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Comparative Evaluation of Four Trityl-Type Amidomethyl Polystyrene Resins in Fmoc Solid Phase Peptide Synthesis

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Abstract: Four trityl-type (i.e. non-substituted trityl-, o-Cl-trityl-, o-F-trityl- and p-CN-trityl-) amidomethyl polystyrene resins were evaluated comparatively, in terms of the stability of the trityl-ester bond in slightly acidic dichloromethane solutions, and the p-CN-trityl-amidomethyl polystyrene resin was found to be the most stable of them. The above resins were applied, in parallel with Wang benzyl-type resin, well known for its stability in mild acidic conditions, to the Fmoc solid phase synthesis of the 43-amino acid residue long bioactive peptide thymosin beta-4. Independent of their differences in acid sensitivity, the resins seemed to function equally well under the conditions used, since pure thymosin beta-4 was obtained with a final yield of approximately 30% from each resin. The trityl-type amidomethyl polystyrene resins were also applied, in parallel with the Wang resin, to the Fmoc solid phase synthesis of a bioactive peptide containing proline at its *C*-terminus, i.e. the *N*-terminal tetrapeptide of thymosin beta-4, AcSDKP. In this case, the best yield (87%) was obtained with the o-Cl-trityl-amidomethyl polystyrene resin, which may be the resin of choice, of those studied, for the Fmoc solid phase peptide synthesis.

Keywords: trityl-type amidomethyl polystyrene resins; Fmoc solid phase peptide synthesis; thymosin beta-4; AcSDKP

INTRODUCTION

Since Merrifield introduced solid phase peptide synthesis (SPPS), various protecting strategies have been developed, the most common being those of Boc/Bzl and Fmoc/t-Bu. Although the Boc strategy [1] is widely used in SPPS, in many cases it has been replaced by the Fmoc approach [2]. The advantages of Fmoc-SPPS include milder reagents, the ability to monitor Fmoc deprotection by UV, and in general high yields of the desired peptide [3].

p-Alcoxy-benzyl-type resins, e.g. the commercially available Wang resin [4] (Scheme 1A), have been applied widely to Fmoc-SPPS. Nevertheless, benzyl-type resins have two main disadvantages. First, the

Abbreviations: ABTS, 2,2′-azinobis-(3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid) diammonium salt; Boc, tert-butoxycarbonyl; Bzl, benzyl; DCM, dichloromethane; DIEA, *N*,*N*′-diisopropyloethylamine; DIC, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*′-dimethylformamide; EDT, ethane-1,2-dithiol; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electron-spray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; ID, internal diameter; IgG, γ-immunoglobulin; PITC, phenylisothiocyanate; RP-HPLC, reverse phase high-performance liquid chromatography; t-Bu, tert-butyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIPS, triisopropylsilane; UV, ultraviolet.

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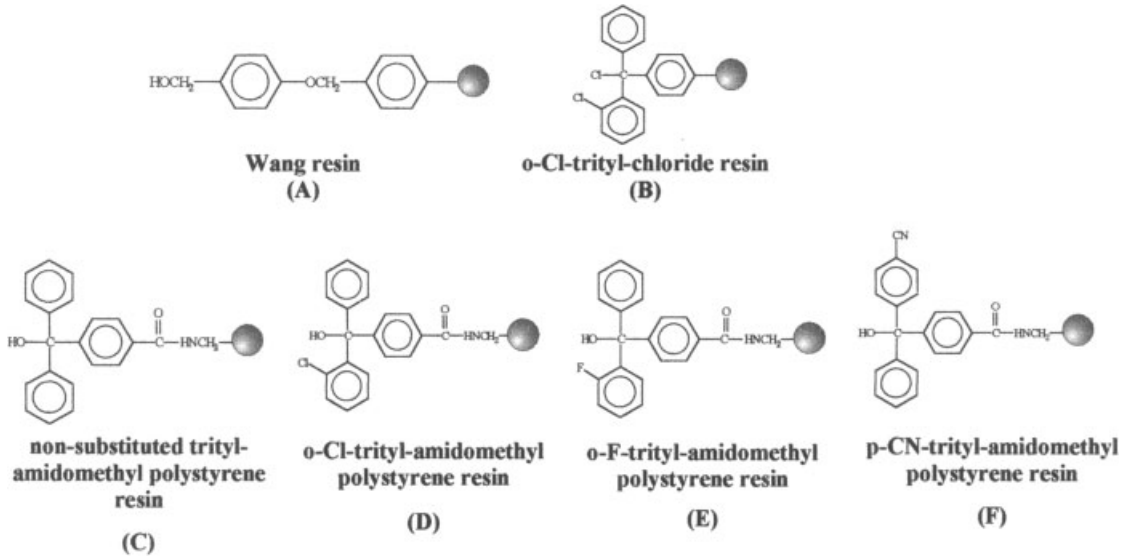
esterification of Fmoc-protected amino acids is often accompanied by a significant degree of racemization and dipeptide formation [5]. Second, increased loss of the *C*-terminal dipeptide, as a diketopiperazine derivative, is often observed [6,7], especially when proline (or glycine) is the *C*-terminal amino acid [8].

Trityl-type resins, such as the commercially available o-Cl-trityl chloride resin (Scheme 1B) described by Barlos *et al.* [5], have also been used successfully in Fmoc-SPPS. An advantage of these resins is that the anchoring of the Fmoc-protected first amino acid on them is easily achieved using the DIEA salt of the amino acid, a procedure avoiding racemization. Also, as reported [9], the o-Cl-trityl chloride resin does not favour the formation of diketopiperazine, due to the steric hindrance of the bulky o-Cl-trityl-group, and this minimizes losses of the *C*-terminal dipeptide. There is little information, however, as to whether other trityl-type resins have the latter advantage. On the other hand, the main disadvantage of the o-Cl-trityl chloride resin, and possibly other trityl-type resins, is that the trityl-ester bond seems to be labile in the mild acidic solutions used during the coupling reaction [10].

During the past decade, our group has prepared in house and gained experience with four trityl-type resins derived from aminomethyl polystyrene, namely non-substituted trityl-amidomethyl polystyrene (which is also commercially available as Bayer's resin), o-Cl-trityl-amidomethyl polystyrene, o-F-trityl-amidomethyl polystyrene and p-CN-trityl-amidomethyl polystyrene resins [11–14], (Scheme 1 C–F), and their performance in Fmoc-SPPS. In the present work, the above trityl-type amidomethyl polystyrene resins were evaluated comparatively in terms of the stability of the trityl-ester bond formed between the resin handle molecule and a Fmoc-protected amino acid in slightly acidic dichloromethane solutions. The trityl-type amidomethyl polystyrene resins were then applied in parallel with the Wang benzyl-type resin well known for its stability in mild acidic solutions to the synthesis of thymosin beta-4, a natural bioactive peptide of 43-amino acid residues. The trityl-type amidomethyl polystyrene resins were also evaluated comparatively, in parallel with the Wang resin, for whether they suffer peptide loss (which can be attributed to diketopiperazine formation) during the Fmoc solid phase synthesis of a bioactive peptide ending with proline, i.e. the *N*-terminal tetrapeptide of thymosin beta-4, AcSDKP.

MATERIALS AND METHODS

The Fmoc-protected amino acids and the cross-linked polystyrene copolymer (100–200 mesh) were purchased from the CBL Company (Patras, Greece). DMF, DCM, THF, TFA and AcCN (analytical



Scheme 1 Wang benzyl-type resin (A), Barlos' o-Cl-trityl-chloride resin (B) and four different trityl-type amidomethyl polystyrene resins (C–F).

grade) were obtained from Merck or Sigma. The aminomethyl polystyrene resin was prepared in our laboratory as previously described [15]. Goat anti-rabbit IgG conjugated to HRP (IgG/HRP) and ABTS were obtained from Sigma. Tween 20 and H_2O_2 were purchased from Merck and Ferak, respectively. The ELISA microplates were obtained from Costar-Corning. Thymosin beta-4 used in the ELISA test (positive control) was obtained from Peninsula. Thymosin beta-10 used in the ELISA test (negative control) was prepared by SPPS as previously described [12]. The polyclonal antiserum used in the ELISA test was raised in New Zealand white rabbits [16] against natural thymosin beta-4, which had been isolated as described previously [16].

General Analytical Methods

Analytical RP-HPLCs were performed on a Waters HPLC System (pump 616, detector 996 PDA) using a LiChrospher RP C18 column (250 × 4.6 mm ID; 5 μm particle size; Merck). The solvent system contained 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). The peptides were eluted by applying 10% solvent B for 10 min, then a linear gradient of 10–20% B in 10 min and, finally, a linear gradient of 20–45% B in 45 min [17]. The flow rate was set at 1.3 ml/min. Peptide peaks were detected photometrically (220 nm).

Preparative RP-HPLCs were performed on a Waters HPLC System (pump 600E, detector UV-484) using a Prep Nova-Pak HR C18 column (300 × 19 mm ID, 6 μm particle size, 60 A, Waters). The solvent system contained 0.05% TFA in water (solvent A) and 60% acetonitrile in solvent A (solvent B). A linear gradient from 0 to 60% solvent B was applied within 50 min, at a flow rate of 8 ml/min. Peptide peaks were detected photometrically (220 nm).

Amino acid analyses were performed using the Waters PICO-TAG amino acid analysis system. Briefly, samples were hydrolysed in vapour-phase HCl (6 M; Pierce) for 1 h at 150 °C. The acid hydrolysate was re-dried using a 2/2/1, v/v/v, mixture of ethanol, water and triethylamine and then derivatized after storage for 20 min at room temperature with a mixture consisting of 7/1/1/1, v/v/v/v, ethanol, triethylamine, water and PITC. The derivatized product was analysed by RP-HPLC on a column (39 × 15 mm ID, 5 μm particle size, Waters) at 38 °C using the PICO-TAG gradient system slightly modified.

For the ESI-MS analysis, test peptide solutions in 50% aqueous acetonitrile (containing 1% acetic acid) were infused into an electrospray interface mass spectrometer (Finnigan AQA Navigator) operating at positive mode, at a flow rate of 100 μl/min, using a HPLC pump (Waters Alliance 2690). In the electrospray source the capillary/skimmer potential difference varied between 70 and 150 V; the other source lenses were held at potentials that optimized the signal intensity. Hot nitrogen gas obtained by a nitrogen generator (Dominick-Hunter UHPLCMS 10) was used for desolvation. The charge of each ion and molecular mass of the peptide were determined by deconvolution algorithms.

Preparation of the Trityl-type Handles

Trityl type handles 4-carboxy-triphenylmethanol, (R,S) 2-chloro-4′-carboxy-triphenylmethanol, (R,S) 2-fluoro-4′-carboxy-triphenylmethanol and the (R,S) 4-cyano-4′-carboxy-triphenylmethanol were performed as described previously [11,14].

Preparation of the Trityl-type Amidomethyl Polystyrene Resins

The corresponding trityl-type handle (2 mmol, 2 mol equiv) and HOBt (2 mmol, 2 mol equiv) were dissolved in an as small as possible volume of DMF. DIC (2 mmol, 2 mol equiv) was added and the mixture was left under stirring at 25 °C for 30 min. Then, it was poured into a suitable SPPS glass vessel with a porous filter, containing aminomethyl polystyrene resin (1 g, 1 mmol/g), and allowed to react for 24 h. Afterwards, the mixture was filtered and the polymer was successively washed with DMF, DCM, methanol (3 × 10 ml, 1 min), Et_2O (2 × 10 ml, 1 min) and then dried under vacuum (P_2O_5) for 18 h.

Anchoring of the First Fmoc-protected Amino Acid on the Trityl-type Resins

A quantity (1 g, 0.75 mmol/g) of each trityl-type resin prepared was added to a SPPS glass vessel with a porous filter. The resin was allowed to swell in DCM for 5 min and filtered. A AcBr/DCM solution (10 ml, 1/1, v/v) was then added and the vessel was placed on a shaker-table and left for 3 h at room temperature. Then, the resin was filtered and washed with anhydrous THF (4 × 10 ml, 2 min) and dry DCM (10 ml, 2 min). A DCM/THF or DMF (1/1, v/v) solution containing the Fmoc-protected amino acid (1.5 mmol,

2 mol equiv) and DIEA (3.75 mmol, 5 mol equiv) was added to the glass vessel and the mixture was allowed to react for 3 h. Then, MeOH (1 ml) was added and the vessel was allowed to react for another 30 min. Afterwards, the mixture was successively washed with DMF, DCM (4 × 10 ml, 1 min), MeOH (2 × 10 ml, 1 min) and diethylether (2 × 10 ml, 1 min). After washings, the resin was dried under vacuum (P_2O_5) for 18 h. Resin loading capacity was monitored by determining photometrically (301 nm, ε: 7800 cm^{-1} M^{-1}) the Fmoc-piperidine derivative (Fmoc adduct) in piperidine/DMF (20%, v/v) solution.

Synthesis of Thymosin Beta-4

Thymosin beta-4 was synthesized in parallel on four different resins, namely on the Fmoc-Ser(t-Bu)-trityl-amidomethyl polystyrene (0.42 mmol/g), Fmoc-Ser(t-Bu)-(o-Cl)-trityl-amidomethyl polystyrene (0.44 mmol/g), Fmoc-Ser(t-Bu)-(p-CN)-trityl-amidomethyl polystyrene (0.40 mmol/g) and Fmoc-Ser(t-Bu)-Wang (0.25 mmol/g) resins. Solid phase peptide synthesis was performed manually. The following side chain — protected amino acids were used: Fmoc-Ser(t-Bu)-OH,Fmoc-Glu(t-Bu)-OH, Fmoc-Asp(t-Bu)-OH, Fmoc-Thr(t-Bu)-OH and Fmoc-Lys(Nε-Boc)-OH. Asparagine and glutamine were used without any side chain-protecting group. Coupling was performed by dissolving an excessive quantity (5 mol equiv) of Fmoc-protected amino acid and HOBt in DMF. The solution was cooled on ice and then DIC (5 mol equiv) was added. The reaction mixture was left on ice (10 min) and then at 25 °C (10 min). Afterwards, it was added to the resin and allowed to react for 3 h (asparagine, glutamine and glycine were added to the resin immediately after remaining for 10 min on ice). Coupling efficiency was checked by using the Kaiser ninhydrin test, which should be negative — indicating the presence of less than 0.05% free amino groups. In the case of a slightly positive Kaiser test, the coupling step was prolonged overnight at 4 °C. In the case of an intensive positive result, the coupling step was repeated (double coupling). If the Kaiser test was positive even after a double coupling, persistent free amino groups were acetylated (capping) using a mixture of Ac_2O/DIEA/DMF (2/1/1, v/v/v, 30 min). Removal of the *N*-terminal Fmoc group (deprotection) was performed within 30 min using a 20% (v/v) solution of piperidine in DMF. After the removal of the Fmoc group of the *N*-terminal amino acid (i.e. serine), the peptide synthesized was acetylated with a mixture of Ac_2O/DIEA/DMF (2/1/1, v/v/v, 30 min). Afterwards, the resin was extensively washed with DMF (4×), DCM (6×) and Et_2O (2×). Then it was dried under vacuum (P_2O_5) for 18 h. The peptide synthesized was cleaved from the resin using an appropriate quantity (~20 ml/g resin) of a mixture consisting of TFA (10 ml), thioanisole (0.5 ml), EDT (0.25 ml), phenol (0.75 g) and H_2O (0.5 ml). The cleavage reaction lasted 2.5 h. At the same time the peptide side chain protecting groups were removed. The reaction mixture was filtered in a round bottom flask and the resin was washed with TFA and TFA/DCM (1/1, v/v) (3 × 2 min). The filtrate was evaporated under vacuum (<35 °C) and the peptide precipitated as a solid material by adding Et_2O. After centrifugation, the procedure was repeated three times. The peptide obtained was dried under vacuum (P_2O_5) for 18 h, dissolved in water and lyophilized. Pure thymosin beta-4 was obtained from the lyophilizate using preparative RP-HPLC.

Synthesis of AcSDKP

The *N*-terminal tetrapeptide of thymosin beta-4 AcSDKP was synthesized on five different resins, namely on the Fmoc-Pro-trityl-amidomethyl polystyrene (0.35 mmol/g), Fmoc-Pro-(o-Cl)-trityl-amidomethyl polystyrene (0.49 mmol/g), Fmoc-Pro-(o-F)-trityl-amidomethyl polystyrene (0.45 mmol/g), Fmoc-Pro-(p-CN)-trityl-amidomethyl polystyrene (0.38 mmol/g) and Fmoc-Pro-Wang polystyrene (0.14 mmol/g) resins. Solid phase peptide synthesis was performed manually. The following protected amino acids were used: Fmoc-Ser(t-Bu)-OH, FmocAsp(t-Bu)-OH and Fmoc-Lys(Boc)-OH. Deprotection and coupling successive steps were performed as described for the synthesis of thymosin beta-4, except that the removal of the lysine Fmoc protecting group lasted 20 min, instead of 30 min. After the removal of the Fmoc group of the *N*-terminal amino acid (i.e. serine), the peptide synthesized was acetylated with a mixture of Ac_2O/DIEA/DMF (2/1/1, v/v/v, 30 min). Cleavage of the AcSDKP peptide from each resin was performed by using appropriate quantity (~10 ml/g resin) of a mixture consisting of TFA/H_2O/TIPS (95/2.5/2.5, v/v/v). The cleavage reaction mixture was allowed to react for 2.5 h and then TFA was removed in a rotary evaporator. In the remaining liquid, appropriate quantity of Et_2O (50–100 ml/g) was added and the AcSDKP peptide precipitated as a white solid material, which

was removed by centrifugation. Then, the peptide was washed with Et_2O (3×), dissolved in H_2O and lyophilized.

Immunochemical Evaluation of Synthetic Thymosin Beta-4 Peptide

ELISA buffers. *Buffer A, pH 7.4*: This consisted of 15 mM KH_2PO_4, 8 mM $Na_2HPO_4{\cdot}2H_2O$, 2.7 mM KCl and 150 mM NaCl. *Buffer B, pH 7.4*: This consisted of 15 mM KH_2PO_4, 8 mM $Na_2HPO_4{\cdot}2H_2O$, 2.7 mM KCl, 150 mM NaCl and 0.05%, v/v, Tween 20. *Buffer C, pH 7.4*: This consisted of 15 mM KH_2PO_4, 8 mM $Na_2HPO_4{\cdot}2H_2O$, 2.7 mM KCl, 150 mM NaCl, 0.05%, v/v, Tween 20 and 0.2%, w/v, BSA. *Buffer D, pH 4.5*: This consisted of 0.1 mM citric acid and 0.1 mM $Na_2HPO_4{\cdot}2H_2O$.

ELISA test. ELISA microwells were coated with a solution of each thymosin beta-4 synthesized — or of control peptides — (100 µl, 0.1 µg/ml, in buffer A) and left at 4 °C overnight. Afterwards, the microwells were washed with buffer A (1 × 350 µl). A 2% BSA solution (200 µl, in buffer B) was then added and the microwells were incubated for 1 h at room temperature. Afterwards, the microwells were washed with buffer B (3 × 350 µl). A sample of a rabbit polyclonal antiserum raised against natural thymosin beta-4 (100 µl, diluted 1/2500, 1/5000, 1/10 000, 1/20 000 or 1/50 000 in buffer C) was added to the microwells (in triplicate) and incubated for 2 h at 37 °C. Next, the microwells were washed with buffer C (3 × 350 µl). Then, a goat anti-rabbit IgG/HRP solution (100 µl, diluted 1/3000 in buffer C) was added to the microwells and incubated at 37 °C for 2 h. The microwells were again washed with buffer C (3 × 350 µl). Finally, an ABTS/H_2O_2 solution (100 µl, 0.1%/0.003% in buffer D) was added to the microwells. The optical absorbance of the coloured product developed (30 min) was measured (405 nm) on an ELISA microtitre plate reader (Model MR 5000, Dynatech Laboratories).

RESULTS AND DISCUSSION

Preparation of the Trityl-type Amidomethyl Polystyrene Resins

The trityl-type amidomethyl polystyrene resins were prepared by coupling the corresponding handle molecule to aminomethyl polystyrene using HOBt/DIC. This approach ensures the preparation of highly pure resins, since the handle molecules had been purified and characterized before being coupled to the aminomethyl polystyrene (or other amino functionalized polymers). This is an advantage over other trityl-type resins, which are prepared after direct synthesis of the trityl-type handle on the polymer.

Stability of the Trityl-ester Bond

Stability of the trityl-ester bond was evaluated following the removal of an arbitrarily selected Fmoc-protected amino acid (Fmoc-Ala-OH) from the corresponding resin, over a period of time, in the presence of suitably selected solutions, which were able to mark out differences in acid sensitivity of the resins studied [(i) TFA/DCM, 0.1%, v/v, (ii) AcOH/TFE/DCM, 1/1/8, v/v/v, and (iii) AcOH/DCM, 10%, v/v]. The removal of Fmoc-Ala-OH from the resin was monitored photometrically (301 nm).

According to the results obtained (Figure 1), in the presence of the most acidic solution (i), the Fmoc-Ala-OH was totally removed from the trityl-type amidomethyl polystyrene resins in less than 2 min (not-substituted trityl-), ~12 min (o-Cl-trityl-, o-F-trityl-) or 30 min (p-CN-trityl-). In the presence of the same solution (i), within 2 min, 21%, 32% and 10% of the Fmoc-Ala-OH was removed from the o-Cl-trityl-, o-F-trityl- and p-CN-trityl- amidomethyl polystyrene resin, respectively (Figure 2). In the presence of the less acidic solution (ii), 100%, 8%, 13% and 3.5% of the Fmoc-Ala-OH was removed, within 15 min, from the non-substituted trityl-,

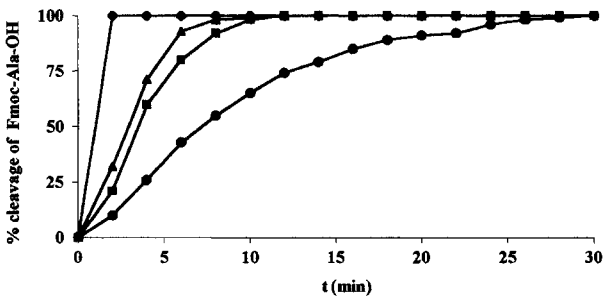


Figure 1 Kinetics of the Fmoc-alanine cleavage from four different trityl-type amidomethyl polystyrene resins in the presence of TFA/DCM, 0.1%, v/v. ♦ non-substituted trityl-amidomethyl polystyrene resin (C); ■ o-Cl-trityl-amidomethyl polystyrene resin (D); ▲ o-F-trityl-amidomethyl polystyrene resin (E); ● p-CN-trityl-amidomethyl polystyrene resin (F).

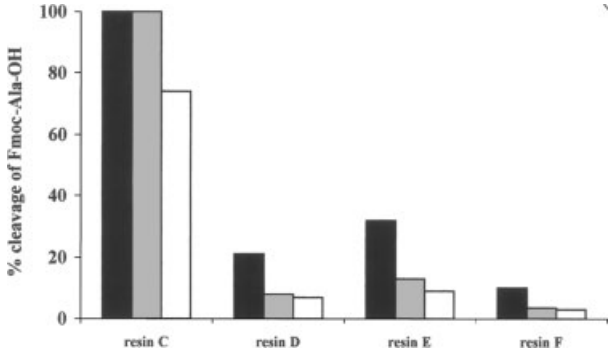


Figure 2 Cleavage of the Fmoc-alanine from four different trityl-type amidomethyl polystyrene resins in the presence of different acidic dichloromethane solutions and at different time intervals. ■ TFA/DCM, 0.1%, v/v, time: 2 min; ■ AcOH/TFE/DCM, 1/1/8, v/v/v, time: 15 min; □ AcOH/DCM, 10%, v/v, time: 30 min; resin C: non-substituted trityl-amidomethyl polystyrene; resin D: o-Cl-trityl-amidomethyl polystyrene; resin E: o-F-trityl-amidomethyl polystyrene; resin F: p-CN-trityl-amidomethyl polystyrene.

o-Cl-trityl-, o-F-trityl- and p-CN-trityl-amidomethyl polystyrene resins, respectively (Figure 2). Finally, in the presence of the least acidic solution (iii), 74%, 7%, 9% and 3% of the Fmoc-Ala-OH was removed, within 30 min, from the non-substituted trityl-, o-Cl-trityl-, o-F-trityl- and p-CN-trityl- amidomethyl polystyrene resins, respectively (Figure 2). According to the above results, the p-CN-trityl amidomethyl polystyrene resin seems to be the most acid stable of the resins studied, followed by the o-Cl-trityl- and the o-F-trityl-amidomethyl polystyrene resins, which showed almost similar stability, and the less stable non-substituted trityl-amidomethyl polystyrene resin.

Application to the Synthesis of Thymosin Beta-4

The above mentioned non-substituted trityl-, o-Cl-trityl- and p-CN-trityl- amidomethyl polystyrene resins were applied to the synthesis of thymosin beta-4. o-F-trityl-amidomethyl polystyrene resin was not included in the present study, because it showed similar trityl-ester bond stability to the o-Cl-trityl- amidomethyl polystyrene resin. For comparison reasons, the Wang benzyl-type resin, which is well known for its stability in mild acidic conditions, was also applied to the synthesis of the same peptide.

Thymosin beta-4 was selected because of its rather long size (43 amino acids, Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES) [18] and, thus, the apparently increased possibility of obtaining a different final percentage yield for peptides synthesized on different resins, depending on the resin acid sensitivity. On the other hand, thymosin beta-4 does not contain special amino acid sequence features, such as a *C*-terminal proline or glycine residue; thus, the yield of its synthesis on different resins is not expected to be seriously influenced by other parameters (e.g. diketopiperazine formation) besides resin acid sensitivity. Another reason for its selection was the experience accumulated by our group on the thymosin peptide family during the past 15 years [12,19–21] and the availability of reagents and methodology for the biological (immunochemical) evaluation of the peptides to be synthesized [16]. On the other hand, since thymosin beta-4 is a natural bioactive peptide of great biochemical [22,23], diagnostic [24] and maybe therapeutic [21,25] interest, its preparation at mg quantities through SPPS is obviously useful and important. Thymosin beta-4 was first synthesized on a hydroxymethylphenylacetamidomethyl (PAM) resin following the Boc-SPPS strategy [17], while synthesis of the peptide or analogues of it following solution methods was later reported [26,27]. Synthesis of thymosin beta-4 by Fmoc-SPPS has been mentioned in an abstract [28], work in which, however, no trityl-type resins were used.

On all resins, thymosin beta-4 was synthesized following the Fmoc strategy and using the DIC/HOBt functioning approach, as described in Materials and Methods. Double coupling, indicating difficult peptide sequences, was necessary for the residues S_1, D_2, K_3, P_4, D_5, I_9, K_{14}, K_{16}, K_{25}, L_{28} and S_{30}. Crude synthetic thymosin beta-4 peptides were obtained at a similar yield (~90%) from all the resins studied, while the analytical HPLC profiles of all crude products were similar as well (Figure 3). As can be observed in Figure 3, the amino acid methionine was oxidized during the preparation of thymosin beta-4, mainly because of the cleavage conditions used, leading to the formation of the oxidized form of the peptide, i.e. thymosin beta-4 sulfoxide [29,30]. After their purification with preparative HPLC, all synthetic thymosin beta-4 peptides were obtained in very pure form, as revealed by analytical HPLC, amino acid analysis and ESI-MS. In addition, all synthetic peptides were obtained at a very satisfactory final yield (~30%, Table 1), much higher than that previously reported (~5%, ~12%) for the Boc-solid phase synthesis [17] of thymosin beta-4 or the liquid phase classical approach [26], respectively. Moreover, the final percentage yield would have been

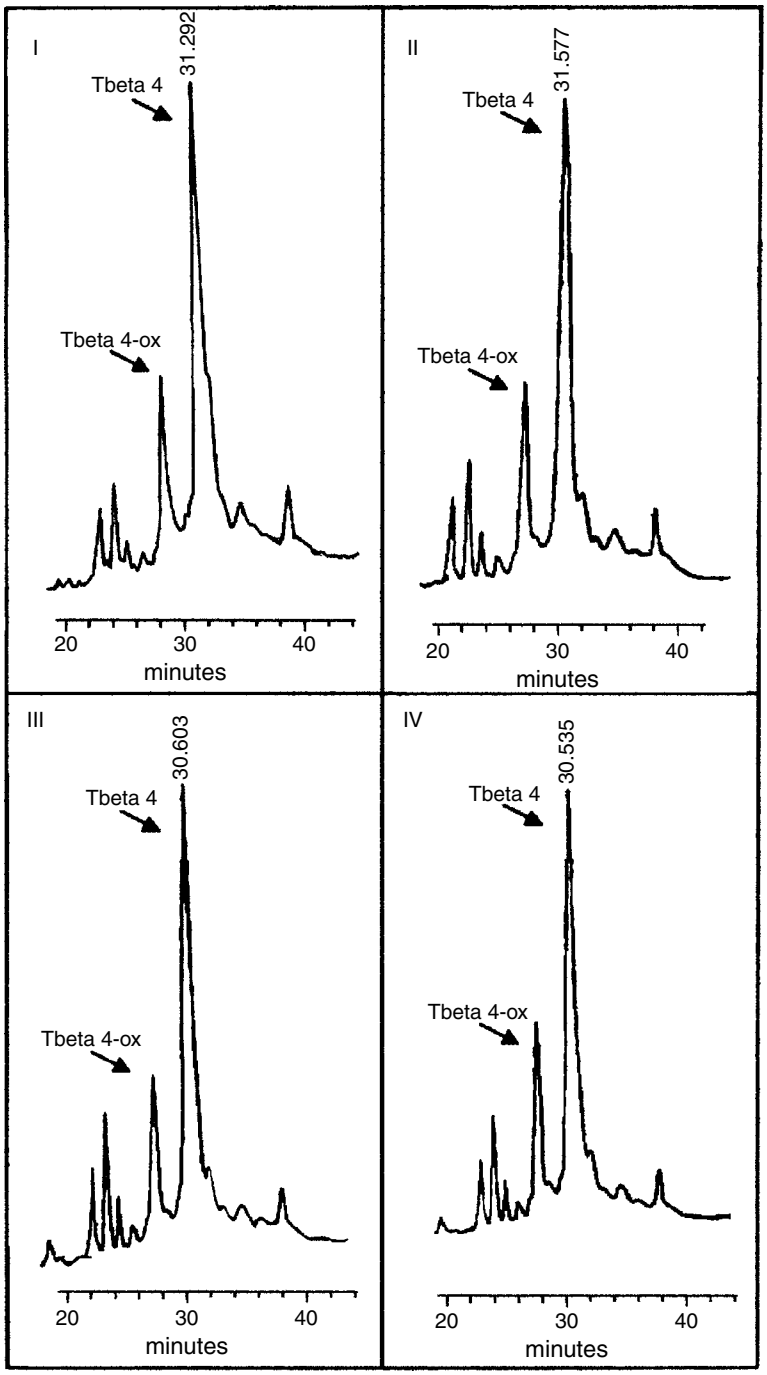


Figure 3 Analytical RP-HPLC profiles of the crude products obtained for thymosin beta-4 peptides synthesized on different resins. I: Wang-resin (A); II: non-substituted trityl-amidomethyl polystyrene resin (C); III: o-Cl-trityl-amidomethyl polystyrene resin (D); IV: p-CN-trityl-amidomethyl polystyrene resin (F).

higher, if the oxidation of thymosin beta-4 sulfoxide had been avoided. These findings agree with previous results of ours [12], according to which thymosin beta-10, i.e. the 43-amino acid residue homologous peptide that accompanies thymosin beta-4 in human tissues, was synthesized by Fmoc-SPPS on the p-CN-trityl-amidomethyl polystyrene resin, with a similarly high final yield.

Synthetic thymosin beta-4 obtained from the four different resins studied were used for coating

Table 1 Solid Phase Synthesis of Thymosin beta-4: Yield Using Different Resins

Resin	Yield of thymosin beta-4 (crude product) (%)	Yield of thymosin beta-4 (pure product) (%)
p-CN-trityl-amidomethyl polystyrene resin (F)	91	30
o-Cl-trityl-amidomethyl polystyrene resin (D)	89	29
Non-substituted trityl-amidomethyl polystyrene resin (C)	85	28
p-alkoxy-benzyl-resin (Wang resin) (A)	91	30

ELISA microwells and then incubated with the anti-thymosin beta-4 antiserum, which had been raised against the natural peptide [16], in various dilutions, from 1/2500 down to 1/50 000. Commercially available thymosin beta-4 and thymosin beta-10 previously synthesized in our laboratory [12] were used as positive and negative control peptides, respectively. All synthetic thymosin beta-4 peptides were recognized by the anti-thymosin beta-4 antiserum, which proves the immunoreactivity of these synthetic products (Figure 4). The titre curves of the synthetic thymosin beta-4 peptides, either those obtained with the trityl- type resins or that obtained with the Wang benzyl-resin, were very similar (Figure 4), which indicates that their immunochemical characteristics correlate very well. The above titre curves were also very similar to that of the commercially available thymosin beta-4. Thymosin beta-10 was hardly recognized by the anti-thymosin beta-4 antiserum, confirming the specificity of the antiserum and, consequently, of the ELISA test.

Taken together, the above results indicate that the differences observed among the trityl-resins studied, concerning their trityl-ester bond stability in slightly acidic solutions, do not seem to influence their overall functioning in Fmoc-SPPS, at least under the conditions used.

Application to the Synthesis of the *N*-terminal Tetrapeptide of Thymosin Beta-4, AcSDKP

According to the literature [8], Fmoc-SPPS of peptides containing proline (or glycine) at their *C*-terminus is often influenced by the formation of diketopiperazine analogues, which are formed as side products mainly during the removal of the Fmoc group from the *C*-terminal dipeptide synthesized on the resin and the next coupling reaction step.

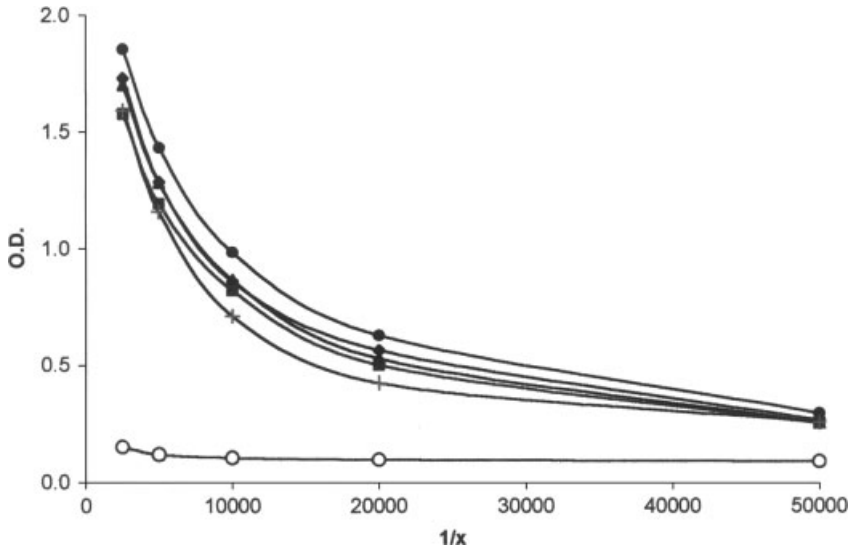


Figure 4 Anti-thymosin beta-4 antiserum titre curves obtained with the test peptides ▲ thymosin beta-4 synthesized on Wang-resin (A); ♦ thymosin beta-4 synthesized on non-substituted trityl-amidomethyl polystyrene resin (C); ■ thymosin beta-4 synthesized on o-Cl-trityl-amidomethyl polystyrene resin (D); ● thymosin beta-4 synthesized on p-CN-trityl-amidomethyl polystyrene resin (F); the positive control peptide, +, commercially available thymosin beta-4, or the negative control peptide, ○, thymosin beta-10.

This leads to *C*-terminal dipeptide loss from the resin and, consequently, to low overall yields. Diketopiperazine formation is especially prominent when alkoxy benzyl type resins are used, while the hindered o-Cl-trityl chloride polystyrene resin [5] has been reported to be convenient for avoiding this side reaction [8]. However, there is limited information concerning whether other trityl-type resins share the same advantage. Thus, it was considered worthwhile to evaluate the performance of the above mentioned non-substituted trityl-, o-Cl-trityl-, o-F-trityl- and p-CN-trityl-amidomethyl polystyrene resins in the synthesis of a bioactive peptide ending with proline, namely the *N*-terminal tetrapeptide of thymosin beta-4, AcSDKP. For comparison reasons, the Wang benzyl-type resin, which is known to suffer diketopiperazine formation during the synthesis of *C*-terminal proline peptides [8], was applied to the synthesis of the same peptide in parallel with the trityl-type resins. The AcSDKP was selected because is very short and, therefore, an ideal model peptide for easily evaluating yield losses during its synthesis; in addition, it is associated with the thymosin peptide family, especially thymosin beta-4, while it has a great biochemical and therapeutic interest itself [31].

On all resins, AcSDKP was synthesized following the Fmoc strategy and using the DIC/HOBt functioning approach, as described in Materials and Methods. Crude synthetic AcSDKP was obtained from the o-Cl-trityl-, o-F-trityl-, non-substituted trityl- and p-CN-trityl- amidomethyl polystyrene resins as well as from the Wang benzyl-type resin at 87%, 77%, 69%, 23% and 33% final yield, respectively (Table 2). Synthetic AcSDKP was obtained in very pure form, as revealed by analytical HPLC, amino acid analysis and ESI-MS. As expected, the percentage yield obtained with the Wang benzyl-type resin was one of the lowest, possibly due to the diketopiperazine formation, as proposed in the literature. If this is the case, it seems that the trityl group which is present in the trityl-type amidomethyl polystyrene resins cannot, by itself, totally prevent the removal of the *C*-terminal dipeptide after the intramolecular aminolysis taking place, thus leading to a non-optimal percentage yield (Table 2, non-substituted trityl-amidomethyl polystyrene resin). However, the o-Cl- as well as the o-F-substitution seems to protect the trityl ester bond at a quite satisfactory extent, leading to the highest yields obtained (87% and 77%, respectively). Interestingly, p-CN-trityl amidomethyl polystyrene resin led to a low percentage yield. This can be associated with increased diketopiperazine formation, maybe favoured by the presence of the stable trityl-oxy anion formed after the p-CN-substitution, which is the best leaving group among those putatively formed by the other trityl-type amidomethyl polystyrene resins.

Table 2 Solid Phase Synthesis of AcSDKP: Yield Using Different Resins

Resin	Yield of AcSDKP (pure product) (%)
o-Cl-trityl-amidomethyl polystyrene resin (D)	87
o-F-trityl-amidomethyl polystyrene resin (E)	77
Non-substituted trityl-amidomethyl polystyrene resin (C)	69
p-CN-trityl-amidomethyl polystyrene resin (F)	23
p-alcoxy-benzyl-resin (Wang-resin) (A)	33

Taken together, the above results indicate that the trityl-type resins studied in this work may function in a different way when applied to the synthesis of special peptide sequences, e.g. *C*-terminal proline peptides. In the latter case, the o-Cl-trityl-amidomethyl polystyrene seems to be the resin of choice.

CONCLUSION

Four trityl-type (non-substituted trityl-, o-Cl-trityl-, o-F-trityl- and p-CN-trityl) amidomethyl polystyrene resins were prepared and evaluated in parallel. Of them, the p-CN-trityl- amidomethyl polystyrene resin seemed to be the most stable in the slightly acidic dichloromethane solutions. The above resins seemed to function equally well when they were applied to the Fmoc-SPPS, including the synthesis of long bioactive peptides, such as thymosin beta-4, despite their slight differences in acid sensitivity. However, the o-Cl-trityl-amidomethyl polystyrene resin seemed to function better than the other trityl-type amidomethyl polystyrene resins when the resins were applied to the synthesis of the

N-terminal bioactive tetrapeptide of thymosin beta-4, AcSDKP, and this may be true for other *C*-terminal proline peptides as well. Thus, the o-Cl-trityl-amidomethyl polystyrene resin may be the resin of choice among those studied in this work for application to the Fmoc solid phase peptide synthesis.

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