Pergamon

Tetrahedron 56 (2000) 3725–3734

TETRAHEDRON

Optimization in Peptide Synthetic Conditions of 1,4-Butanediol Dimethacrylate Cross-Linked Polystyrene Resin and its Efficiency in Solid Phase Peptide Synthesis

M. Roice,[a] K. S. Kumar[b,*] and V. N. Rajasekharan Pillai[a]

[a]*School of Chemical Sciences, Mahatma Gandhi University, Kottayam, Kerala 686560, India*
[b]*Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram 695014, India*

Received 18 December 1999; revised 22 February 2000; accepted 9 March 2000

Abstract—A 1,4-butanediol dimethacrylate cross-linked polystyrene (PS–BDODMA) support was prepared by aqueous suspension polymerization. The C-terminal amino acid incorporation, N_α-Fmoc and Boc-deprotection, acylation reactions and the removal of the target peptide from the support were optimized. The efficiency of the support was demonstrated by synthesizing leucyl-alanyl-glycyl-valine, acyl carrier protein fragment (65–74) and a 25-residue peptide fragment designed from the NS1 region of hepatitis C viral polyprotein under optimal reaction conditions. The efficiency of the polymer support was compared with commercially available Merrifield and Sheppard resins by synthesizing the same peptides under the identical conditions. The purity of these peptides was checked by HPLC and the structures of the peptides were established by amino acid analysis and MALDI TOF MS. © 2000 Elsevier Science Ltd. All rights reserved.

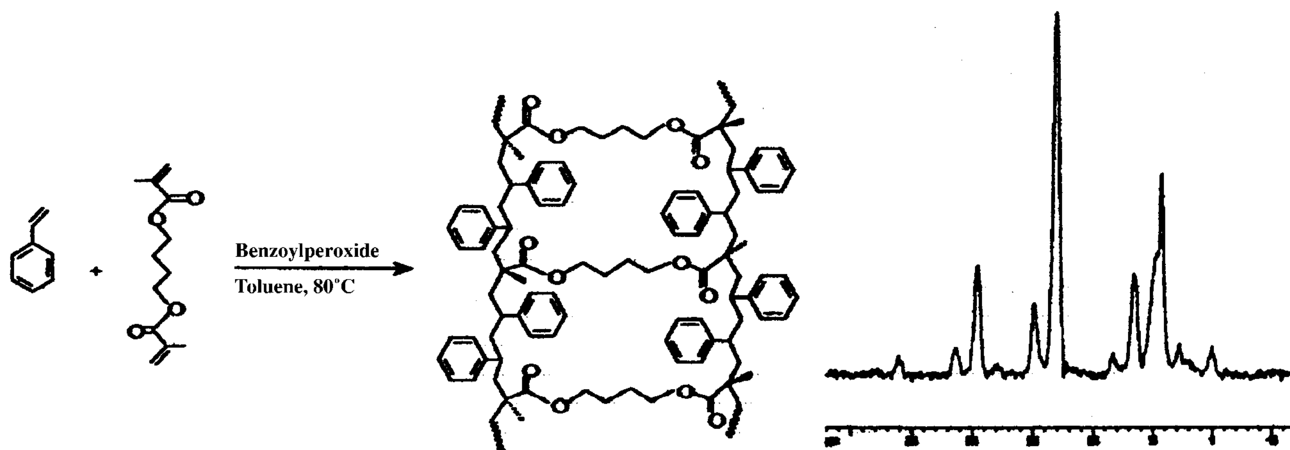
Introduction

Synthesis of biologically active peptides for research and clinical purposes requires strategies that enable side-product-free synthesis of every intermediate and allow analytical procedures to control the reactions and purity of sequential peptides. The main challenge in peptide synthesis is to establish synthetic routes to homogeneous products of defined covalent structure. The use of polymeric resins as a solid support for the synthesis of peptides has become popular ever since divinylbenzene cross-linked polystyrene (PS–DVB) was introduced by Merrifield in 1963.[1] Synthesis of peptides with high purity and homogeneity using PS–DVB resin has been a challenging problem because of the rigidity, hydrophobicity and physicochemical incompatibility of the polymer with the growing peptide chain.[2] The rate of incorporation of respective amino acid residues in the N-terminus of the growing peptide chain has been found to decrease with increase in chain length. The strong hydrophobic macromolecular environment of the polymer can persuade the growing peptide chain to adopt unfavorable conformations that lead to low yield and purity of the target peptides.[3] The influence on mass transport of reagents, solvation of the polymer as well as the peptide and reaction rates of acylation and deprotection have been mostly unfavorable because of physicochemical incompatibility of the polymer network with the growing peptide chain. A series of styrene-based resins like polyethyleneglycol–polystyrene, tentagel graft resin, tetraethyleneglycol diacrylate cross-linked polystyrene and hexanediol diacrylate cross-linked polystyrene were developed to minimize the drawbacks of PS–DVB resin.[4–7] Non-styrene based resins such as polyamides, cotton, chitin and other carbohydrates, polyethyleneglycol–polyamide, POEPOP and POEPS resins and cross-linked ethoxylate acrylate resin have also been developed and employed for the synthesis of peptides.[8–13]

The efficiency of a solid support depends upon its mechanical stability, swellability and compatibility with a range of hydrophilic and/or hydrophobic solvents. Since PS–DVB resin is highly hydrophobic, one way to develop a better styrene based support for peptide synthesis is by reducing the hydrophobicity of the polystyrene matrix. This can be attuned by introducing a 1,4-butanediol dimethacrylate cross-linker of various cross-linking density to hydrophobic styrene. In this report we describe the synthesis, optimization of reaction conditions and utility of PS–BDODMA resin as a support for peptide synthesis. The efficiency of the support was compared with Merrifield and Sheppard resins by synthesizing peptides Leu-Ala-Gly-Val, acyl carrier protein (ACP, 65–74) fraction and a 25-residue peptide from the NS1 region of hepatitis C viral polyprotein.[14] The high purity and nearly quantitative yield illustrates that the temporary amino deprotection and acylation reactions were driven to completion under the optimal conditions.

Keywords: polymer support; optimization of reaction conditions; SPPS.
* Corresponding author. Fax: +91-71-329472;
e-mail: rgcbt@md2.vsnl. net.in

0040–4020/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0040-4020(00)00224-6



Scheme 1. Synthesis of PS–BDODMA polymer and ^{13}C CP-MAS NMR of PS–BDODMA.

Results and Discussion

The chemical nature and topographical structure of the polymer matrix are the important parameters determining the physicochemical properties required for efficient peptide synthesis. The chemical nature and the mole percentage of the cross-linking agent provide the desired mechanical integrity and polarity to the resin. The PS–BDODMA resin bead showed a smooth spherical appearance when tested under a scanning electron microscope. The functionalized and the peptidyl-resin do not show significant changes in the morphological character that indicate the comparable mechanical stability of PS–BDODMA resin with PS–DVB resin. The higher hydrophilic nature of the cross-linker compared to divinylbenzene render it to be physicochemically compatible with the resin-bound peptide.[15] The support was synthesized by aqueous suspension polymerization of styrene with BDODMA using toluene as diluent and benzoyl peroxide as radical initiator (Scheme 1). A series of resins with 1, 2, 3 and 4 mol% BDODMA were synthesized (Table 1). The insoluble polymer was obtained as spherical uniform beads of 100–200-mesh size. Reproducible results were obtained by adjusting the amount of suspension stabilizer, the amount of diluent, reaction temperature, geometry of the reaction vessel and the stirring rate. The IR (KBr) spectrum of the polymer showed a sharp band at 1720 cm^{-1} corresponding to the ester carbonyl of the cross-linker besides the usual peaks of polystyrene. Solid-state ^{13}C CP-MAS NMR spectrum of the resin showed an intense peak at 130.48 ppm that corresponds to the aromatic polystyrene carbons and a small peak at 145.30 ppm for C-3 carbon of styrene. The peak at 42.78 ppm corresponds to the backbone methylene carbon of the polymer. The methylene carbon of the cross-linking agent appears as a small peak at 67.47 ppm.

Table 1. Preparation of PS–BDODMA by aqueous suspension polymerization

Mol% of BDODMA in feed	Amount of styrene (g)	Amount of BDODMA (g)	Yield of polymer (g)
1	10.41	0.23	7.60
2	10.20	0.45	8.34
3	10.10	0.68	8.82
4	9.99	0.91	9.26

The chloromethyl functional group was introduced to the resin by means of chloromethylation using chloromethyl methyl ether (CMME, a dreadful carcinogen!) and $ZnCl_2$/THF as catalyst. The chlorine capacity of the resin was determined by Volhardt's method.[16] The reaction can be controlled and a chloromethyl resin of the desired chlorine capacity can be prepared by adjusting the amount of reagent and catalyst, temperature and time duration of the reaction. The IR (KBr) spectrum showed bands at 670 and 1420 cm^{-1} for C–Cl stretching and 1250 cm^{-1} for H–C–Cl vibration. The ^{13}C CP-MAS NMR spectrum showed peaks at 48.30 ppm for methylene carbon of the chloromethyl group and a small peak in the region 135.6 ppm for C-6 carbon of polystyrene ring. The chloromethyl resin was converted to aminomethyl resin by treating with potassium phthalimide followed by hydrazinolysis. The capacity of the resin was determined by the picric acid method.[17] The resin showed characteristic IR (KBr) absorption at 1720, 1480 (ester) and 1520 cm^{-1} (amino). The ^{13}C CP-MAS NMR spectrum showed a peak at 58.93 ppm for methylene carbon of aminomethyl group and a small peak at 136.42 ppm for C-6 of polystyrene ring (Fig. 1b). The chloromethyl resin was converted to hydroxymethyl resin by heating with potassium acetate followed by hydrazinolysis. The IR spectrum of resin–KBr showed absorption at 1720 and 1490 cm^{-1} corresponding to the ester group of the cross-linker and absorption at 3400 cm^{-1} corresponding to the hydroxyl functional group. ^{13}C CP-MAS NMR spectrum showed a peak at 62.21 ppm for methylene carbon of hydroxymethyl group (Fig. 1a).

PS–BDODMA support showed excellent swelling properties in all types of solvents that are used in polypeptide synthesis. The solvent absorption was determined by placing 2% PS–BDODMA (1 g) in a glass sintered stick (G3) by immersing in solvents. After 48 h it was transferred to a centrifuge tube and excess solvent was removed. The stick and its contents were then weighed. A blank experiment was performed using an empty sintered stick. The solvent absorption (mL/g) of dry 2% PS–BDODMA and 1% PS–DVB (bracket) in different solvents are acetone

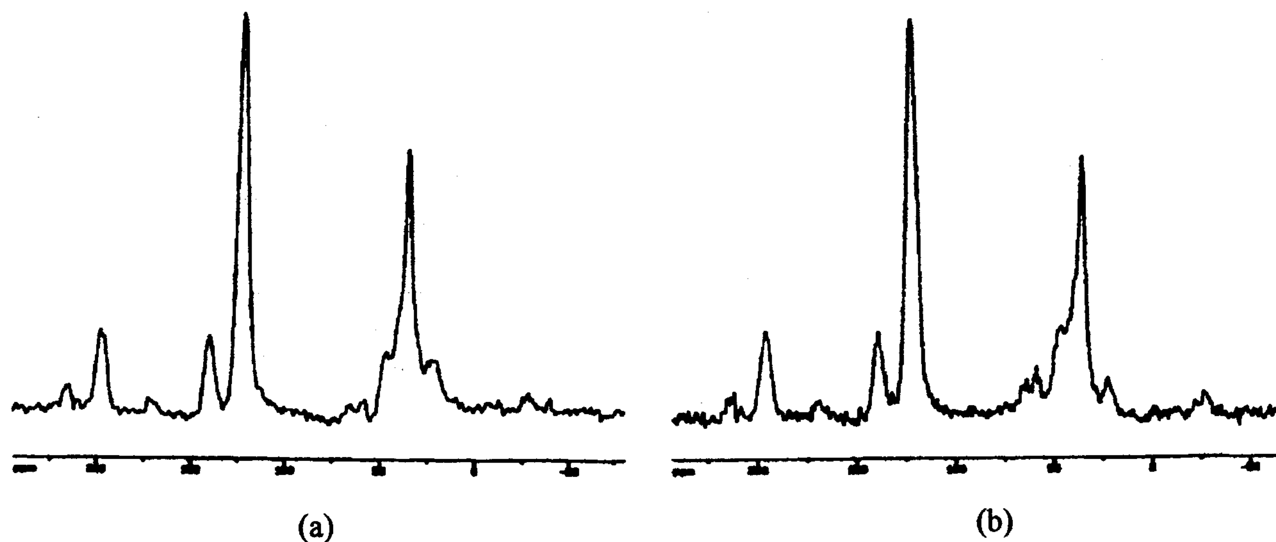


Figure 1. ^{13}C CP-MAS NMR of (a) hydroxymethyl PS–BDODMA and (b) aminomethyl PS–BDODMA.

2.6 (2.5); MeOH 1.2 (1.8); DCM 8.5 (4.3); THF 7.8 (5.2); toluene 6.2 (4.7); NMP 8.7 (5.2); chloroform 8.6 (4.3); DMF 7.3 (2.8); dioxane 6.1 (3.5) and pyridine 6.2 (4.2). The stability of the support in various reagents and solvents was tested by IR spectroscopy. The polymer does not show any change in its IR spectrum even after 48 h of treatment with TFA, 20% piperidine in DMF, aqueous NaOH, hydroxylamine in aqueous methanol and liquid ammonia.[15]

A 4-hydroxymethylphenoxyacetic acid (HMPA) linker was attached to the aminomethyl resin by active ester coupling. The resulting hydroxymethylphenoxyacetamidomethyl resin (PS–BDODMA–HMPA) and hydroxymethyl PS–BDODMA resins were used as supports for peptide synthesis. Time-dependent percentage incorporation of the C-terminal amino acid of the respective peptides (Fmoc-Val, Fmoc-Ala and Fmoc-Gly) on these resins was compared with the PS–DVB–HMPA resin and hydroxymethyl PS–DVB resin (Fig. 2). The MSNT coupling method was used for the covalent bonding of the C-terminal Fmoc-amino acid to the respective resins. In a typical reaction, the above resins having approximately the same hydroxyl capacity were treated with 2 equiv. of MSNT, Fmoc-amino acid and 1.5 equiv. of *N*-methylimidazole. PS–BDODMA–HMPA resin required 20 min for the quantitative reaction, whereas PS–DVB–HMPA resin required 38 min under the same synthetic conditions. Hydroxymethyl derivatives of PS–BDODMA and PS–DVB resins required 24 and 45 min, respectively, for optimum reaction. The time dependent incorporation of C-terminal Boc-amino acids (Boc-Val, Boc-Ala and Boc-Gly) to chloromethyl PS–BDODMA and PS–DVB resins were also compared (Fig. 3). In each reaction, 2 mmol excess of the cesium salt of the respective Boc-amino acid was used for the esterification reaction under the identical conditions. The results showed that for the PS–BDODMA resin 100% incorporation has taken place in 8 h whereas the PS–DVB resin required 24 h for the quantitative incorporation. This discrepancy appears to be due to the flexible, hydrophilic BDODMA cross-linker of the resin that can allow free interaction between the reactive centers on the support and the respective amino acids and reagents in

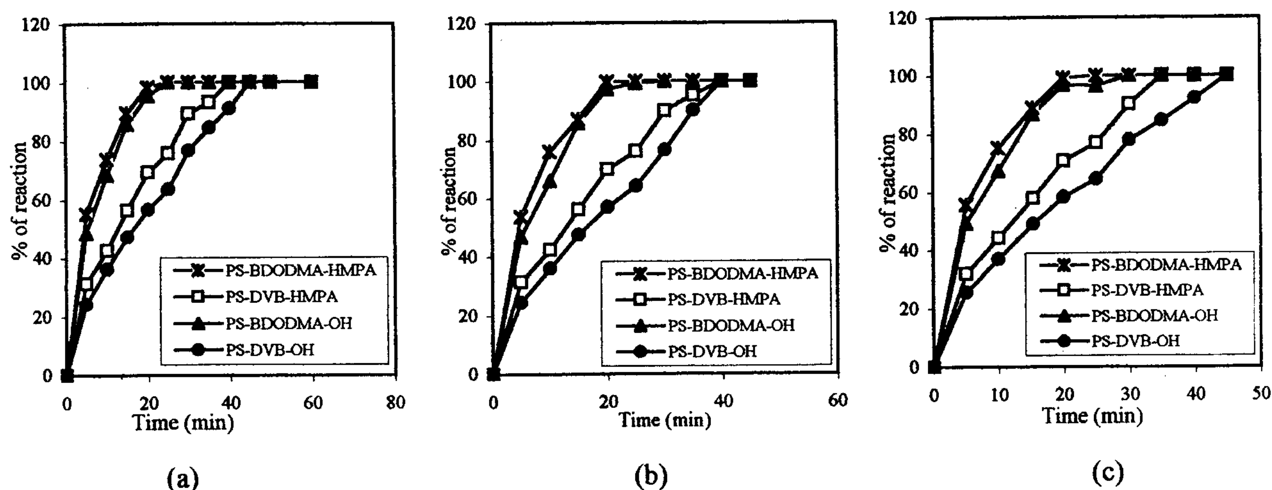


Figure 2. Time-dependent incorporation of C-terminal amino acids: (a) Fmoc-Val; (b) Fmoc-Ala; (c) Fmoc-Gly.

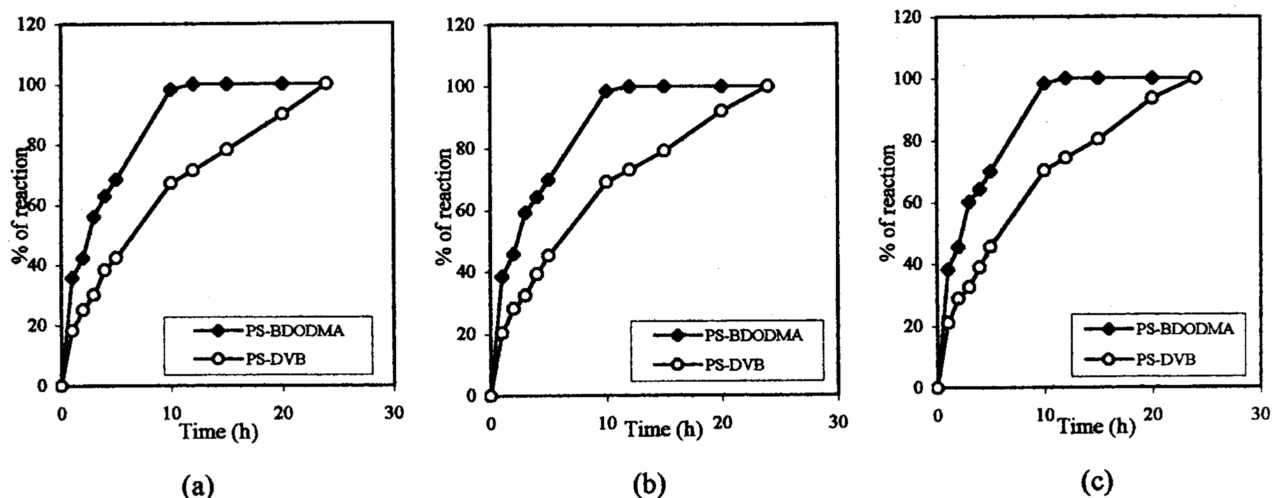


Figure 3. Time-dependent incorporation of C-terminal amino acids: (a) Boc-Val; (b) Boc-Ala; (c) Boc-Gly.

DMF. The higher rate of incorporation of C-terminal amino acids for HMPA functionalized resins could be due to the spacer effect that enables the functional groups to project away from the macromolecular environment of the polymer.

A time-dependent study of Fmoc removal using 5, 10, 15, 20 and 25% piperidine in DMF from the C-terminal amino acid of PS–BDODMA–HMPA resin showed that the percentage cleavage in unit time increases with base concentration (Fig. 4a). The time difference for quantitative Fmoc removal was negligible when compared with the time dependent cleavage using 20 and 25% base concentrations. A 20% piperidine/DMF was therefore used as the N_α deprotection reagent throughout the synthesis to avoid any side reactions and racemization at higher base concentrations (Fig. 4b). The rate of cleavage was determined by measuring the optical density (OD) of the dibenzofulvene–piperidine adduct. The PS–BDODMA resin required 14 min whereas the PS–BDODMA–HMPA resin required only 9 min for quantitative removal. PS–DVB required 30 min for 100% Fmoc-removal whereas PS–DVB–HMPA resin required only 15 min. N_α-Boc protection was removed by adding 30% TFA/DCM. The rate of Boc-removal was determined by measuring the free amino group of the resin at different time intervals using the picric acid method. The PS–BDODMA resin required 14 min whereas the PS–DVB resin required 20 min for quantitative removal of Boc group (Fig. 4c). These results show that the reaction rate in the PS–BDODMA resin is approximately twice that in the PS–DVB resin. The higher solvation and swelling characteristics of this resin in the reaction medium may enhance the free interaction of the protected amino acids and cleavage reagent improving the reaction rate.

Merrifield's model peptide (Leu-Ala-Gly-Val) was synthesized on PS–BDODMA–HMPA, PS–BDODMA and PS–DVB resins. A comparative time-dependent acylation at different temperatures was carried out on these resins to find out the most efficient support for peptide synthesis. Among the resins the extent of reaction in the first 10 min was higher for PS–BDODMA–HMPA and showed about a 10% increase in coupling efficiency compared to about 7%

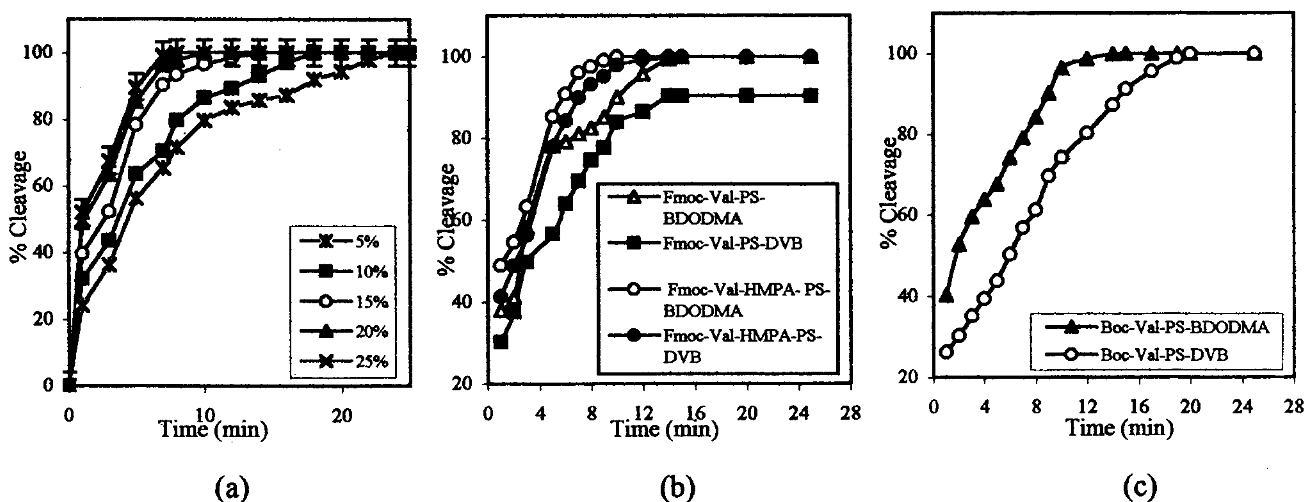


Figure 4. (a) Time-dependent Fmoc removal from C-terminal Fmoc-Val attached PS–BDODMA–HMPA resin with various concentrations of piperidine in DMF. (b) Time-dependent Fmoc removal of the C-terminal Fmoc-Val from various supports using 20% piperidine in DMF. (c) Time-dependent Boc removal of the C-terminal Boc-Val from various supports using 30% TFA in DCM.

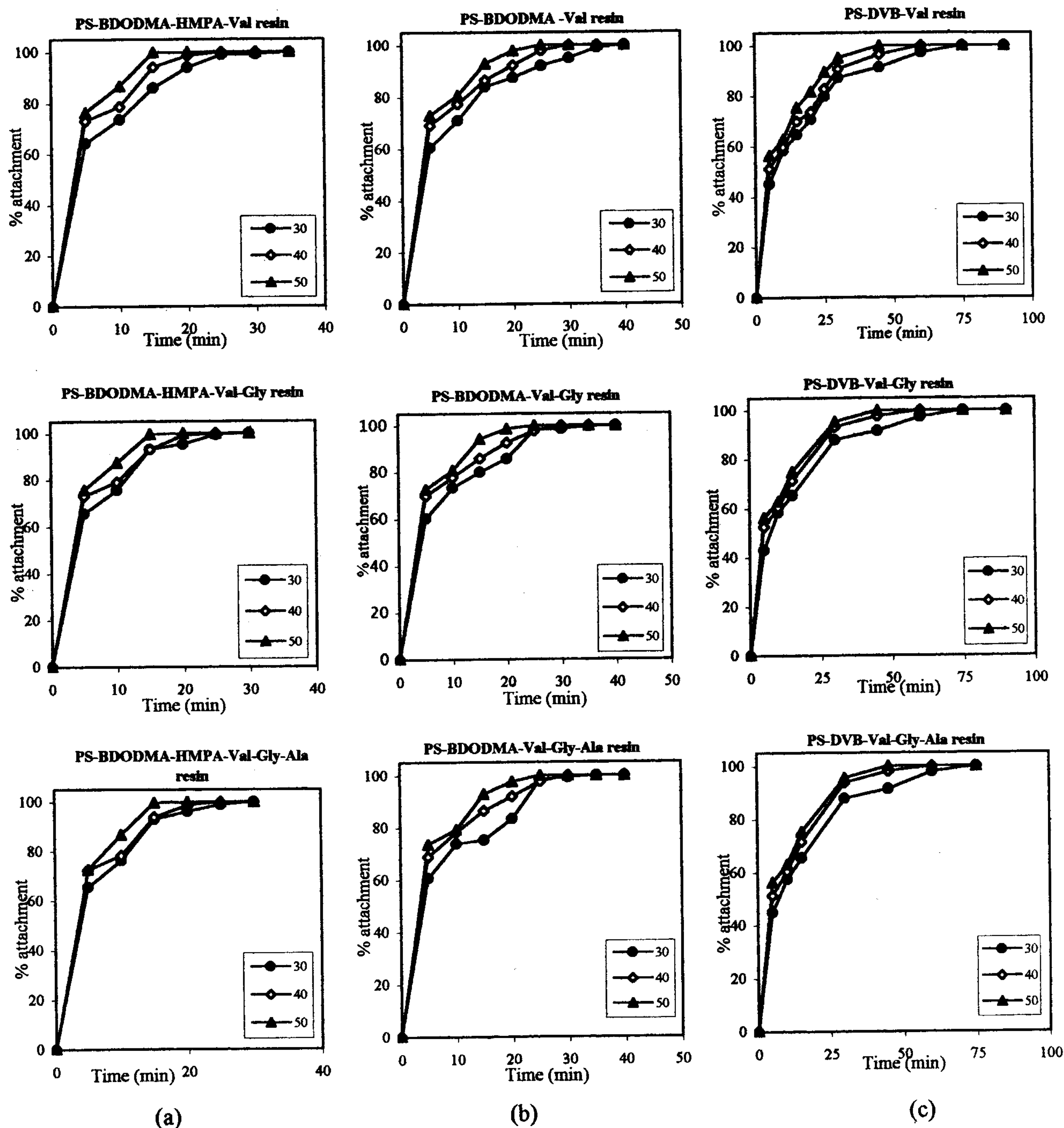


Figure 5. Time-dependent amino acid incorporation of Fmoc-Val, Fmoc-Gly and Fmoc-Ala at 30, 40 and 50°C to: (a) PS–BDODMA–HMPA resin; (b) PS–BDODMA resin; (c) PS–DVB resin.

in PS–BDODMA and 5% in PS–DVB resins for every 10°C rise in temperature (Fig. 5). A comparative time-dependent removal of the peptide at room temperature (30°C) showed 97% cleavage in 2 h from the PS–BDODMA–HMPA resin whereas the PS–BDODMA resin required 18 h for 94% cleavage and the PS–DVB resin required 20 h for 85% cleavage (Fig. 6a). The peptide cleavage reaction at 40°C gave 94% yield in 1 h from the PS–BDODMA–HMPA resin whereas the PS–BDODMA resin required 8 h for about 92% cleavage and the PS–DVB resin required 12 h for about 83% cleavage (Fig. 6b). All the studies reveal the superiority of the PS–BDODMA–HMPA resin as solid support for polypeptide synthesis.

The efficiency of the PS–BDODMA–HMPA resin was further compared with HMPA attached Sheppard and Merrifield resins by synthesizing the model tetra peptide and ACP fragment using Fmoc-amino acids. The percentage incorporation of amino acids of the model peptide in these resins was monitored under identical conditions by estimating the capacity of the intermediate peptidyl resins (Fig. 6c). The amino capacity of the final peptidyl PS–BDODMA–HMPA resin showed that 99.4% of theoretically estimated peptide chains were retained in the resin. The yield of the crude peptide from 200 mg of starting resin was 98% of the theoretically calculated value (including the resin removed for the estimation of amino capacity in the intermediate

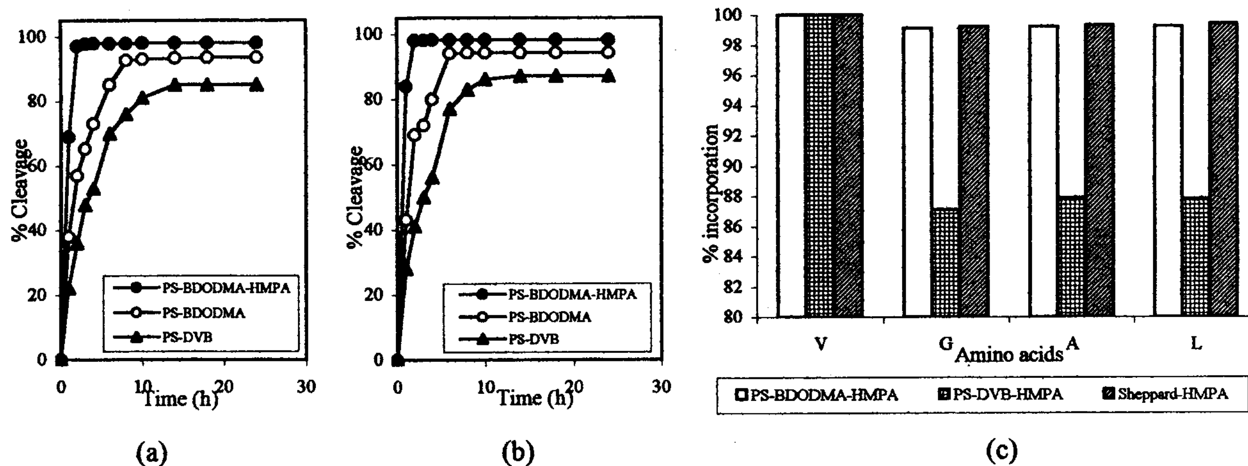


Figure 6. Time-dependent cleavage of tetra peptide (Leu-Ala-Gly-Val) from different resins: (a) cleavage at 30°C; (b) cleavage at 40°C; (c) % incorporation of amino acids in the peptide to different resins.

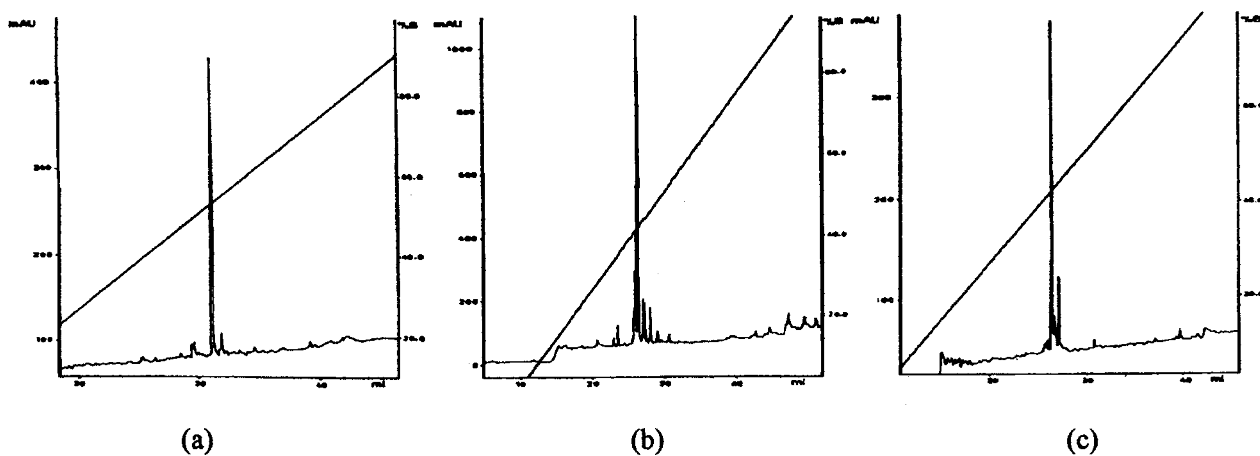


Figure 7. HPLC time-course analysis of peptide synthesized on: (a) PS–BDODMA resin; (b) PS–DVB resin; and (c) Sheppard resin using the buffer (A) 0.5 mL of TFA in 100 mL water and (B) 0.5 mL of TFA in 100 mL CH_3CN/H_2O (4:1). Flow rate: 0.5 mL/min. Gradient: 0% B in 5 min, 100% B in 45 min.

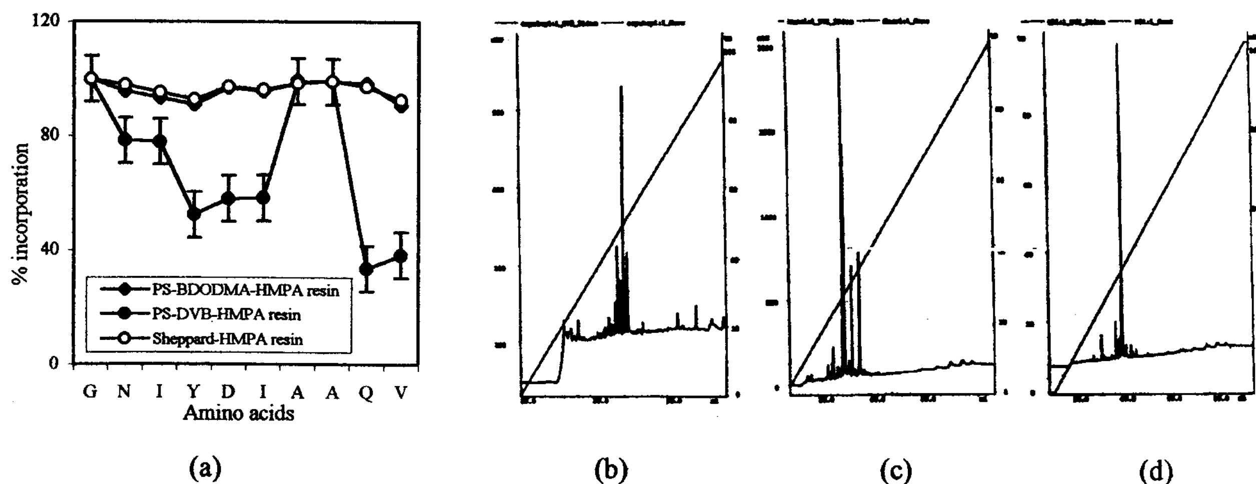


Figure 8. (a) Percentage incorporation of respective amino acids of ACP fragment on different supports; HPLC time-course analysis of the peptide synthesized on (b) Sheppard–HMPA resin, (c) PS–DVB–HMPA resin and (d) PS–BDODMA–HMPA resin using the buffer (A) 0.5 mL TFA in 100 mL water and (B) 0.5 mL TFA in 100 mL acetonitrile/water (4:1). Flow rate: 0.5 mL/min. Gradient: 0% B in 5 min, 100% B in 45 min.

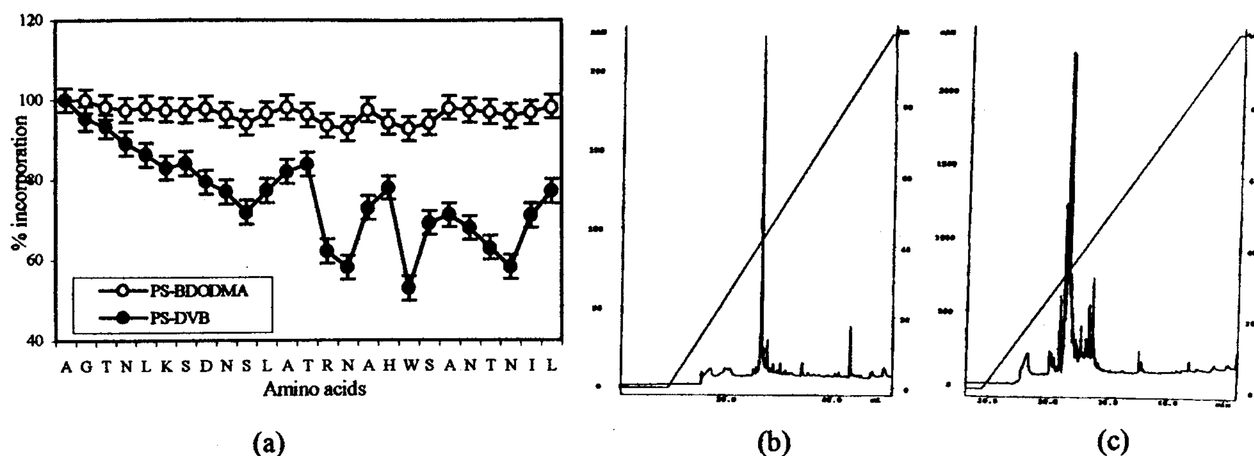


Figure 9. (a) Percentage incorporation of respective amino acids of 25 residue NS1 peptide on different supports; HPLC time-course analysis of the peptide synthesized on (b) PS–BDODMA–HMPA resin and (c) PS–DVB–HMPA resin using the buffer (A) 0.5 mL TFA in 100 mL water and (B) 0.5 mL TFA in100 mL acetonitrile/water (4:1). Flow rate: 0.5 mL/min. Gradient: 0% B in 5 min, 100% B in 45 min.

steps). The HPLC profile of the peptide synthesized on PS–BDODMA–HMPA showed only one major peak and the percentage of pure peptide calculated from the peak area was 91.3 whereas that from the Merrifield resin was only 68 (Fig. 7). Percentage incorporation studies of amino acids of ACP showed that coupling reactions proceed quantitatively in PS–BDODMA–HMPA and Sheppard resins (Fig. 8a). The yield of the crude peptide from 200 mg of the starting: (i) PS–BDODMA–HMPA resin was 96%; (ii) PS–DVB–HMPA was 65%; and (iii) Sheppard–HMPA was 95%. From the HPLC profile the peak area corresponding to ACP fraction showed that the PS–BDODMA–HMPA yielded about 71% of pure peptide, the PS–DVB–HMPA resin yielded only about 38% of pure peptide and the Sheppard–HMPA resin yielded about 67% of pure peptide under the same synthetic conditions (Fig. 8b–d).

A 25-residue peptide corresponding to the NS1 region of hepatitis C viral polyprotein was synthesized on the PS–BDODMA–HMPA resin and the Merrifield resin under identical conditions in order to demonstrate the superior efficiency of the PS–BDODMA–HMPA resin. The percentage incorporation of amino acid residues was determined by estimating the amino capacity of the peptidyl resin after each acylation reaction. Though the coupling reactions are highly sequence dependent, the present study clearly showed that acylation steps proceed more effectively on the PS–BDODMA–HMPA resin compared to the PS–DVB–HMPA resin (Fig. 9a). The HPLC time course analysis of peptides synthesized on PS–DVB–HMPA support showed several peaks. Amino acid analysis of each peak fractions showed that the major peak at 37.8% B concentration was the target peptide. The yield of the peptidyl resin from 200 mg of the starting PS–BDODMA–HMPA and PS–DVB–HMPA resin (including the resin used for intermediate amino estimations) was 294 mg (expected yield calculated based on the degree of functionalization of the resin is 101.3 mg) and 267 mg (theoretical 296 mg), respectively. The HPLC time course analysis of peptides synthesized on PS–BDODMA–HMPA support showed only one major peak corresponding to the target peptide indicating the superiority of the resin over the Merrifield resin in synthesizing medium to larger peptides. The percentage of the pure peptide in the crude mixture was 88% as calculated from the HPLC peak area whereas the percentage of the pure peptide synthesized on the Merrifield resin was only 47% (Fig. 9b and c).

All these results support the positive role of the comparatively higher hydrophilic flexible cross-linking of the support in facilitating the synthetic reactions. In solvents like DMF, NMP and DCM, the polypeptide chain and the polymer network could be highly solvated. The coupling reactions, N_α amino deprotection reactions and the final peptide cleavage from the support are very fast and this could be achieved within few minutes. A 2% cross-linked support could be a good substitute for other polystyrene based solid supports for solid phase peptide synthesis.

Experimental

Materials

Styrene, BDODMA, HMPA, polyvinyl alcohol (PVA, MW~75000), and cesium carbonate were purchased from Aldrich Chemical Company, USA. 4-Dimethylamino pyridine (DMAP), piperidine, 2-(1H-benzo triazol-1-yl) 1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), Fmoc-amino acids and Boc-amino acids were purchased from Novabiochem Ltd, UK. Thioanisole, ethanedithiol, diisopropylethylamine (DIEA) and methyl cellosolve were purchased from Sigma Chemicals Company, USA. Chloromethyl methyl ether (CMME) was prepared using the literature procedure.[18] All solvents used were of HPLC grade purchased from E. Merck (India) and SRL (India). IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C CP-MAS solid-state NMR spectra were measured using a Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. A fine powder of the polymer beads was used, and a KelF rotor was employed for MAS. The samples were rotated with a spectral width of 25 kHz, the CP time was 22 min and the number of scans was in the range of 200–300. HPLC was performed on a Pharmacia Akta purifier instrument using a C-18 reverse phase semi prep. HPLC column. The amino acid analysis

was carried out on an LKB 4151 Alpha plus amino acid analyzer. For this the peptide was hydrolyzed using 6 N HCl in a Pyrex glass tube fused under nitrogen for 15 h at 130°C. Mass spectra of peptides were performed in a Kratos MALDI TOF MS instrument.

Butanediol dimethacrylate cross-linked polystyrene copolymer

Inhibitors are removed from styrene and BDODMA by washing with 1% NaOH solution, distilled water and drying over calcium chloride. A four-necked reaction vessel equipped with a thermostat, Teflon stirrer, water condenser and nitrogen inlet and a dropping funnel were used. A net volume of 1% solution of PVA (MW~75000) was prepared by dissolving PVA (1.1 g) in double distilled water (110 mL), and was added to the reaction vessel. A mixture of styrene, BDODMA and benzoyl peroxide (0.5 g) dissolved in toluene (10 mL) were added to the reaction vessel while stirring the aqueous solution at 2000 rpm. A slow stream of nitrogen was bubbled into the reaction mixture. The temperature of the reaction mixture was maintained at 80°C using a thermostated oil bath and the reaction was allowed to continue for 6 h. The copolymer obtained was washed thoroughly with hot water (to remove the stabilizer), acetone (3×50 mL), benzene (3×50 mL), toluene (3×50 mL), soxhlet extracted with DCM and MeOH, and dried in vacuum.

Chloromethyl resin

2% PS–BDODMA resin (5 g) was swollen in DCM and, after about 1 h, excess DCM was decanted. A mixture of chloromethyl methyl ether (30 mL), DCM (10 mL) and 1 M $ZnCl_2$ in THF (1 mL) was added and the suspension was refluxed at 45°C for 1 h. The resin was filtered and washed with THF (3×50 mL), THF/water (1:1, 3×50 mL), THF/3 N HCl (1:1, 3×50 mL), THF (3×50 mL) and methanol (3×50 mL) and dried in vacuum. The chlorine capacity= 0.22 mmol/g resin as estimated by Volhardt's method.[16]

Aminomethyl resin

Chloromethyl PS–BDODMA resin (0.22 mmol Cl/g, 2 g) was swollen in *N*-methyl pyrrolidone (NMP) (50 mL). After 2 h the resin was filtered and stirred with potassium phthalimide (407 mg, 2.2 mmol) in NMP (20 mL) at 120°C for 12 h. The resin was filtered, washed with NMP (3×50 mL), dioxane (3×50 mL) and methanol (3×50 mL). The dried resin was suspended in ethanol (20 mL) and refluxed with hydrazine hydrate (100 μL, 2.2 mmol). After 8 h, the resin was filtered, washed with hot ethanol (3×50 mL) and methanol (3×50 mL) and dried in vacuum. The amino capacity of the resin=0.23 mmol/g as estimated by the picric acid titration method.[17]

Hydroxymethyl resin

Chloromethyl PS–BDODMA resin (0.22 mmol Cl/g, 1 g) was stirred with potassium acetate (216 mg, 2.2 mmol) in methyl cellosolve (20 mL) at 130°C. After 48 h, the acetoxy resin was filtered, washed with DCM (3×50 mL) and NMP (3×50 mL). The acetoxy resin was suspended in hydrazine hydrate (100 μL, 2.2 mmol) in NMP (6 mL) for 72 h. The resin was filtered, washed with NMP (3×50 mL), DCM (3×50 mL), ethanol (3×50 mL), methanol (3×50 mL) and dried in vacuum. The resin (100 mg) was acetylated with acetic anhydride–piperidine mixture (1:4, 3 mL). After 6 h, the above mixture was refluxed with distilled water (10 mL) for 3 h, cooled and filtered. The acetic acid formed was back titrated with NaOH (0.1 N). Resin capacity=0.21 mmol OH/g.

PS–BDODMA–HMPA resin

Pre-swollen aminomethyl PS–BDODMA resin (0.5 g, 0.11 mmol) in DMF was shaken with HMPA (60 mg, 0.33 mmol), HBTU (131 mg, 0.33 mmol), HOBt (44.5 mg, 0.33 mmol) and DIEA (57 μL, 0.33 mmol) mixture. After 1 h, the resin was filtered, washed with DMF (6×30 mL), DCM (6×30 mL), methanol (6×30 mL), ether (6×30 mL), and dried in vacuum. Resin capacity=0.22 mmol OH/g.

Esterification of Boc-amino acid to chloromethyl PS–BDODMA resin

Boc-amino acid (2 equiv.) was dissolved in 4:1 ethanol/ water and a 1 M solution of Cs_2CO_3 was added drop by drop till the pH was become 7.0. The solvent was removed by azeotropic distillation with benzene, and the white powdery solid was kept over P_2O_5 under vacuum. The cesium salt of Boc-amino acid was dissolved in dry DMF (1 mL) and shaken with pre-swollen chloromethyl PS–BDODMA resin (1 g, 0.22 mmol) in DMF. About 5 mg of the resin was withdrawn from the reaction mixture in every 1 h interval up to 24 h and each aliquot was washed with DMF (6×25 mL), DMF/ water (4:6, 6×25 mL), DMF (6×25 mL), DCM (6×25 mL), ether (6×25 mL) and dried under vacuum. The Boc-protection was removed by 30% TFA/DCM in 30 min and the resin was washed with DCM (6×25 mL) and ether (6×25 mL). The free amino group in the resin was estimated by the picric acid titration method.[17]

Esterification of Fmoc-amino acid to PS–BDODMA resin

The C-terminal Fmoc-amino acid (2 equiv. with respect to hydroxyl capacity of the resin), MSNT (2 equiv.) in dry DCM (1 mL) and *N*-methylimidazole (1.5 equiv.) was shaken with pre-swollen hydroxymethyl PS–BDODMA (0.5 g, 0.1 mmol) resin and PS–BDODMA–HMPA resin (0.5 g, 0.11 mmol) in DCM. About 5 mg of the resin was withdrawn from the reaction mixture every 5 min up to 1 h. The Fmoc-amino acid attached resin (10 mg) was mixed with 3 mL 20% piperidine in DMF for 30 min. The percentage incorporation was estimated by measuring the OD of dibenzofulvene–piperidine adduct at 290 nm. The results obtained were also confirmed by the picric acid titration method.

Time-dependent Boc-deprotection

The Boc group was removed from the Boc-amino acid attached resin using 30% TFA/DCM solution. About 5 mg of the resin was withdrawn from the reaction mixture in 2 min interval for 30 min and the resin was washed with

DCM (5×25 mL), MeOH (5×25 mL), ether (5×25 mL) and dried. Accurately weighed amino acid attached resin was treated with 0.1 M picric acid and the amount of free amino group was estimated by measuring the OD of picrate adsorbed on the resin at 358 nm.

Time-dependent Fmoc-deprotection

Fmoc-amino acid resin (250 mg) was treated with 20% piperidine/DMF (10 mL). About 5 mg of the resin was withdrawn from the reaction mixture at 1 min intervals up to 30 min and the resins were washed with DMF (5×10 mL), MeOH (5×10 mL), ether (5×10 mL) and dried. Accurately weighed resin was treated with 0.1 M picric acid and the extent of Fmoc deprotection was measured from the OD of the picrate adsorbed on the resin at 358 nm. This was further confirmed by suspending 10 mg of the partially Fmoc cleaved resin in 3 mL 20% piperidine in DMF for 30 min. The percentage cleavage was estimated by measuring the OD of dibenzofulvene–piperidine adduct at 290 nm.

Time-dependent coupling of amino acids at different temperatures

Fmoc-protection of the C-terminal amino acid attached resin was removed by suspending the resin in 20% piperidine in DMF (10 mL) for 10 min. The resin was washed with DMF (6×10 mL). Fmoc amino acid (3 equiv. relative to the amino capacity), HOBt (5 equiv.) and HBTU (3 equiv.) in DMF (2 mL) were shaken with the resin. About 5 mg of the resin were withdrawn from the reaction mixture every 5 min up to 1 h. The resin was washed with DMF (6×10 mL), MeOH (6×10 mL), ether (6×10 mL) and dried. The Fmoc content in the resin was determined by measuring the OD of the dibenzofulvene–piperidine adduct. The same protocol was used for the optimization of the coupling rate of amino acids at 40 and 50°C. The reaction was carried out by wrapping the reaction vessel in thermolyne heating tape and was regulated with a rheostat.

Detachment of peptide from the PS–BDODMA support

The cleavage times of the peptide from hydroxymethyl PS–BDODMA and PS–BDODMA–HMPA resins were optimized. 50 mg of the Leu-Ala-Gly-Val resins were treated separately with TFA (2.85 mL) and water (150 μL) for 1 h to 18 h at 30 and 40°C. The yield of peptide was calculated by comparing the weight of the peptidyl resin and the amount of peptide obtained.

Attachment of C-terminal Fmoc-amino acid to HMPA resins

HMPA resins (200 mg) were swollen in dry DCM (25 mL) in a round bottomed flask and, after 1 h, the excess DCM was removed. Fmoc-amino acid (2 equiv.) and *N*-methylimidazole (1.5 equiv.) were dissolved in dry DCM (5 mL) containing 3 drops of dry THF, and shaken with the resin in a septum-stoppered flask with an N_2 balloon attached. MSNT (2 equiv.) was dissolved in dry DCM (5 mL) and injected to the reaction mixture. After 20 min, the reactants were washed out with DCM (6×30 mL) and DMF (6×30 mL). The extent of reaction was determined by adding 20% piperidine in DMF (3 mL) to a known amount of resin (10 mg). After 20 min the optical density of the solution was measured at 290 nm. From the OD value the amino capacity of the resin was calculated. A second coupling was performed for PS–DVB–HMPA resin for quantitative reaction. The capacities of the resins used for the different peptide synthesis are: PS–BDODMA–HMPA resin, 0.19 mmol Val/g, 0.2 mmol Gly/g, 0.19 mmol Ala/g; PS–DVB–HMPA resin, 0.18 mmol Val/g, 0.2 mmol Gly/g, 0.18 mmol Ala/g; Sheppard–HMPA resin, 0.21 mmol of Val/g, 0.22 mmol of Gly/g.

General procedure for peptide synthesis

Peptides were synthesized manually in a silanized glass-reaction vessel of 15 mL volume with a sintered filter at one end. Pre-swollen C-terminal amino acid attached resin (1 equiv.) in DMF was shaken with respective Fmoc-amino acid (2.5 equiv.), HOBt (4 equiv.), HBTU (2.5 equiv.) and DIEA (2.3 equiv.) in DMF (3 mL). After 35 min, the resin was filtered and washed with DMF (6×10 mL). Acylation reactions were monitored by the Kaiser test. N_α Fmoc protection was removed by suspending the resin in 20% piperidine in DMF (10 mL) for 10 min. The resin was washed with DMF (5×10 mL). After the synthesis, the peptidyl resin was washed with DMF (5×10 mL), MeOH (5×10 mL) and ether (5×10 mL), and dried in vacuum.

Removal of peptide from the polymer support

The peptidyl resin was suspended in a mixture of TFA (2.55 mL), thioanisole (150 μL), ethanedithiol (75 μL), phenol (75 μL) and double distilled water (150 μL). The mixture was kept at room temperature for 2 h. The reaction mixture was filtered and the resin was washed with TFA (1 mL) followed by DCM (2×5 mL). The filtrate, along with the washings, was vacuum-evaporated at 40°C to one quarter of the original volume. The peptide was precipitated by adding ice-cold ether (10 mL). The precipitate was washed thoroughly with cold ether (5×10 mL) to remove all the scavengers. The peptide was dissolved in glacial acetic acid and re-precipitated by adding ice-cold ether. The precipitate was again washed with ether (5×10 mL).

Leu-Ala-Gly-Val

The yield of crude peptide from the PS–BDODMA resin is 13.4 mg (98%, based on the C-terminal Val incorporated to the resin). Amino acid analysis: Val, 1.0 (1); Gly, 0.98 (1); Ala, 1.0 (1); Leu, 1.02 (1). MALDI TOF MS: *m/z* 359.5 $[(M+H)^+$, 100%], 380.1 $[(M+Na)^+$, 53%] $C_{16}H_{30}N_4O_5$ requires M^+ 358.43. The yield of crude peptide from the PS–DVB resin=10.9 mg (85%) and the Sheppard resin=14.7 mg (98%).

Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly (ACP peptide)

The yield of crude peptide from the PS–BDODMA resin=40.1 mg (96%). Amino acid analysis: Gly, 1.06 (1); Ile, 1.98 (2); Tyr, 0.68 (1); Asp, 2.03 (2); Ala, 2.07 (2); Glu, 0.96 (1); Val, 1.0 (1). The low value of Tyr is due to its partial degradation. Gln and Asn were hydrolyzed to Glu and Asp. MALDI TOF MS: *m/z* 1046.63 $[(M+H)^+$, 100%]

$C_{47}H_{74}N_{12}O_{16}$ requires M^+ 1045.12. The yield of crude peptide from the PS–DVB resin=27 mg (65%) and the Sheppard resin=39.7 mg (95%).

Leu-Ile-Asn-Thr-Asn-Ala-Ser-Trp-His-Ala-Asn-Arg-Thr-Ala-Leu-Ser-Asn-Asp-Ser-Lys-Leu-Asn-Thr-Gly-Ala

The yield of crude peptide from the PS–BDODMA resin=99 mg (98%). Amino acid analysis: Ala, 4.12 (4); Gly, 1.02 (1); Thr, 2.85 (3); Leu, 3.06 (3); Lys, 0.91 (1); Ser, 2.74 (3); Asp, 5.7 (6); Arg, 0.93 (1); His, 0.89 (1); Ile, 1.10 (1). Trp is destroyed during acid hydrolysis. The high value of Asp is due to the hydrolysis of five Asn present in the peptide. MALDI TOF MS: *m/z* 2670.02 [$(M+H)^+$, 100%] $C_{112}H_{181}N_{37}O_{39}$ requires M^+ 2668.92. The yield of crude peptide from the PS–DVB resin=79 mg (82%).

References

1. Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
2. Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161–214.
3. Sheppard, R. C. *Peptides 1971*; Nesvadba, H., Ed.; North Holland: Amsterdam, 1973, p 111.
4. Zalipzky, S.; Chang, J. L.; Albercio, F.; Barany, G. *React. Polym.* **1994**, *22*, 243–258.
5. Hellerman, H.; Lucas, H. W.; Maul, J.; Pillai, V. N. R.; Mutter, M. *Makromol. Chem.* **1983**, *184*, 2603–2617.
6. Renil, M.; Nagaraj, R.; Pillai, V. N. R. *Tetrahedron* **1994**, *50*, 6681–6688.
7. Varkey, J. T.; Pillai, V. N. R. *J. Pept. Res.* **1998**, *51*, 49–54.
8. Arshady, R.; Atherton, E.; Clive, D. L. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1981**, 529–537.
9. Englebretsen, D. R.; Harding, D. R. K. *Int. J. Pept. Protein Res.* **1994**, *43*, 546–554.
10. Neugebauer, W.; Williams, R. E.; Barbier, J. R.; Brzezinski, R.; Willick, G. *Int. J. Pept. Protein Res.* **1996**, *47*, 269–275.
11. Meldal, M. *Tetrahedron Lett.* **1992**, *33*, 3077–3080.
12. Renil, M.; Meldal, M. *Tetrahedron Lett.* **1996**, *37*, 6185–6188.
13. Kempe, M.; Barany, G. *J. Am. Chem. Soc.* **1996**, *118*, 7083–7093.
14. Conry-Cantilena, C. *TIBTECH* **1997**, *15*, 71–75.
15. Roice, M.; Kumar, K. S.; Pillai, V. N. R. *Macromolecules* **1999**, *32*, 8807–8815.
16. Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*; Pierce Chemical Company: Rockford, IL, 1984; Vol. 2.
17. Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248–249.
18. Carpino, L. A.; Giza, C. A.; Carpino, B. A. *J. Am. Chem. Soc.* **1959**, *81*, 955–960.