol), BOP (0.57 g, 1.29 mmol) and DIPEA (0.62 ml, 3.66 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/methanol, 30/1, v/v) yielded 1.2 g (95%) of pure **7**. R_F 0.46 (system E).

Fmoc-Thr(Bu^t)-Aib-Leu-Aib-OBu^t (8)

Removal of the Fmoc group from **11** (0.37 g, 0.64 mmol) by 0.1 M NaOH in dioxane/methanol/-water was carried out as described above. To the suspension of the residue containing **11a** in 6 ml DMF, were added Fmoc-Thr(Bu^t)-OH (0.31 g, 0.77 mmol), BOP (0.34 g, 0.77 mmol) and DIPEA (0.37 ml, 2.18 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/methanol, 40/1, v/v) yielded 0.41 g (87%) of pure **8**. M.p. = 78°C; R_F 0.55 (system E); $[\alpha]_D^{22} = -6.8$ ($c = 2$, CH_2Cl_2).

Fmoc-Gln-Aib-Hyp(Bu^t)-Aib-Pro-OBu^t) (9^I)

Removal of the Fmoc group from **12** (0.14 g, 0.17 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. To the suspension of the residue containing **12a** in 2 ml DMF, were added **19^I** (92 mg, 0.25 mmol), BOP (110 mg, 0.25 mmol) and DIPEA (0.12 ml, 0.68 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/methanol, 30/1, v/v) yielded 116 mg (75%) of pure **9^I**. M.p. = 153°C; R_F 0.54 (system E).

Fmoc-[2-^{2}H-Gln]-Aib-Hyp(Bu^t)-Aib-Pro-OBu^t (9^{II})

Compound **9^{II}** was prepared according to the same procedure as described for the synthesis of **9^I** using **12** (42 mg, 0.07 mmol), **19^{II}** (31 mg, 0.08 mmol), BOP (35 mg, 0.08 mmol) and DIPEA (0.040 ml, 0.23 mmol). Purification on a silica gel column (eluent: dichloromethane/methanol, 30/1, v/v) followed by gel filtration on Sephadex LH-20 (eluent: MeOH) afforded the pure pentapeptide **9^{II}**. Yield: 45 mg (75%). M.p. and R_F parameters are identical to those for **9^I**.

Fmoc-[4,4-$^2H_2$-Gln]-Aib-Hyp(Bu^t)-Aib-Pro-OBU^t (9^{III})

Compound **9^{III}** was prepared according to the same procedure as described for the synthesis of **9^I** using **12** (98 mg, 0.17 mmol), **19^{III}** (92 mg, 0.25 mmol), BOP (110 mg, 0.25 mmol) and DIPEA (0.12 ml, 0.68 mmol). Purification on a silica gel column (eluent: dichloromethane/methanol, 30/1, v/v) followed by gel filtration on Sephadex LH-20 (eluent: MeOH) afforded the pure pentapeptide **9^{III}**. Yield: 117 mg (75%). M.p. and R_F parameters are identical to those for **9^I**.

Fmoc-Ile-Gln(Trt)-D-Iva-OBu^t (10)

Removal of the Fmoc group from **13** (1.14 g, 1.49 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. To the suspension of the residue containing **13a** in 10 ml DMF were added Fmoc-Ile-OH (0.64 g, 1.80 mmol), BOP (0.80 g, 1.80 mmol) and DIPEA (0.87 ml, 5.1 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/methanol, 40/1, v/v) yielded 1.26 g (96%) of pure **10**. R_F 0.50 (system D).

Fmoc-Aib-Leu-aib-OBu^t (11)

Removal of the Fmoc group from **14** (0.56 g, 1.14 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. To the suspension of the residue containing **14a** in 6 ml DMF were added Fmoc-Aib-OH (0.6 g, 1.9 mmol), BOP (0.8 g, 1.9 mmol), DMAP (15 mg, 0.1 mmol) and DIPEA (0.9 ml, 5.0 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/methanol, 40/1, v/v) yielded 0.65 g (98%) of pure **11**. M.p. = 118°C; R_F 0.55 (system E); $[\alpha]_D^{22} = -31.8$ ($c = 2$, CH_2Cl_2).

Fmoc-Aib-Hyp(Bu^t)-Aib-Pro-OBu^t (12)

Removal of the Fmoc group from **15** (0.84 g, 1.30 mmol) by 0.1 M NaOH in dioxane/methanol/water was carried out as described above. To the suspension of the residue containing **15a** in 6 ml DMF were added Fmoc-Aib-OH (0.65 g, 2.00 mmol), BOP (0.88 g, 2.00 mmol), DMAP (12 mg, 0.13 mmol) and DIPEA (0.91 ml, 5.3 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/methanol, 30/1, v/v) yielded 0.68 g (71%) of pure **12**. M.p. = 130°C; R_F 0.49 (system A); $[\alpha]_D^{22} = -16.0$ ($c = 1$, CH_2Cl_2).

Fmoc-Gln(Trt)-D-Iva-OBu^t (13)

Fmoc-Gln(Trt)-OH (1.02 g, 1.67 mmol) and BOP (0.74 g, 1.67 mmol) were added to **16a** (0.29 g, 1.40 mmol) dissolved in DMF (5.0 ml). Subsequently, DIPEA (0.81 ml, 4.74 mmol) was added and the reaction mixture was stirred for 30 min. The reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/ether, 10/1, v/v) yielded 1.05 g (98%) of pure **13**. M.p. = 169°C; R_F 0.23 (system C); $[\alpha]_D^{22} = -9.2$ ($c = 0.5$, MeOH).

Fmoc-Leu-Aib-OBu^t (14)

Compound **14** was prepared according to the procedure described above for the synthesis of dipeptide **13** using Fmoc-Leu-OH (0.85 g, 2.4 mmol), **17a** (0.32 g, 2.0 mmol), BOP (1.06 g, 2.4 mmol) and DIPEA (0.99 ml, 5.8 mmol) in 6 ml DMF. After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. Purification on a silica gel column (eluent: dichloromethane/ether, 98/2, v/v) yielded 0.9 g (98%) of pure **14**. M.p. = 75°C; R_F 0.59 (system C); $[\alpha]_D^{22} = -18.4$ ($c = 1$, CH_2Cl_2).

Fmoc-Hyp(Bu^t)-Aib-Pro-OBu^t (15)

Removal of the Fmoc group from **18** (1.1 g, 2.3 mmol) by 0.1 M NaOH in dioxane/methanol/

water was carried out as described above. To the suspension of the residue containing **18a** in 12 ml DMF, were added Fmoc-Hyp(Bu^t)-OH (1.1 g, 2.8 mmol), BOP (1.2 g, 2.8 mmol) and DIPEA (1.3 ml, 7.9 mmol). Purification on a silica gel column (eluent: dichloromethane/methanol, 40/1, v/v) yielded 1.30 g (89%) of pure **15**. M.p. = 105°C; R_F 0.50 (system D); $[\alpha]_D^{22} = -63.4$ ($c = 1$, CH_2Cl_2).

HCl · H-(R)-Iva-OBu^t (16a)

To R-isovaline **16** (0.6 g, 5 mmol) suspended in a mixture of 15 ml of *tert*-butyl alcohol and 15 ml of *tert*-butyl acetate, H_2SO_4 (5 g) was added, resulting in a clear solution. After stirring for 2 h, the reaction mixture was neutralized with 1 M Na_2CO_3. The product was extracted three times with dichloromethane. To the organic layer 100 mol% of a 0.1 M solution of HCl in dichloromethane (prepared by bubbling dry HCl into dry dichloromethane then diluting to 0.1 M with additional dichloromethane) was added and the mixture was concentrated. The residue was crystallized by trituration with anhydrous ether and the crystals of pure **16a** were filtered off. Yield 0.6 g (56%). R_F 0.55 (system B).

HCl · H-Aib-OBu^t (17a)

Compound **17a** was synthesized as described above for the preparation of **16a** using α-aminoisobutyric acid **18** (1.03 g, 10 mmol), 30 ml of *tert*-butyl alcohol, 30 ml of *tert*-butyl acetate and 10 g of H_2SO_4. Yield: 1.14 g, (58%). R_F 0.63 (system B).

Fmoc-Aib-Pro-OBu^t (18)

BOP (3.3 g, 7.5 mmol), DIPEA (2.1 ml, 12.5 mmol) and DMAP (0.06 g, 0.5 mmol) were added to the solution of Fmoc-Aib-OH (2.44 g, 7.5 mmol) in 5 ml of CH_2Cl_2, HCl · H-Pro-OBu^t (1.0 g, 5.0 mmol). After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and work-up was carried out as described for the preparation of **4**. The product was purified on silica gel column (eluent: dichloromethane/ether, 1/1, v/v) yielded 2.3 g (95%) of pure **18**. M.p. = 78°C; R_F 0.38 (system C); $[\alpha]_D^{22} = -54.6$ ($c = 1$, CH_2Cl_2).

Fmoc-[2-^{2}H-Gln]-OH (19^{II})

A solution of Fmoc-OSu (0.41 g, 1.2 mmol) in 3 ml of acetonitrile was added to [2-^{2}H]-L-Gln (0.15 g, 1.0 mmol) dissolved in a mixture of 3.0 ml water and 0.07 ml of triethylamine. The pH of the reaction mixture was maintained at 8.5–9.0 by the addition of triethylamine. After stirring for 30 min to the reaction mixture 1.2 ml of 1.5 M HCl was added. The crystallized product was collected by filtration, washed with water and methanol to result 0.40 g (97%) of pure **19^{II}**. R_F 0.54 (system B).

Fmoc-[4,4-$^2H_2$-Gln]-OH (19^{III})

Compound **19^{III}** was prepared from [4,4-$^2H_2$]-L-Gln (50 mg, 0.34 mmol), following the same procedure described for the preparation of **19^{II}**. Yield: 120 mg (97%). R_F 0.54 (system B).

RESULTS AND DISCUSSION

Stepwise Synthesis of Fragments

The (1–4), (5–9) and (10–16) fragments of zervamicin IIB were prepared stepwise using Fmoc amino acids. For the coupling of the Fmoc amino acids, *in situ* activation with BOP was chosen because of the ease of use, fast reactions and the general lack of side reactions. However, the coupling of Fmoc-Aib-OH on to the *N*-alkylated amino acid H-Pro-OBu^t, using the BOP coupling reagent, failed owing to intrinsic steric hindrance of these amino acids. In order to find the optimal conditions for the coupling of an Aib residue on to proline we examined four different reaction conditions for the formation of Fmoc-Aib-Pro-OBu^t from Fmoc-Aib-OH and H-Pro-OBu^t. The following coupling methods were tested: (i) BOP; (ii) BOP in the presence of DMAP as a catalyst; (iii) Fmoc-Aib-Cl; and (iv) the recently described Fmoc-Aib-F [29]. The results clearly indicate that coupling of the Aib residue onto H-Pro-OBu^t using BOP as a coupling reagent was inferior. The three other coupling methods, Fmoc-Aib-OH/BOP/DMAP, Fmoc-Aib-F and Fmoc-Aib-Cl, led to good yields (95–98%). However, the rates of the reaction using Fmoc-Aib-Cl and Fmoc-Aib-OH/BOP/DMAP reagents were superior compared with that of Fmoc-Aib-F. For the synthesis of zervamicin we further used the BOP/DMAP activation for couplings of Fmoc-Aib-OH because of the ease of use and that both reagents, BOP and DMAP, are commercially available.

The coupling reactions were carried out with 1.2 equivalents of the Fmoc amino acids for 30 min at room temperature but for the incorporation of the sterically hindered Aib residues 1.5 equivalents of Fmoc-Aib-OH was adopted.

The Fmoc group was removed by reaction with 0.1 M NaOH in dioxane/methanol/water (30/9/1, v/v/v). The molar ratio between NaOH and Fmoc-group was 3:1. The functional side chains of the Fmoc amino acids were protected by the acid-labile protective groups as follows: *tert*-butyloxycarbonyl (Boc) for Trp, trityl (Trt) for the Gln-3 and *tert*-butyl (Bu^t) for Thr and Hyp residues.

The (1–4) and (5–9) fragments were synthesized starting from the C-terminal Iva and Aib *tert*-butyl esters **16a** and **17a**, respectively, prepared in turn from R-Iva and Aib, respectively, using a mixture of *tert*-butyl acetate/*tert*-butyl alcohol (1/1, v/v) in the presence of sulphuric acid.

Initially, attempts to synthesize the C-terminal heptapeptaibols $\mathbf{3a^{I–III}}$ (fragment 10–16) stepwisely starting from the C-terminal L-Phl failed and the elongation procedure was terminated at the tripeptide stage. During removal of the Fmoc group from Fmoc-Aib-Pro-Phl spontaneous cleavage of the Pro-Phl peptide bond occurred with the release of free Phl, as was judged by thin-layer chromatography. For this reason, the desired heptapeptaibols $\mathbf{3a^{I–III}}$ were synthesized using an alternative approach where unprotected L-Phl was coupled to the Fmoc-protected hexapeptides $\mathbf{6a^{I–III}}$ (Scheme 2). In this strategy a Pro residue is present at the C-terminus of the hexapeptides $\mathbf{6a^{I–III}}$ and therefore we have no risk of racemization during the fragment condensation. The protected hexapeptides $\mathbf{6^{I–III}}$ were prepared from the C-terminal H-Pro-OBu^t by stepwise elongation with Fmoc amino acids. Specifically, deuterium-labelled [2-^{2}H]- and [4,4-$^2H_2$]-L-Gln were prepared according our approach described in [24]. The synthesis of their Fmoc-protected derivatives $\mathbf{19^{II–III}}$ was achieved using Fmoc-OSu in a mixture of acetonitrile/water/triethylamine [31]. The Gln derivatives have been incorporated into peptide without protection of the carboxamide side chain. In order to prevent the possible intramolecular cyclization of the N-terminal Gln residue after the Fmoc group removal to pyroglutamate [32, 33] a large excess of Fmoc-Hyp(Bu^t)-OH (3 equivalents) was adopted. Removal of the three *tert*-butyl groups from $\mathbf{6^{I–III}}$, which contain the acid-labile Aib^{12}–Hyp^{13} and Aib^{14}–Pro^{15} peptide bonds, was achieved without use of scavengers by means of TFA/CH_2Cl_2 (1/1, v/v) for 30 min affording $\mathbf{6a^{I–III}}$ in 90% yields. After removal of the acid-labile side-chain protecting groups, the N^{α}-protected hexapeptides $\mathbf{6a^{I–III}}$ were coupled in DMF on to L-Phl using the BOP reagent. The partially unprotected compounds $\mathbf{3^{I–III}}$ are poorly soluble in the sodium hydroxide reagent and removal of the Fmoc group from $\mathbf{3^{I–III}}$ was achieved with 40% diethylamine in DMF. The heptapeptaibols $\mathbf{3a^{I–III}}$ were subsequently purified by gel-filtration on Sephadex LH-20 and semi-preparative RP-HPLC.

Care should be taken for the acid deprotection of Trp-containing peptides because of facile alkylation of the indole ring by carbocations formed during the deprotection by TFA [34–37]. Therefore, removal of the acid-labile protective groups from the tetrapeptide **4** and nonapeptide **2** was performed using a mixture of TFA/water/triisopropylsilane (90/5/5, v/v/v) for 30 min, the latter being an effective scavenger [38]. It should be noted that trialkylsilanes may cause reduction of Trp residues [39], converting the side-chain indole to an indoline. Hence, the use of the more hindered triisopropylsilane was necessary [38].

The homogeneity of the synthesized zervamicin's peptide fragments (1–4) **4a**, (5–9) **5a** and (10–16) $\mathbf{3a^{I–III}}$ was fully confirmed by RP-HPLC, mass spectrometry and NMR spectroscopy.

Fragment Condensations

The critical steps in the strategy developed were the two fragment condensations. The difficult condensations involving the N-acetylated tetrapeptide **4a** with the pentapeptide *tert*-butyl ester **5a** and the N-acetylated nonapeptide **2a** with the heptapeptaibol $\mathbf{3a^{I}}$ failed using BOP as a coupling reagent and DMAP as a catalyst. This problem could be solved by the application of the recently described coupling reagent CF_3-PyBOP, which enhances yields for the incorporation of the Aib residue onto sterically hindered amino acids in solution [27]. The tetrapeptide **4a**, which contains an Iva residue at the C-terminus, was coupled to the pentapeptide *tert*-butyl ester **5a** overnight, using equimolar amounts of the two peptides yielding **2** in 80% yield. Removal of the two Bu^t protecting groups from **2** by TFA/H_2O/triisopropylsilane (90/5/5, v/v/v) for 30 min followed by HPLC purification resulted in the nonapeptide fragment **2a** in 74% yield. The final condensations between **2a** (which contains at the C-terminus an Aib residue) and the fully unprotected C-terminal heptapeptaibols $\mathbf{3a^{I–II}}$ were also carried out using CF_3PyBOP. Zervamicin IIB $\mathbf{1^{I}}$ and the isotopically labelled zervamicins IIB $\mathbf{1^{II}}$ and $\mathbf{1^{III}}$ have been obtained in 87% yield. Both labelled zervamicins $\mathbf{1^{II}}$ and $\mathbf{1^{III}}$ have been prepared in 40% overall yield based on isotopically labelled L-Gln residues. Comparison of HPLC behaviour of native and

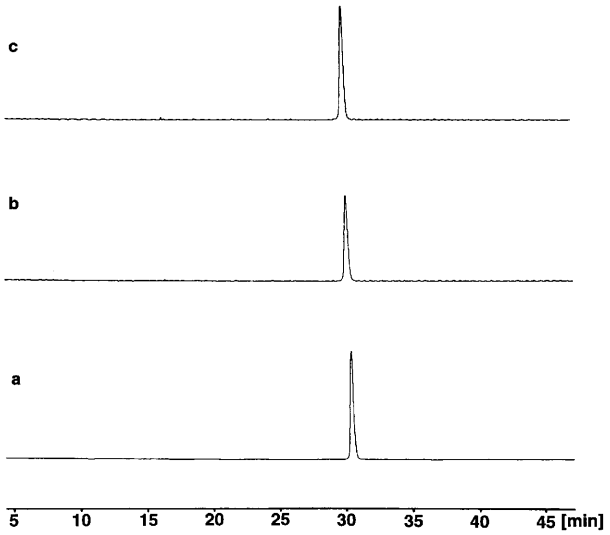


Figure 2 Comparison of HPLC retention behaviour of native and synthesized zervamicins IIB: **(a)** native zervamicin IIB; **(b)** synthesized zervamicin IIB; **(c)** co-injected synthetic and native zervamicin IIB. The peptides were separated on a Spherisorb ODS-2 (5 μm) Pharmacia column (250 × 4 mm), employing a gradient of 50% MeOH to 100% MeOH over 40 min (0.4 ml/min).

synthesized zervamicins is given in Figure 2. FAB mass spectrometric and 600 MHz ^{1}H NMR spectroscopic characterizations of the zervamicins specifically labelled with deuterium at two positions of Gln^{11} confirm the homogeneity of the final products.

^{1}H-NMR Spectroscopic Characterization of Zervamicins

The 600 MHz ^{1}H-NMR spectra of both the synthetic zervamicin IIB and the native peptide are shown in Figure 3. Clearly, there is a good agreement in line

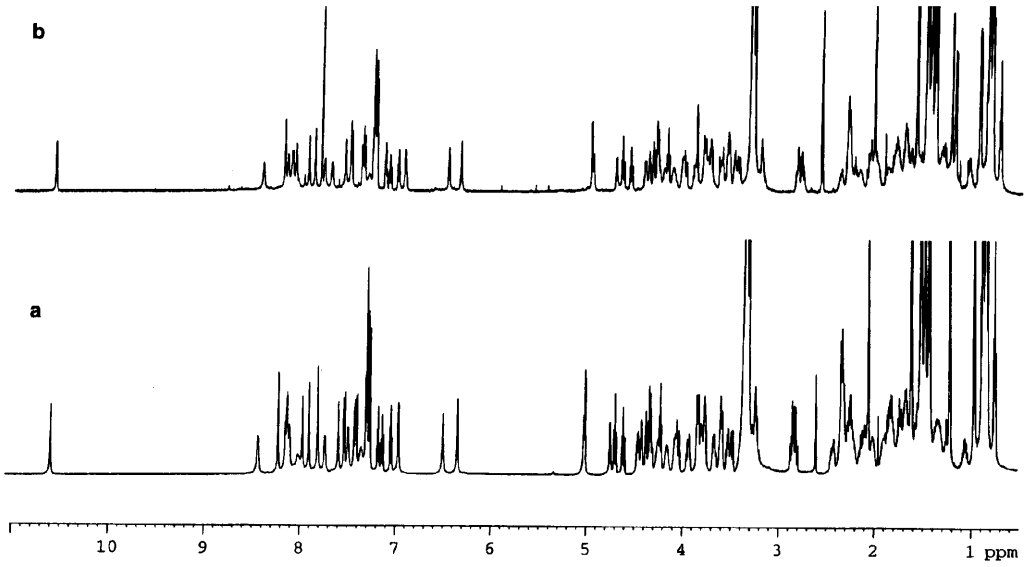


Figure 3 600 MHz ^{1}H NMR spectra of natural zervamicin IIB (a) and synthetic zervamicin IIB (b). The spectra are recorded in deuterated chloroform/dimethyl sulphoxide (80/20, v/v) at $T = 283$ K.

Table 1 ^{1}H-resonance Assignments for Zervamicin IIB in Deuterated Chloroform/Dimethyl Sulphoxide (80/20, v/v) at $T = 283$ K

Residue	Resonance assigned	Chemical shift values
Ac	CH_3	2.05
Trp-1	NH	8.08
	NH_{indole}	10.56
	$C^{\alpha}H$	4.43
	$C^{\beta}H_2$	3.22
	$CH_{arom.}2'$	7.24
	4′	7.50
	5′	7.02
	6′	7.11
	7′	7.37
Ile-2	NH	7.99
	$C^{\alpha}H$	3.74
	$C^{\beta}H$	1.68
	$C^{\gamma}H_2$	1.33, 1.07
	$C^{\gamma}H_3$	0.84
	$2 \times C^{\delta}H_3$	0.83
Gln-3	NH	8.41
	$C^{\alpha}H$	3.82
	$C^{\beta}H_2$	2.24, 2.01
	$C^{\gamma}H_2$	2.42
	$N^{\varepsilon}H_2$	*trans* 7.47, *cis* 6.47
Iva-4	NH	7.95
	$C^{\beta}H_2$	2.25, 1.67
	$C^{\beta}H_3$	1.44
	$C^{\gamma}H_3$	0.75
Ile-5	NH	7.78
	$C^{\alpha}H$	3.78
	$C^{\beta}H$	1.89
	$C^{\gamma}H_2$	1.34, 0.96
	$C^{\gamma}H_3$	0.84
	$2 \times C^{\delta}H_3$	0.83
Thr-6	NH	7.71
	$C^{\alpha}H$	3.75
	$C^{\beta}H$	4.23
	$C^{\beta}OH$	4.73
	$C^{\gamma}H_3$	1.22
Aib-7	NH	7.57
	$2 \times C^{\beta}H_3$	1.50, 1.47
Leu-8	NH	7.33
	$C^{\alpha}H$	4.14
	$C^{\beta}H_2$	1.82, 1.55
	$C^{\gamma}H$	0.84
	$2 \times C^{\delta}H_3$	1.79, 1.65
Aib-9	NH	8.11
	$2 \times C^{\beta}H_3$	1.62, 1.51
Hyp-10	$C^{\alpha}H$	4.59
	$C^{\beta}H_2$	2.34, 1.86
	$C^{\gamma}H$	4.40
	$C^{\gamma}OH$	5.00
	$C^{\delta}H_2$	4.01, 3.58
Gln-11	NH	8.13
	$C^{\alpha}H$	4.32
	$C^{\beta}H_2$	2.24, 2.11
	$C^{\gamma}H_2$	2.35
	$N^{\varepsilon}H_2$	*trans* 6.94, *cis* 6.32
Aib-12	NH	7.88
	$2 \times C^{\beta}H_3$	1.61, 1.50
Hyp-13	$C^{\alpha}H$	4.68
	$C^{\beta}H_2$	2.32, 1.74
	$C^{\gamma}H$	4.35
	$C^{\gamma}OH$	4.99
	$C^{\delta}H_2$	3.82, 3.50
Aib-14	NH	8.20
	$2 \times C^{\beta}H_3$	1.50, 1.41
Pro-15	$C^{\alpha}H$	4.33
	$C^{\beta}H_2$	2.08, 1.49
	$C^{\gamma}H_2$	1.80, 1.74
	$C^{\delta}H_2$	3.91, 3.65
Phl-16	NH	7.40
	$C^{\alpha}H$	4.03
	$C^{\beta}H_2$	2.84
	$C^{\beta}H_2OH$	3.59, 3.52
	$C^{\beta}OH$	4.20
	$CH_{arom.}4'$	7.15
	2′,3′,5′6′	7.29–7.26

Table 1 (*continued*)

positions and intensities of ^{1}H-resonances in the spectra of these two compounds. A shift of some ^{1}H-resonances for amide and hydroxyl proton signals of the two peptides are attributed to the presence of different amounts of residual water or a concentration effect in the samples. We performed the assignment of the resonances in the ^{1}H-NMR spectra of the two peptides which clearly confirmed their structures and homogeneities. Furthermore, the presence of a single set of ^{1}H-resonances in the spectrum of synthetic zervamicin IIB is a strong indication that the molecule is enantiomerically pure. The assignments of all ^{1}H resonances in zervamicin IIB (Table 1) have been achieved using two-dimensional COSY and NOESY NMR experiments and followed established procedures reviewed by Wüthrich [40]. Once the assignment of ^{1}H-resonances of zervamicin IIB is completed, the position of deuterium labels in the isotopically labelled peptide [4,4-$^2H_2$-Gln^{11}]-zervamicin IIB is determined by a disappearance of the γ-proton resonances of the Gln-11 residue at 2.35 ppm (Figure 4). The α-region of the ^{1}H-NMR spectrum of zervamicin IIB is rather crowded and it was impossible to demonstrate the absence of the H^{α} proton signal in the spectrum of the labelled [2-^{2}H-

Gln[11]]-zervamicin IIB (dissolved in deuterated chloroform/dimethyl sulphoxide). However, the ^{1}H-NMR spectrum of this labelled zervamicin IIB in methanol is well resolved (Figure 5). Clearly, there is a complete disappearance of one proton signal at 4.38 p.p.m. (double doublet) in the α-region of the spectrum of [2-^{2}H-Gln[11]]-zervamicin IIB compared with the spectrum of native zervamicin IIB which is attributed to the replacement of the α-proton of Gln-11 by deuterium. The line positions and intensities of all other ^{1}H-resonances in the spectra of two compounds are in excellent agreement with each other.

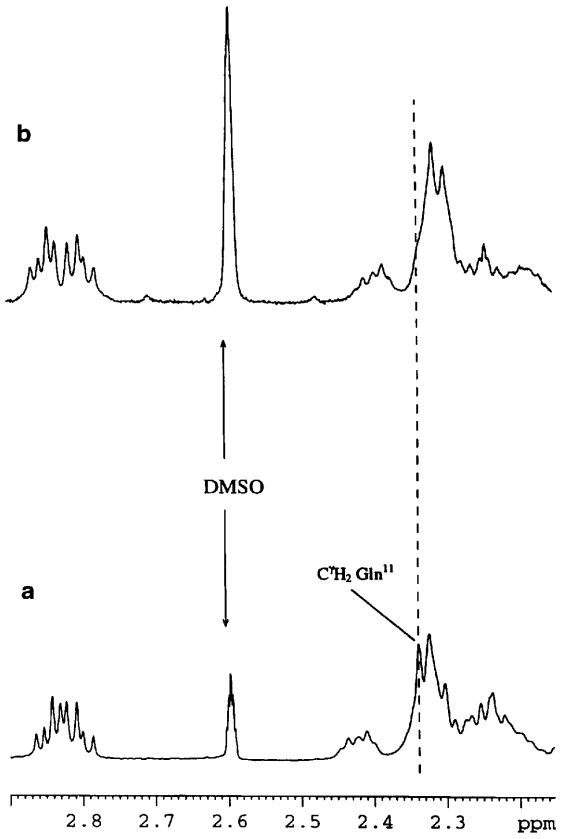


Figure 4 Comparison of the 600 MHz ^{1}H-NMR spectra of natural zervamicin IIB (a) and [4,4-^{2}H$_2$-Gln[11]]-zervamicin IIB (b), (aliphatic region). The spectra are recorded in deuterated chloroform/dimethyl sulphoxide (80/20, v/v) at $T = 283$ K.

FAB Mass Spectrometric Characterization of Zervamicins

Figure 6 presents the high molecular mass region of the positive ion FAB mass spectra of natural zervamicin IIB and its synthesized isotopically

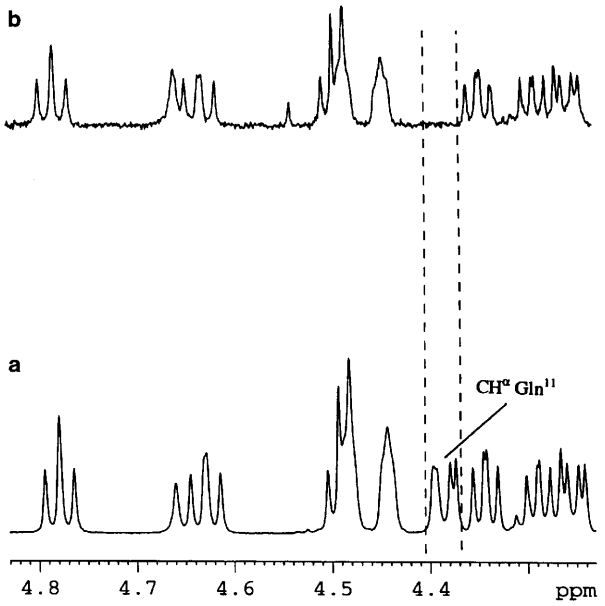


Figure 5 Comparison of the 600 MHz ^{1}H-NMR spectra of natural zervamicin IIB (a) and [2-^{2}H-Gln[11]]-zervamicin IIB (b), (H$^\alpha$ region). The spectra are recorded in deuterated methanol at $T = 293$ K.

labelled analogues [2-^{2}H-Gln[11]]- and [4,4-^{2}H$_2$-Gln[11]]-zervamicin IIB. Upwards mass shifts of 1 and 2 mass units for $(M + Na)^+$ molecular ions of the deuterium-labelled [2-^{2}H-Gln[11]]- (m/z 1862.0) and [4,4-^{2}H$_2$-Gln[11]]-zervamicin IIB (m/z 1863.0), respectively, compared with the value of this ion for native zervamicin IIB (m/z 1861.0) can be clearly seen. From these peaks the isotopic enrichments of the [2-^{2}H-Gln[11]]- and [4,4-^{2}H$_2$-Gln[11]]-labelled zervamicins IIB were found to be, within experimental error, $98 \pm 2\%$. These isotope enrichments are the same as in the starting compounds, the isotopically labelled L-Gln residues. This proves that no scrambling or dilution of labelled material has taken place during peptide synthesis. Furthermore, only a cluster of one molecular ion type is present in the FAB mass spectra of the synthetic peptaibols, which argues for the homogeneity of the synthesized peptides.

CONCLUSIONS

For the first time the syntheses of the 16-residue peptaibol antibiotic zervamicin IIB and its isotopically analogues have been described. The labelled peptaibols have been synthesized in 40% overall yield based on the isotopically labelled L-Gln starting materials.

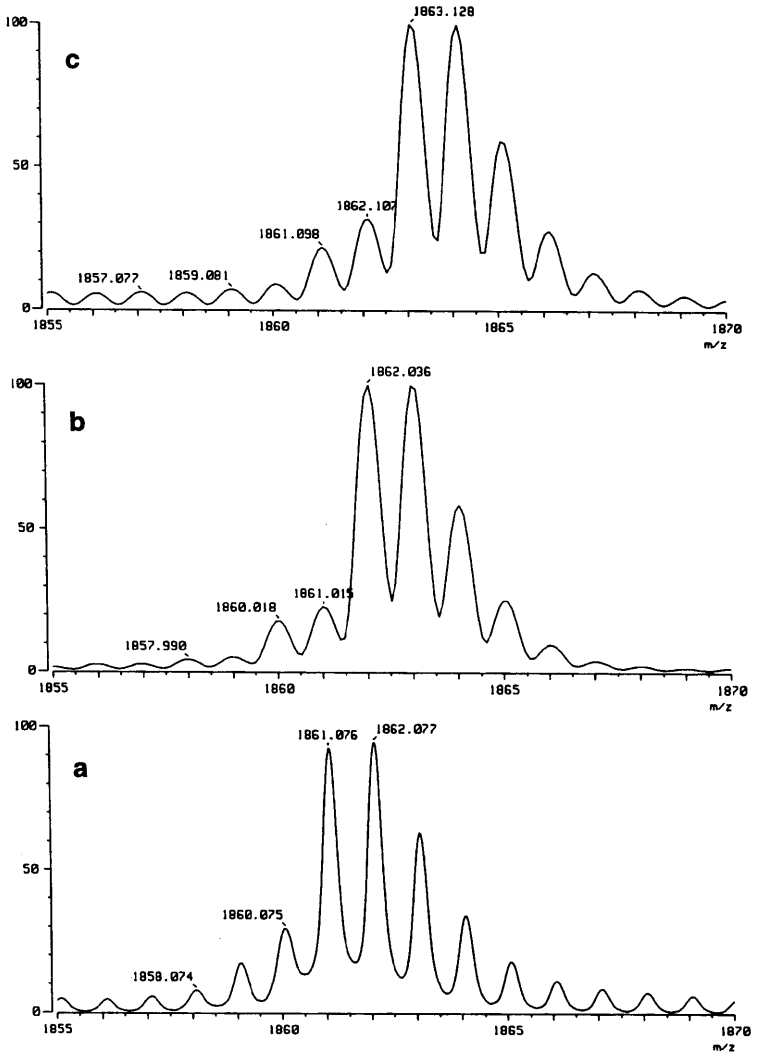


Figure 6 The high-molecular mass region of the positive ion FAB mass spectra of native zervamicin IIB (a) and its isotopically labelled analogues, [2-^{2}H-Gln^{11}]-zervamicin IIB (b) and [4,4-$^{2}H_2$-Gln^{11}]-zervamicin IIB (c).

The method described features not only the synthesis of zervamicin IIB specifically labelled at Gln^{11} but also allows isotope labelling at any other position or combination of positions. The successful synthesis of zervamicin IIB described in the present study strongly suggests that other peptaibols can be synthesized similarly. The marked efficiency of the CF_3-PyBOP coupling reagent in the fragment condensations of peptides containing the C-terminal α,α-dialkylated amino acids, Iva and Aib, has been demonstrated.

Based on a comparison between the 600 MHz NMR spectrum of the synthetic zervamicins IIB with that of the native one isolated from the fungi *Emericellopsis salmosynnemata*, we conclude that the synthesized peptides have the correct structure. For the first time, the ^{1}H-resonances of zervamicin IIB have been assigned (to be published elsewhere). The positions of the deuterium atoms in the labelled zervamicins IIB have been clearly demonstrated by ^{1}H-NMR spectroscopy. The homogeneity of the synthetic isotopically labelled zervamicins IIB has been proven by FAB mass spectrometry. The isotope enrichments of [2-^{2}H-Gln^{11}]- and [4,4-$^{2}H_2$-Gln^{11}]-zervamicin IIB have been determined as 98 ± 2%. These isotope enrichments are, within experimental error, the same as in the starting compounds, the isotopically labelled L-Gln residues.

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