

Solid-phase peptide synthesis using a new PS-TTEGDA resin: Synthesis of pardaxin (1-26)

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Tetraethyleneglycol diacrylate (4%)-crosslinked polystyrene has been used as solid support for peptide synthesis. This resin undergoes extensive swelling in a broad range of solvents with varying polarity. The resin beads after chloromethylation have been used in the synthesis of 26-residue peptide corresponding to the hydrophobic amino terminal region of pardaxin from *Pardachirus pavoninus* (H-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-OH). The first amino acid Boc-Leu is attached to the chloromethyl resin by cesium salt method in a capacity of 1.8 mmol/g and the remaining amino acids are incorporated into this support following the standard solid-phase methodology of peptide synthesis. The completely deprotected peptide is cleaved from the resin by trifluoroacetic acid, isolated in solid form, purified by FPLC and characterised by amino acid analysis and gas phase protein sequencing. The free peptide has an α -helical conformation as revealed by CD measurement. This synthesis illustrates the application of the novel flexible support for the synthesis of 26-residue bio-active peptide.

Development of new resins with optimum hydrophilic-hydrophobic balance has been a challenge for polymer chemists ever since the introduction of solid phase peptide synthesis by Merrifield¹. Even though divinylbenzene-crosslinked polystyrene is being widely used as solid support for peptide synthesis, the purity criteria is not satisfied. The success of solid phase organic reaction is often affected by the choice of polymeric support, with regard to the mechanical stability, swellability and compatibility with a range of hydrophilic and/or hydrophobic solvents. This led to the development of various hydrophilic polymeric supports for peptide synthesis²⁻⁷. Polyethyleneglycol grafted polystyrene resins are being used as solid support in peptide synthesis³⁻⁵. We have designed and synthesised tetraethyleneglycol diacrylate (TTEGDA)-cross-linked polystyrene as a flexible, highly swelling and mechanically stable polymer support for solid-phase peptide synthesis (SPPS)^{8,9}. A unique family of crosslinked supports which include triethyleneglycol dimethacrylate¹⁰, hexanediol diacrylate¹¹ and butanediol dimethacrylate¹²-crosslinked polystyrene resins

were tested and applied for SPPS from this laboratory. The PS-TTEGDA resin has an open net-work of polymer chains allowing easy mass transport, and polyoxyethylene crosslinks impart a hydrophilic character to the polymer support. This makes the polymer matrix ideal for carrying out gel phase peptide synthesis.

Pardaxin is a shark repellent peptide toxin isolated from the secretion of the Red Sea Moses sole fish¹³. Pardaxin exhibits pathological and pharmacological effects like smooth muscle contraction in guinea pig ileum, activation of acetyl/cholinesterase virion destruction and lysis of red blood cells. These effects are presumed to arise from their ability to perturb the membranes. In this paper, we report the utility of PS-TTEGDA resin for the synthesis of a 26-residue peptide corresponding to the N-terminal region of pardaxin¹⁴. It was observed that even high-capacity chloromethylated resins could be used effectively for the solid-phase synthesis in the case of this new resin^{9a-c}. The stepwise coupling and deprotection steps in this synthetic strategy were observed to proceed in near-quantitative yield supporting the positive role of the hydrophilic and flexible polyoxyethylene crosslinks in facilitating the synthetic reactions. This will also explain the ability of the support to accommodate

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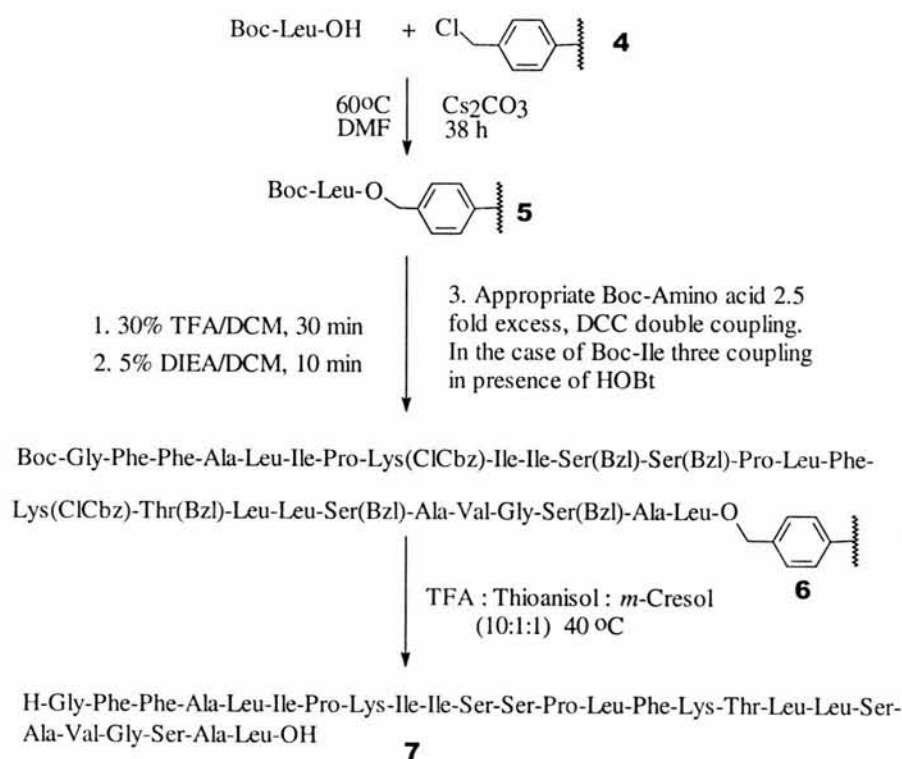
the growing peptide chain even at high capacity. The completely deprotected peptide was cleaved from the resin using anhydrous trifluoroacetic acid (TFA) and purified by Fast Protein Liquid Chromatography (FPLC). The purified peptide which was isolated in 20% overall yield was characterised by amino acid analysis and N-terminal sequencing. This cross-linked polymer serves as a new class of insoluble but highly solvating resins for solid-phase peptide synthesis.

Suspension polymerisation has been proved to be the most useful technique for synthesising crosslinked polymeric supports, principally because of the extremely convenient physical form of the beaded product which lends itself for further conversion¹⁵. The topography of the polymer matrix is determined by the chemical nature of monomers and the mole percentage of crosslinking agent. The crosslinking provides the desired mechanical integrity for the resin. PS-TTEGDA (4%) co-polymer **3** was prepared by suspension polymerisation. The resin was refluxed with TFA at 75 °C for 6 hr to remove any linear polystyrene. There was no weight loss even after prolonged TFA treatment. The ester bonds of the crosslinking agent were found to be stable under these conditions as confirmed by IR spectra. The bands at 1720 cm⁻¹, 1480 cm⁻¹ (ester) and 1150 cm⁻¹ (ether) remained intact. It is possible that the ester linkages in the interior of the crosslinked matrix resist the

hydrolysis due to the relative inaccessibility for the hydrolytic reagents¹⁶. A comparative study of swelling properties of DVB and TTEGDA crosslinked polystyrene supports showed that PS-TTEGDA resin has an enhanced swelling capacity in a wide range of solvents^{9a,c}. The resin was sieved and beads of size ranging from 100-200 mesh were used for functionalization. The polymer beads were chloromethylated using ZnCl₂ catalyst to obtain chloromethyl resin¹⁷ **4** (2.1 mmol Cl/g).

Application of PS-TTEGDA Resin

The polymer beads **3** of uniform size (100-200 mesh) were used in SPPS. Boc-Leu was attached to the chloromethyl resin **4** using the cesium salt procedure¹⁸. Stirring of the resin during this step caused problem of clogging during the synthesis. Hence, stirring was avoided. There was no residual chlorine after the reaction was over as indicated by Volhard's method¹⁹. The peptide was assembled by the stepwise incorporation of Boc-amino acids using the DCC coupling procedure manually.^{19,20} Double coupling procedure using 2.5-fold molar excess of Boc-amino acid was followed throughout the synthesis. HOBt was used along with DCC for coupling of Boc-Ile. Boc-group was deprotected using 30% TFA-CH₂Cl₂ and stepwise synthesis was carried out as shown in **Scheme I**. Progress of the coupling



Scheme I – Synthesis of pardaxin (1-26) using PS-TTEGDA resin

reaction was monitored by the semi-quantitative ninhydrin method²¹ and coupling yields averaging 99.8% were obtained in each coupling. After the synthesis, the resin showed nearly a five-fold weight increase which is in agreement with the molecular weight of the peptide. Cleavage of the completely deprotected peptide was accomplished by treatment with trifluoroacetic acid at 40 °C in the presence of thioanisole and *m*-cresol for 24 hr under completely anhydrous conditions.^{22,23} The yield of acidolytic cleavage was about 95% which was determined by the measurement of the remaining peptide still bound to the resin. The cleaved peptide was isolated by filtration and evaporation of the filtrate under vacuum. The peptide **7** was precipitated as a white powder using cold dry ether. The white residue was thoroughly washed with cold ether and solid product reprecipitated out from methanol-ether mixture. The crude product was further purified by FPLC to obtain 26-residue pure peptide **7** with an overall 20% yield based on starting Leu-polymer **5** (Figure 1). A single major peak was obtained at 58% acetonitrile (time 39 min) which was collected and lyophilised to get the pure peptide **7**. Amino acid analysis: Found (Cal) : Leu, 4.91 (5); Ala, 2.87 (3); Ser, 3.51 (4); Gly, 2.07

(2); Val, 0.97 (1); Thr, 0.904 (1); Lys, 2.00 (2); Phe, 3.02 (3); Pro, 2.1 (2); Ile, 2.35 (3). The values of Ile, Ser are slightly low due to partial cleavage of Ile-Ile and Ser-Ser bond under the hydrolytic conditions used. The peptide **7** in methanol showed a circular dichroism curve with a strong positive peak at 195 nm and two strong negative bands at 209 nm and 222 nm indicating a α -helical structure (Figure 2). This observation is an indirect proof to demonstrate the optical purity of the peptide.

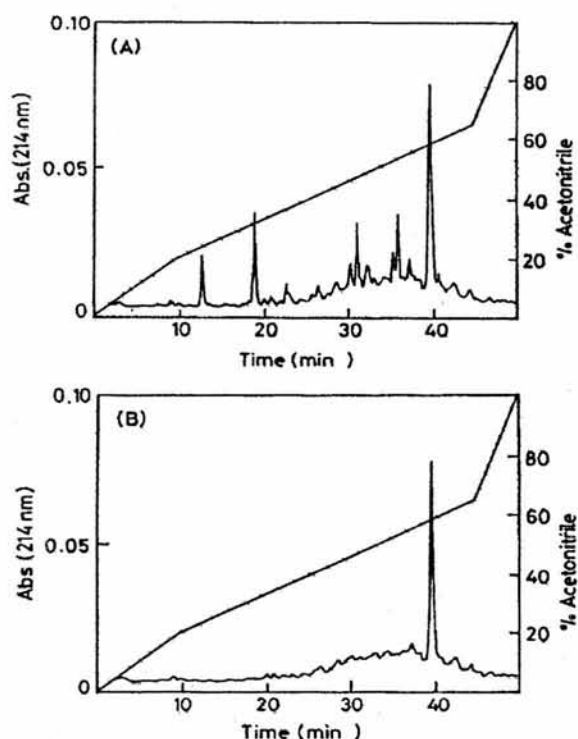


Figure 1 — Analytical FPLC of total crude (A) and purified (B) 26-residue peptide. A Pharmacia C-18 column was used. Elution was carried out with a gradient of MeCN on 0.1% TFA as indicated in the figure at 0.5 mL/min flow rate.

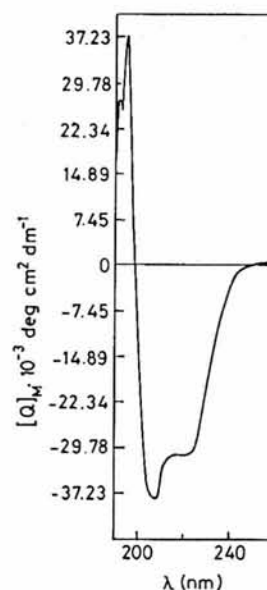


Figure 2 — CD spectrum of Pardaxin (1-26) in methanol

The peptide was further characterised by Edman degradation on an Applied Biosystem gas phase protein sequencer. With pure peptide **7** the analysis was possible only up to thirteenth residue from the N-terminal. This may be due to the hydrophobic nature of the peptide which got washed off from the solid support. Therefore the resin-bound peptide **6** was sequenced using a different program on the protein sequencer (03R.REZ program).²⁴ Sequence analysis confirmed the purity of the target peptide supporting the FPLC profile.

In summary a new resin was developed for peptide synthesis based on systematic analysis of solvation reactivity characteristics of functionalized crosslinked polymeric support considering the existing problems in solid-phase synthesis. This resin could be conveniently prepared by suspension polymerisation of styrene, and TEGDA serves as a new class of polymer support for peptide synthesis. These supports are extremely stable under all conditions of peptide

synthesis and they have the added advantage of high swelling and increased reactivity in aminoacylation and deprotection steps driving the gel phase reaction nearly to completion.

Experimental Section

General. Amino acids, dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt) were purchased from Sigma Chemical Co, USA. Styrene, TTEGDA, TFA, and di-isopropylethylamine (DIEA) (distilled over ninhydrin) were obtained from Aldrich Chemical Co., USA, and thioanisole from Fluka. Chloromethyl methyl ether was prepared using literature procedure.²⁵ Amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyser. For this the peptide was hydrolysed using 6N HCl-TFA (2:1) in a pyrex glass tube fused under nitrogen for 15 hr at 130 °C. Edman degradation was performed on an Applied Biosystem gas phase protein sequencer Model 470 A. Solvents, reagents and phenylthiohydantoin (PTH) standards were obtained from Applied Biosystems. CD spectrum was recorded on a Jasco J 500 A spectropolarimeter attached to a Jasco DP-501 N data processor using quartz cell.

PS-TTEGDA crosslinked resin 3. In a typical experiment a four-necked reaction vessel equipped with a thermostat, teflon stirrer, water condenser and nitrogen inlet was used. Polyvinyl alcohol (Mol. wt 72000; 0.5 g) dissolved in double distilled water (200 mL), calcium sulfate (5 mg) and calcium phosphate (10 mg) were placed in the vessel. A mixture of styrene **1** (25.5 g) tetraethyleneglycol diacrylate **2** (1.5 g) and benzoylperoxide (0.5 g) dissolved in benzene (20 mL) was added to the vessel while stirring the aqueous solution at 400 rpm. The temperature was maintained at 80 °C using a thermostated water-bath. The entire reaction was carried out under a slow stream of nitrogen. After 20 hr, the solvent-embedded copolymer beads were washed free of stabiliser and un-reacted monomers by treating with distilled water, acetone, chloroform and methanol. The copolymer **3** was further purified by refluxing with TFA to remove any linear impurity for 6 hr. The polymer beads were filtered, washed with CH₂Cl₂ and CH₃OH, and dried under vacuum at 40 °C for 10 hr to yield 25 g of dry beads (92.5%). After chloromethylation,^{9a,c} chlorine capacity of the resin was determined by pyridine fusion method and excess silver chloride estimated by modified Volhard's titration against standard ammonium thiocyanide using ferric alum as indicator¹⁹. Chlorine capacity of resin **4** was estimated to be 2.1 mmol/g.

Synthesis of pardaxin (1-26) hydrophobic segment.

Boc-Leu resin 5: Cesium Boc-Leucinate. Boc-Leu (0.95 g, 5 mmoles) was dissolved in ethanol-water (4:1) (7 mL) and a 1M solution of Cs₂CO₃ added to it dropwise until pH was 7.0. The solvent was removed by azeotropic distillation with benzene and the resulting white solid kept overnight over P₂O₅ under vacuum. Boc-LeuO.Cs (5 mmoles) was dissolved in dry DMF (7 mL) and chloromethyl resin (1 g, 2.1 mmoles) swelled in DMF added to it. The mixture was kept at 40 °C for 15 hr with occasional shaking. The resin was washed with DMF (3 × 1 min), DMF-water (9:1) (5 × 2 min), DMF-water (4:6) (5 × 2 min), DMF (3 × 1 min), CH₂Cl₂ (3 × 2 min) and finally with ether. The product resin **5** was dried under vacuum for 8 hr (1.5 g). The amino acid analysis of hydrolysate gave 1.8 mmol of Leu/g resin.

Stepwise incorporation of amino acid residues
The target peptide chain was assembled on Boc-Leu resin **5** using the remaining Boc-amino acids. Boc-Ser(Obz), Boc-Lys(ClCbz) and Thr(Bzl) were the side chain amino acids used for synthesis. For each step, two couplings with a 2.5-fold molar excess of Boc-amino acid was required to drive the reaction to completion. Boc-Ile was coupled using DCC and HOBt active ester in a mixture of DMF and DCM while all other amino acids were coupled in DCM. The extent of substitution in the resin as well as in the coupling were monitored by picric acid test or by semi-quantitative ninhydrin method. One cycle of synthesis for 160 mg (0.3 mmol) resin consists of the following operations. (i) CH₂Cl₂ wash: (5 mL × 3 × 1 min); (ii) deprotection: 30% TFA-CH₂Cl₂ (5 mL × 1 × 30 min); (iii) CH₂Cl₂ wash: (5 mL × 3 × 1 min); (iv) pre-wash: 5% TEA-CH₂Cl₂ wash (5 mL × 1 × 1 min); (v) neutralisation: 5% DIEA-CH₂Cl₂ (5 mL × 1 × 10 min); (vi) CH₂Cl₂ wash: (5 mL × 6 × 1 min); (vii) equilibration with Boc-amino acids (0.75 mmole) in CH₂Cl₂ followed by addition of 155 mg (0.75 mmole) DCC in CH₂Cl₂ (115 mg, 0.75 mmole HOBt in DMF in the case of Ile): total volume 7 mL × 45 min; (viii) 33% ethanol-CH₂Cl₂ wash: (5 mL × 4 × 1 min). Steps (vii) and (viii) were repeated for second coupling. At the end of the synthesis the resin was washed with CH₂Cl₂ (5 mL × 4 × 1 min), CH₂Cl₂-MeOH (1:1) (5 mL × 8 × 1 min) and MeOH (5 mL × 4 × 1 min), and dried under vacuum to yield 750 mg of the peptide resin **6**.

TFA cleavage of the peptide. The resin-bound peptide **6** (300 mg) was suspended in TFA (6 mL). Thioanisole (0.6 mL) and *m*-cresol (0.6 mL) were

added and the suspension was allowed to stand in an oil-bath at 40 °C for 18 hr. The resin was filtered, and washed with TFA (2 mL $\times$ 2). The filtrate was evaporated under reduced pressure to remove TFA. The residue was cooled in an ice-bath, and ice-cold dry ether added to precipitate the free peptide **7** completely. The peptide (130 mg) was washed thoroughly with ice-cold ether (10 mL $\times$ 8) to remove all the scavenging reagents. The resin was kept for a second cleavage under the same conditions as described above to yield an additional crop of 55 mg of peptide. The peptide was dissolved in methanol and again reprecipitated with cold ether to afford 182 mg of the crude product.

Purification. The crude peptide **7** (Figure 1A) was purified by Fast Protein Liquid Chromatography (FPLC, Pharmacia) using a C-18 reverse phase column. The peptide (5 mg) was dissolved in 0.5 mL methanol and injected into Analytical C-18 RPC in small portions. The solvent systems used were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). A single major peak was obtained at 58% acetonitrile (time, 39 min) which was collected and evaporated to get 1 mg pure peptide (Figure 1B). More amount of peptide was purified according to the same procedure.

Automatic Edmann degradation of peptide. The PTH derivatives were transferred automatically from the sequencer-conversion vessel to an on-line Applied Biosystem PTH Analyser Model 120 A. The PTH derivatives of free and side-chain protected amino acids were separated on an Applied Biosystems PTH C-18 column (2.1 mm $\times$ 29 cm) at a flow-rate of 200 μ L/min at 55 °C. The following solvents were used for elution: (A) 5% aqueous THF containing 30 mL of 3M sodium acetate buffer at pH 3.8 and 7 mL of 3M sodium acetate buffer at pH 4.6 /litre, (B) MeCN containing 500 nmol *N,N'*-dimethyl-*N'*-phenylthiourea (DMPTU) per litre. The elution was programmed at %B as follows: 10% at 0 min; 14% at 2 min; 40% at 20 min; 60% at 25 min; 60% at 45 min; 0% at 55 min. With the pure peptide the resolution was possible only up to 13 residue from the N-terminal. This appears to be due to hydrophobic nature of the peptide which got washed off from the solid support. Therefore, the resin bound pardaxin **6** was sequenced on 470 A Applied Biosystem gas phase sequencer using 03R REZ program. This run was designed for sequencing resin bound peptides synthesised by solid phase peptide synthesis method. This was carried out at a high cartridge temperature (50 °C) while the flash temperature remained the same as in other runs (55 °C). The increased temperature of the cartridge helped to promote both cleavage and extraction of PTH amino acids.

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References

- 1 Merrifield R B, *J Am Chem Soc*, **85**, **1963**, 2149.
- 2 Atherton E, Clive D L J & Sheppard R C, *J Am Chem Soc*, **97**, **1975**, 6584.
- 3 (a) H Becker H -W Lucas, J Maul, V N R Pillai, H Anzinger & M Mutter, *Makromol Chem Rapid Commun*, **3**, **1982**, 217.
(b) H Hellerman, H -W Lucas, J Maul, V N R Pillai & M Mutter, *Makromol Chem*, **184**, **1983**, 2603.
- 4 Rapp W, Zhang L, Habich R & Bayer E, *Peptides, Proc of 20th Eur Pept Symp*, edited by G Jung (Walter de Gruyter and Co, Berlin) 1989, p.199.
- 5 Zalipsky S, Alberico F & Barany G, *Peptides: Structure and function, Proc of the 9 th Am Pept Symp*, edited by C M Deber, V J Hruby & K D Kopple (Pierce: Rockford, IL) 1985, p 257.
- 6 (a) Meldal M, *Tetrahedron Lett*, **33**, **1992**, 3077.
(b) Renil M & Meldal M, *Tetrahedron Lett.*, **37**, **1996**, 6185.
- 7 Kempe M & Barany G, *J Am Chem Soc*, **118**, **1996**, 7083.
- 8 Renil M & Pillai V N R, *Polymer Science: Contemporary Themes*, Vol. 1, edited by S Sivaram (Tata McGraw-Hill, New Delhi) 1991, p 471; *Chem Abstr*, **114**, **1991**, 207807m.
- 9 (a) Renil M, Nagaraj R & Pillai V N R, *Tetrahedron*, **50**, **1994**, 6681.
(b) Renil M & Pillai V N R, *Proc Indian Acad Sc (Chem Sci)*, **106**, **1994**, 1123.
(c) Renil M & Pillai V N R, *J Appl Polym Sci*, **61**, **1996**, 1585.
(d) Santosh Kumar K & Pillai V N R, in: *Macromolecules Current trends*, Vol. II, edited by S Venkatachalam, R Ramaswamy, V C Joseph and V N Krishnamurthy (Allied Publishers Limited, N Delhi) 1994, p 691.
- 10 Chandy M C & Pillai V N R, *Indian J Chem*, **36B**, **1997**, 303.
- 11 (a) Arunan C & Pillai V N R, *Macromolecules Current trends*, Vol. II, edited by S Venkatachalam, R Ramaswamy, V C Joseph and V N Krishnamurthy (Allied Publishers Limited N Delhi) 1994, 714.
(b) Latha K S & Pillai V N R, *Proc of 4 th Kerala Science Congress, Trichur*, **1992**, 181.
(c) Varkey J T & Pillai V N R, *J Pept Res*, **51**, **1998**, 49.
- 12 Michael R & Pillai V N R, *unpublished results*.
- 13 Saberwal S & Nagaraj R, *Biochim Biophys Acta*, **984**, **1989**, 360.
- 14 Renil M & Pillai V N R, *Proc of 4th Kerala Science Congress, Trichur*, 1992.
- 15 *Polymer Supported Reactions in Organic Synthesis*, edited by P Hodge and D C Sherrington (John Wiley Sons, New York) 1980.
- 16 Horak D, Straka J, Stokr J, Schneider B, Tennikova T B & Svec F, *Polymer*, **32**, **1991**, 1135.
- 17 Feinberg R S & Merrifield R B, *Tetrahedron*, **30**, **1974**, 3209.
- 18 Gisin B F, *Helv Chim Acta*, **56**, **1973**, 1476.
- 19 Stewart J M & Young J D, *Solid-phase Peptide Synthesis*, 2nd edn (Pierce Chemical Company, Rockford IL) 1984, 54, 113

- 20 Barany G & Merrifield R B, in: *The Peptides*, Vol.2, edited by E Gross and J Meienhofer (Academic Press, New York) 1979, pp.1-284,
- 21 Sarin V K, Kent S B H, Tam P J & Merrifield R B, *Anal Biochem*, 117, **1981**, 147.
- 22 Bodanszky M & Bodanszky A, *Int J Peptide Protein Res*, 23, **1984**, 287.
- 23 Merrifield R B, *Recent Prog Horm Res*, 23, **1964**, 451.
- 24 *Applied Biosystems Users Bulletin Protein Sequencer* (Applied Biosystems, Foster City, CA) 1985.
- 25 Marvel C S & Porter P K, *Org Synth Coll Vol 1*, (John Wiley & Sons, New York) 2nd edn, 37; **Caution CMME is Carcinogenic.**