Solid-phase synthesis of thymosin β_{10} using a *p*-cyanotrityl resin. Chemical characterization and immunochemical control of the synthetic peptide †

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Thymosin β_{10} (43 amino acids) was synthesized following the Fmoc-solid-phase strategy, using a new *p*-cyanotrityl resin. This resin shows enhanced acid stability compared with the usual trityl ester resins, thus providing selective peptide synthesis with high yield and purity. Crude $T\beta_{10}$ was purified with gel filtration and semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). The purified synthetic peptide was shown to co-elute with recombinant $T\beta_{10}$ in an analytical RP-HPLC system. The amino acid analysis data were in agreement with those reported for the literature primary structure of natural $T\beta_{10}$. The electrospray ionization mass spectrometry (ESIMS) data showed almost identical average mass ($M_{r,exp}$)-values for the synthetic or the recombinant product, which were in agreement with the molecular mass calculated on the basis of the proposed primary structure for the peptide. Furthermore, the synthetic $T\beta_{10}$ exhibited similar immunochemical characteristics compared with the recombinant material in an *in vitro* ELISA test using rabbit polyclonal anti-$T\beta_{10}$ antiserum.

Introduction

Thymosins comprise a family of related peptides, the first of which were originally isolated from calf thymus.[2] Initially, they were divided into three classes, namely alpha-, beta- and gamma-thymosins, according to their isoelectric point.[3] The beta-thymosin family (β-thymosins) consists of small, acidic peptides, with approximately 45 amino acid residues each, which have been highly conserved during evolution.[4] In mammals, two highly homologous β-thymosins are usually present in one species. The main mammalian β-thymosin is thymosin β_4 ($T\beta_4$), which is accompanied by thymosin β_{10} ($T\beta_{10}$) in man, horse, cat, rabbit and mouse, thymosin β_9 ($T\beta_9$) in ox, thymosin β_9^{met} ($T\beta_9^{met}$) in pig or thymosin β_{13} ($T\beta_{13}$) in whale.[5]

Among β-thymosins, thymosin β_4 and thymosin β_{10} are considered of special interest, due to their occurrence in human tissues. $T\beta_4$, a 43 amino acid residue peptide,[3] was thought to be a specific thymic hormone, but is now believed to have a more general function which may be associated with cell proliferation.[5] In the early nineties, $T\beta_4$ was reported to inhibit the salt-induced polymerization of G-actin.[6] This finding has rekindled interest in research concerning β-thymosins.[7]

Thymosin β_{10}, which was first isolated in 1983,[8] consists of 43 amino acid residues.[9] Its primary structure differs from that of $T\beta_4$ in 11 amino acid residues, four of which are located in adjacent sites at the peptide C-terminus, consisting the 39–42 sequence.

† Abbreviations used are in accordance with the rules in ref. 1. Other abbreviations: ABTS, 2,2′-azinobis-(3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid) diammonium salt; DIPC, *NN*′-diisopropylcarbodiimide; DIEA, *NN*-diisopropylethylamine; DMF, *NN*-dimethylformamide; EDT, ethane-1,2-dithiol; ELISA, enzyme-linked immunosorbent assay; Fmoc, fluoren-9-ylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IgG, γ-immunoglobulin; KLH, keyhole limpet haemocyanin; TFA, trifluoroacetic acid.

As in case of $T\beta_4$, no precursor molecule has been found for $T\beta_{10}$, indicating that $T\beta_{10}$ is the primary translation product.[9] Although less investigated than those of $T\beta_4$, the biological role and functions of $T\beta_{10}$ seem to be associated with cell growth and proliferation. Since the high levels of thymosin β_{10} mRNA found in human foetal brain[10] decline dramatically after parturition,[11] $T\beta_{10}$ seems to play a significant role in the development of nervous-system tissues. These findings imply that $T\beta_{10}$ plays an essential role in human neuroembryogenesis. In human kidney, $T\beta_{10}$ and, to a lesser extent, $T\beta_4$ were found to decrease with age, while their mRNAs increased in renal carcinomas.[12] In another study, increased levels of $T\beta_{10}$ mRNA correlated with high metastatic character in human cutaneous melanomas.[13] On the other hand, recombinant $T\beta_{10}$ was reported to show an inhibitory activity on F-actin formation, similar to that of $T\beta_4$, through binding to G-actin with a K_d of 0.7–1.0 × 10^{-6} mol dm^{-3}.[14]

The above reported important, although not yet completely elucidated, functions of $T\beta_{10}$ emphasize the need for further investigation concerning this peptide. Such investigation would be greatly facilitated if large amounts of $T\beta_{10}$ could be easily provided. Until now, however, $T\beta_{10}$ (mainly of recombinant origin) can be commercially obtained from very few sources in only low quantities and at rather high prices.

We have previously reported the isolation of $T\beta_4$ from bovine tissues using a large-scale chromatofocusing method.[15] However, the fact that only tiny amounts of $T\beta_{10}$ are present in animal tissues, usually much lower than those of $T\beta_4$, and the difficulties in developing a scaled up isolation method for $T\beta_{10}$, capable of completely separating $T\beta_{10}$ from $T\beta_4$, led us to develop a protocol for the chemical synthesis of $T\beta_{10}$.

Syntheses of thymosin β_4, thymosin α_1, or other thymic factors have been described in the literature.[16–20] In this paper we report, for the first time to our knowledge, the synthesis of $T\beta_{10}$. This was performed according to the previously reported amino acid sequence for natural $T\beta_{10}$ and followed the general principles of the Fmoc-solid-phase synthesis of peptides.[21] The key point in the synthetic strategy was the use of a *p*-cyanotrityl

β_{10}: *N*-Acetyl-Ala-Asp-Lys-Pro-Asp-Met-Gly-Glu-Ile-Ala-Ser-Phe-Asp-
β_4: *N*-Acetyl-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-

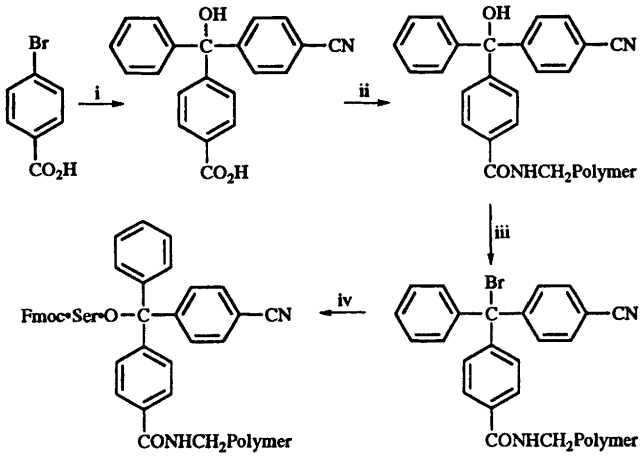
β_{10}: -Lys-Ala-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Thr-Leu-Pro-
β_4: -Lys-Ser-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-

β_{10}: -Thr-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Arg-Ser-Glu-Ile -Ser-OH
β_4: -Ser-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH

Amino acid sequence of $T\beta_{10}$ and $T\beta_4$. Not-in-common amino acid residues are underlined.

resin as the solid support. In general, the trityl-type resins are preferred for solid-phase peptide synthesis because they are stable to nucleophiles such as piperidine.[22] The *p*-cyanotrityl resin used in this work has additional stability to acids, which makes the peptide-anchoring bond less sensitive to acidic conditions. Thus, most of the usually observed losses were eliminated and the peptide synthesized was obtained in high yield.

After it had been cleaved from the resin, crude $T\beta_{10}$ was purified by gel filtration and semi-preparative RP-HPLC. The purified synthetic peptide was shown to co-elute with commercially available recombinant $T\beta_{10}$ in an analytical RP-HPLC system. The amino acid analysis data and the ESIMS results were in agreement with those reported in the literature primary structure for natural $T\beta_{10}$. Furthermore, the synthetic $T\beta_{10}$ exhibited similar immunochemical characteristics compared with the recombinant material in an *in vitro* ELISA test using rabbit polyclonal anti-$T\beta_{10}$ antiserum.



Scheme 1 *Reagents:* i, BuLi, 4-cyanobenzophenone; ii, HOBt, DIPC, DMF, aminomethylpolystyrene; iii, AcBr, CH_2Cl_2; iv, Fmoc-Ser-OH, DIEA, CH_2Cl_2

Results and discussion

Solid-phase synthesis and purification of thymosin β_{10}

The trityl-type resin-handle 4-carboxy-4′-cyanotriphenylmethanol used in this work was prepared using the method described for the 4-carboxy-2′-fluoro(chloro)triphenylmethanol handle, slightly modified.[22] The aminomethyl resin was prepared by direct amidomethylation of copoly(styrene–1% 1,3-divinylbenzene) resin, as previously described.[23] The handle was linked to the aminomethyl resin by the HOBt/DIPC method (Scheme 1). The bromo-4-cyanotrityl resin was esterified with Fmoc-Ser(Bu^t)-OH using DIEA. After deprotection of the amino group, the capacity of the H-Ser(Bu^t)-4-cyanotrityl resin was measured by the Kaiser ninhydrin test and found to be equal to 0.56 mmol g^{-1}.

The resin (72 mg) was placed in a reaction vessel. The peptide sythesis was carried out by sequentially repeating the well established protocols for solid-phase peptide synthesis. The yield of the coupling reaction was checked using the Kaiser ninhydrin test. Even a brownish or slightly bluish colour of the resin was interpreted as incomplete reaction and the coupling step was prolonged (overnight, 4 °C). If, despite the overnight prolongation, the Kaiser test was still slightly positive, the remaining free amino groups were capped by treatment with acetic anhydride–DIEA (2:1, v/v). Afterwards, the Kaiser test was always negative. Such incomplete coupling problems were first addressed by Kent[24] and shown to be sequence-dependent, due to intermolecular hydrogen-bonded aggregates. In $T\beta_{10}$ synthesis, sequence-dependent incomplete coupling was observed at residues Thr 33, Thr 30, Leu 28, Lys 16, Ala 15 and Lys 3. An acetyl group was incorporated into the peptide N-terminus by using the same procedure as in the capping with acetic anhydride–DIEA (2:1, v/v).

The acetylated tritetracontapeptide was cleaved from the resin and the amino acid side chains were deprotected by treatment with a cocktail of TFA, water and scavengers (TFA 60%, water 5%, thioanisole 5%, phenol 5%, EDT 2.5%, CH_2Cl_2 22.5%). Washing of the resin with TFA–CH_2Cl_2 (6:4, v/v) led to a solution which, after removal of the organic solvent and precipitation with cold diethyl ether, gave the crude peptide (250 mg).

The crude peptide was dissolved in water and chromatographed on a Sephadex G-25 column (4 × 50 cm), equilibrated with 10% acetic acid in water. The peptide was eluted with 10% acetic acid. The eluted fractions were monitored by measuring their absorbance at 254 nm and by analytical RP-HPLC. The eluted fractions containing the peptide were pooled and lyophilized to give thymosin β_{10} (135 mg, 67% yield based on the capacity of the resin).

The peptide was further purified by semi-preparative RP-HPLC. Detection of the peptide was achieved with a variable-multi-wavelength detector set at 220 nm. The major peak was lyophilized to give the purified product (80 mg, 40% final yield).

Chemical characterization of the synthetic thymosin β_{10}

The elution profile of the purified synthetic $T\beta_{10}$ on analytical RP-HPLC is shown in Fig. 1(a). The peptide was eluted at 15.71 min as a single peak with a solvent system containing 0.05% TFA in water (Solvent A) and 60% acetonitrile in solvent A (solvent B). The gradient used was linear, 0–100% B over a period of 20 min. As shown in Fig. 1(b), when recombinant $T\beta_{10}$ (product of Bachem) was injected into the column under the same conditions as above, a single peak was eluted at 15.74 min. When synthetic and recombinant $T\beta_{10}$ were mixed in an equimolar final concentration and then injected together into the analytical HPLC column, they co-eluted at 15.67 min [Fig. 1(c)].

The purified synthetic peptide $T\beta_{10}$ was hydrolysed with 6 mol dm^{-3} HCl for 1 h at 150 °C. The acid hydrolysate was then derivatized with phenyl isothiocyanate (PITC), according to an established protocol proposed by Waters. The derivatized product was analysed with a PICO-TAG amino acid analysis system (Waters). Comparison with amino acid standards (Pierce) gave the amino acid analysis data shown in Table 1. For comparison purposes, the theoretical amino acid content of natural thymosin β_{10}, as deduced from cDNA molecular cloning analysis,[9] and our amino acid analysis data for recombinant thymosin β_{10} (Bachem) are also listed. The amino acid compositions of all three peptides appear to be identical.

Table 1 Amino acid analysis of the synthetic $T\beta_{10}$ and the recombinant $T\beta_{10}$ (Bachem)

Amino acid	Theoretical[a] composition	β_{10} (Synthetic)	β_{10} (Bachem)	Amino acid	Theoretical[a] composition	β_{10} (Synthetic)	β_{10} (Bachem)
Asx	4	4.05	3.97	Pro	2	2.01	1.94
Glx	9	9.08	9.12	Met	1	1.03	0.82
Ser	3	2.97	2.34	Ile	3	2.93	2.90
Gly	1	1.18	1.00	Leu	2	2.07	2.02
Arg	1	1.16	1.01	Phe	1	1.11	1.00
Thr	5	4.90	4.54	Lys	8	8.29	8.14
Ala	3	3.21	2.91				

[a] Theoretical composition was deduced from cDNA molecular cloning analysis.[9]

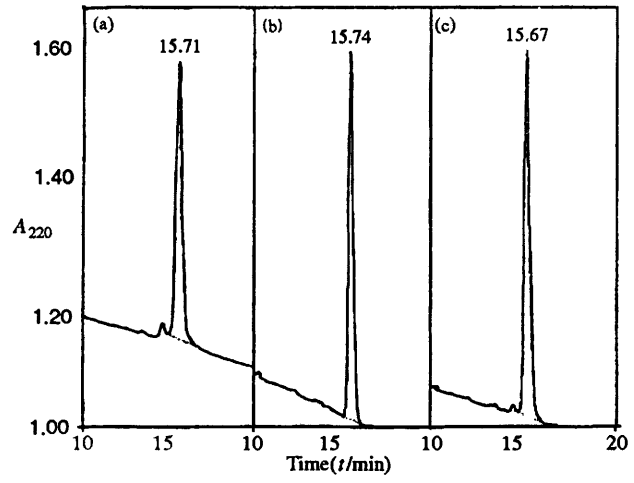


Fig. 1 Analytical RP-HPLC chromatograms showing (a) pure synthetic $T\beta_{10}$, (b) recombinant $T\beta_{10}$, (c) an equimolar mixture of both

The synthetic peptide $T\beta_{10}$ and the recombinant $T\beta_{10}$ (Bachem), as control, were analysed by ESIMS. According to this gentle ionization method[25,26] the peptide solution is sprayed in the presence of a high electric field, leading to the formation of analyte ions, often bearing multiple charges. Fig. 2 shows the electrospray mass spectra of the synthetic $T\beta_{10}$ (A) and the recombinant $T\beta_{10}$ (B). The major ions were the 6+ and 5+ charged states at m/z 823.65 and 988.25 for synthetic $T\beta_{10}$ or 823.65 and 988.30 for recombinant $T\beta_{10}$ (Bachem). Other related minor ions, 7+ and 4+, were also present. The charge on each ion and the molecular mass of the peptide were determined by deconvolution algorithms. The synthetic $T\beta_{10}$ molecular mass obtained ($M_{r,exp}$ = 4936.08) was almost identical with the molecular mass obtained for recombinant $T\beta_{10}$ (Bachem) ($M_{r,exp}$ = 4936.21) and very close to the average molecular mass calculated ($M_{r,cal}$ = 4937) on the basis of the peptide primary structure.

Immunochemical control

A conjugate between recombinant $T\beta_{10}$ (product of Peninsula) and keyhole limpet haemocyanin (KLH) was prepared by using 25% aq. glutaraldehyde. A New Zealand White rabbit was then immunized with the $T\beta_{10}$-KLH conjugate emulsified with Complete Freund's Adjuvant, according to the method of Vaitukaitis.[27] The first booster injection was given six weeks after the first immunization, whereas subsequent injections were given at 4-week intervals. Antiserum was collected 10 to 12 days after each booster injection.

In the immunoreactivity test (Fig. 3), synthetic $T\beta_{10}$ and, for comparison purposes, recombinant $T\beta_{10}$, either from Peninsula or from Bachem, were used for coating the ELISA microwells and consequently treated with the anti-$T\beta_{10}$ antiserum in various dilutions, from 1/15 000 down to 1/100 000. Thymosin β_4, isolated as previously described,[15] was used as negative control. Two different preparations of the anti-$T\beta_{10}$ antiserum

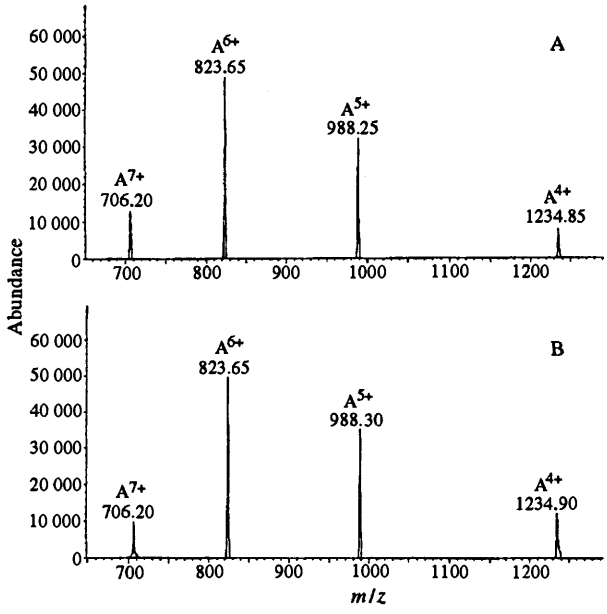


Fig. 2 Deconvolution mass spectra of (A) synthetic $T\beta_{10}$, (B) recombinant $T\beta_{10}$

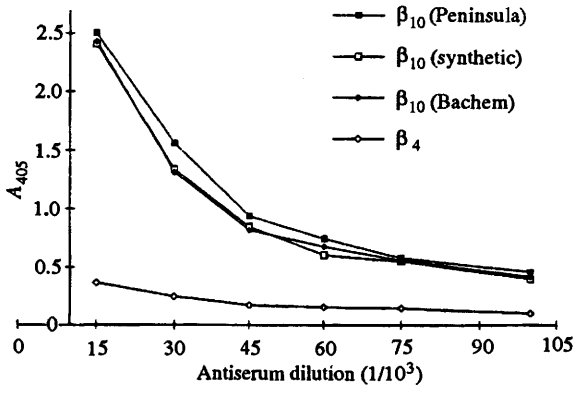


Fig. 3 Anti-$T\beta_{10}$ antiserum titre curves obtained with synthetic $T\beta_{10}$ recombinant $T\beta_{10}$ or $T\beta_4$

were used, *i.e.* antiserum obtained after the first or the fourth booster injection, respectively. The latter antiserum preparation gave the highest titre curves for every $T\beta_{10}$-peptide tested. With both antiserum preparations, however, the titre curves obtained using either synthetic or recombinant $T\beta_{10}$ were very similar (Fig. 3). These findings show that the immunochemical characteristics of the synthetic $T\beta_{10}$ correlate very well with those of the recombinant peptides studied, indicating stereochemical similarity. Isolated $T\beta_4$ was hardly recognized by the anti-$T\beta_{10}$ antiserum, confirming the specificity of the antiserum and, consequently, of the ELISA test used in these studies.

Conclusions

In the present work, we synthesized the 43-amino acid residue peptide thymosin β_{10} by a solid-phase method, using a new *p*-cyanotrityl resin. The yield of the final purified product was 40%, based on the capacity of the resin used. The purified synthetic peptide was shown to be chemically and immunologically indistinguishable from the natural/recombinant peptide.

The high overall yield obtained (40%) can be attributed to the use of the *p*-cyanotrityl resin-handle, which enhanced the stability of the anchoring bond between the peptide and the solid support to acidic media, thus minimizing chain losses that are usually observed in the process of peptide synthesis.

The major problem we encountered during this stepwise solid-phase synthesis was the occurrence of 'difficult sequences' in couplings, where the incorporation of the amino acid residue added could not be complete. Such sequence-dependent coupling problems were previously shown to be caused by intermolecular hydrogen-bonded aggregates.

Amino acid analysis of the synthetic $T\beta_{10}$ indicated that it has the same amino acid composition with that previously deduced from cDNA molecular cloning analysis for the natural peptide. The electrospray mass spectra of the synthetic and the recombinant products were almost identical and in agreement with the average molecular mass calculated on the basis of the proposed primary structure. Furthermore, the fact that the synthetic and the recombinant product (Bachem) co-elute when injected together in an analytical RP-HPLC system indicated that the synthetic peptide is chemically identical with the control material.

The immunochemical characteristics of the synthetic $T\beta_{10}$ were evaluated in an *in vitro* ELISA test, which was based on the use of a rabbit polyclonal anti-recombinant $T\beta_{10}$ antiserum. In this test, the synthetic $T\beta_{10}$ exhibited similar immunochemical characteristics compared with recombinant material obtained from two different sources. Isolated $T\beta_4$ was hardly recognized by the anti-$T\beta_{10}$ antiserum, which indicates the specificity of the antiserum and, consequently, of the ELISA test used in these studies.

Experimental

Materials

Fmoc-protected amino acids were obtained from CBL-Patras. Analytical-grade reagents were obtained from Merck or Sigma. TFA, sequential grade, was obtained from Sigma. Water and acetonitrile for HPLC were obtained from Lab-Scan (HPLC grade).

Aminomethyl-polystyrene resin and the 4-carboxy-4′-cyanotriphenylmethanol handle were prepared as described elsewhere.[22,23]

6 mol dm^{-3} HCl, PITC and amino acid standards for amino acid analysis were purchased from Pierce.

KLH, glutaraldehyde, goat anti-rabbit IgG conjugated to HRP (IgG/HRP) and ABTS were obtained from Sigma. Complete Freund's Adjuvant (CFA) was a product of Gibco. Tween 20 and H_2O_2 were purchased from Merck and Ferak, respectively. The ELISA microplates were obtained from Costar.

Recombinant $T\beta_{10}$ used for raising the polyclonal antiserum against $T\beta_{10}$ was a product of Peninsula. Recombinant $T\beta_{10}$ used as control material in HPLC and the amino acid analysis as well as in the immunoreactivity test was purchased from Peninsula or Bachem. $T\beta_4$ used as a negative control in the immunoreactivity test was isolated from bovine tissues as previously described.[15]

General methods

All analytical or semi-preparative RP-HPLC were performed on a Waters HPLC System equipped with two Waters 650 pumps, a Rheodyne 200 mm^3 manual injector and a Waters 484 multi-wavelength detector.

Analytical RP-HPLC was performed on a LiChrospher RP 18 analytical column (250 × 4,6 mm I.D.; 5 μm particle size; Merck). The solvent system contained 0.05% TFA in water (solvent A) and 60% acetonitrile in solvent A (solvent B). A linear gradient was applied from 0 to 100% B during 20 min, at a flow rate of 1.2 cm^3 min^{-1}. Detection of the peptide was set at 220 nm.

Semi-preparative RP-HPLC was performed on a Nucleosil C_{18} column (250 × 10 mm I.D.; 7 μm particle size; Macherey-Nagel). Solvent A was 0.05% TFA in water and solvent B was 60% acetonitrile in solvent A. A linear gradient was applied from 10 to 50% B during 50 min, at a flow rate of 5 cm^3 min^{-1}. Detection of the peptide was set at 220 nm.

Gel filtration was performed on a Sephadex G-25 column (4 × 50 cm), equilibrated with 10% acetic acid in water. The peptide was eluted with 10% acetic acid at a flow rate of 35 cm^3 h^{-1}. The eluted fractions were monitored by measuring their absorbance at 254 nm (Pharmacia) and by analytical HPLC.

Amino acid analysis was performed using the PICO-TAG amino acid analysis system (Waters). Samples were hydrolysed in vapour-phase HCl (6 mol dm^{-3} HCl; Pierce) for 1 h at 150 °C. The acid hydrolysate was then re-dried with a 2:2:1 mixture of ethanol, water and triethylamine and derivatized by storage for 20 min at room temperature with a mixture consisting of 7:1:1:1 ethanol, triethylamine, water and PITC. The derivatized product was analysed by RP-HPLC on a 5 μm particle size Waters column (3.9 cm × 15 mm) at 38 °C by using modifications of the PICO-TAG gradient system.

For the ESIMS analysis, control peptide (recombinant $T\beta_{10}$, Bachem) or test peptide (synthetic $T\beta_{10}$) solutions of 0.5 mg cm^{-3} in 50% aq. methanol (containing 1% acetic acid) were infused at a flow rate of 3 mm^3 min^{-1}, using a Harvant syringe pump, into an electrospray interface mass spectrometer (ES Engine HP-5989A). In the electrospray source the spray needle was grounded: voltages of −4.5, −3.5 and −3.0 kV were applied to the capillary, end plate and cylindrical electrodes, respectively. The capillary/skimmer potential difference varied between 150 and 300 V; the other source lenses were held at potentials that optimized the signal intensity. Hot nitrogen gas was used for desolvation. The charge on each ion and the molecular mass of the peptide were determined by deconvolution algorithms.

Preparation of the antiserum against $T\beta_{10}$ (anti-$T\beta_{10}$ antiserum)

A conjugate between recombinant $T\beta_{10}$ (Peninsula) and KLH was prepared and used as immunogen according to the following procedure: $T\beta_{10}$ [0.4 mg in 0.1 mol dm^{-3} phosphate buffer, consisting of 0.084 mol dm^{-3} $Na_2HPO_4 \cdot 2H_2O$ and 0.016 mol dm^{-3} KH_2PO_4, pH 7.4 (150 mm^3)] was pipetted into a reaction vessel containing KLH [0.6 mg in 0.1 mol dm^{-3} phosphate buffer, pH 7.4 (60 mm^3)] and then 25% aq. glutaraldehyde (12 mm^3) was added. After gentle agitation, first for 3 h at room temperature and then for 12 h at 4 °C, the reaction mixture was diluted with 150 mmol dm^{-3} NaCl to a final concentration of 80 μg $T\beta_{10}$ cm^{-3}. The diluted mixture was used for immunization without any special purification.

A New Zealand White rabbit was immunized with 40 μg of recombinant $T\beta_{10}$, as the KLH-conjugate emulsified with Complete Freund's Adjuvant, according to the method of Vaitukaitis.[27] The first booster injection was given six weeks after the first immunization, whereas subsequent injections were given at 4-week intervals. Antiserum was collected 10–12 days after each booster injection.

ELISA test

ELISA microwells were coated with aq. $T\beta_{10}$ or $T\beta_4$ (0.1 μg cm^{-3}; 200 mm^3) and dried overnight at 50 °C. A sample (200 mm^3) of the antiserum against $T\beta_{10}$, diluted 1/15 000, 1/30 000,

1/45 000, 1/60 000, 1/75 000 or 1/100 000 with buffer B, pH 7.4 (consisting of 15 mmol dm^{-3} KH_2PO_4, 8 mmol dm^{-3} $Na_2HPO_4 \cdot 2H_2O$, 2.7 mmol dm^{-3} KCl, 150 mmol dm^{-3} NaCl, 0.2% bovine serum albumin and 0.05%, v/v, Tween 20) were added to the microwells (in triplicate) and incubated for 2 h at 37 °C. Then the liquid was discarded and the microwells were washed three times with buffer A, pH 7.4 (consisting of 15 mmol dm^{-3} KH_2PO_4, 8 mmol dm^{-3} $Na_2HPO_4 \cdot 2H_2O$, 2.7 mmol dm^{-3} KCl, 150 mmol dm^{-3} NaCl and 0.05%, v/v, Tween 20). Next, an IgG/HRP solution (200 mm^3; diluted 1/1000 in buffer B) was added to each microwell and incubated for 2 h at 37 °C. After the liquid had been discarded, the wells were again washed three times with buffer A. Finally, an ABTS/H_2O_2 (0.1%/0.003%) solution in buffer C, pH 4.5 [(200 mm^3); citrate/phosphate buffer (consisting of 0.1 mol dm^{-3} citric acid and 0.1 mol dm^{-3} $Na_2HPO_4 \cdot 2H_2O$)] were pipetted into each microwell. The absorbance was measured after development (30 min) in a microtiter plate reader (Model MR 5000, Dynatech Laboratories, Torrance, USA) at 405 nm.

Solid-phase synthesis

The aminomethyl resin was prepared by direct amidomethylation of copoly(styrene–1% 1,3-divinylbenzene) resin as described.[23] The trityl-type handle 4-carboxy-4′-cyanotriphenylmethanol was prepared in a similar way to that described elsewhere for the 4-carboxy-2′-fluoro(chloro)-triphenylmethanol handle.[22] The handle used in this work was linked to the aminomethyl resin by the HOBt/DIPC method. The bromo-4-cyanotrityl resin was esterified with Fmoc-Ser(Bu^t)-OH using DIEA. After deprotection of the amino group, the capacity of the H-Ser(Bu^t)-4-cyanotrityl resin was measured by the Kaiser ninhydrin test and found to be 0.56 mmol g^{-1}.

Synthesis of the peptide was carried out manually by using Fmoc amino acids. The side chain functional groups of aspartic acid and glutamic acid were protected as *tert*-butyl esters, Fmoc-Glu(OBu^t)-OH and Fmoc-Asp(OBu^t)-OH, threonine and serine as *tert*-butyl ethers, Fmoc-Thr(Bu^t)-OH and Fmoc-Ser(Bu^t)-OH, lysine as its *tert*-butoxycarbonyl derivative, Fmoc-Lys(Boc)-OH, and arginine as the 2,2,5,7,8-pentemethylchromane-6-sulfonyl derivative, Fmoc-Arg(Pmc)-OH.

A sample of the resin (72 mg) was placed in a reaction vessel. Amino acids were coupled as their free acids (10 mol equiv.) by addition of DIPC (10 mol equiv.) and HOBt (10 mol equiv.). The couplings were performed in DMF. After coupling, the resin was rinsed with DMF, before the success of the coupling was checked with the Kaiser ninhydrin test. When the Kaiser test was, even slightly, positive, the resin was acetylated by treatment (10 min) with acetic anhydride–DIEA (2:1, v/v).

Deprotection of the Fmoc group was achieved by repetitive treatment (10 min) with 20% piperidine in DMF. After final removal of the Fmoc group, the resin was washed successively with DMF and propan-2-ol.

An acetyl group was incorporated into the peptide N-terminus by using the same procedure as in capping with acetic anhydride–DIEA (2:1, v/v).

The resin-bound peptide was removed from the resin and amino acid side chains were deprotected by treatment (2 h) with a cocktail of TFA, water and scavengers (TFA 60%, thioanisole 5%, phenol 5%, water 5%, EDT 2.5%, CH_2Cl_2 22.5%). After washing of the resin with TFA–CH_2Cl_2 (6:4, v/v) and removal of the organic solvents, the crude product was precipitated with cold diethyl ether and dissolved in water to be purified.

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