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Synthesis of 25 Residue Peptide Designed From Ns2 Protein Fraction of Hepatitis C Virus Polyprotein on Newly Designed Flexible PS-NVP-HDODA Resin

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ABSTRACT
25 residue peptide designed from ns2 protein fraction of hepatitis c virus polyprotein on newly designed flexible PS-NVP-HDODA resin.The protein was grown in a stepwise method from the resin . High efficiency of the resin in protein synthesis was proved beyond doubt by the estimation of purity of peptide synthesised.

I. INTRODUCTION

Merrifield's 1963 contribution to science served to foment, decades later, an explosion in the field of polymer supported organic synthesis. He envisaged that covalent anchoring of the peptide chain to an insoluble and inert polymer should greatly improve the quality and quantity of the target peptide.Merrifield's solid phase method possesses several limitations and have been critically discussed by several authors.Solid support plays an important role in determining the purity and homogeneity of the peptide. In order to achieve this, the reagents and solvents must be freely accessible to the growing resin bound peptide chain. Divinylbenzene crosslinked polystyrene (DVB-PS) support has been widely used in solid phase peptide synthesis with considerable success. However, low penetration of the reagents, difficulty in selecting a good solvent for both polymer and growing peptide,and low rates of acylation and deprotection are the major limitations of using the support.So a new resin with flexibility and swellability was introduced.The new resin contains flexible cross linker HDODA and Hydrophilic NVP contents

II. MATERIALS AND METHODS

Styrene, 4-(Dimethylamino) pyridine (DMAP), cesium carbonate, Sheppard resins (Novasyn® KA 125), dicyclohexyl carbodiimide (DCC), 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), Boc and Fmoc-amino acids, HMPA, HOBt, and MSNT were purchased from Novabiochem Ltd., UK. Thioanisole, 1, 6-hexanediol diacrylate (HDODA), ethanedithiol, diisopropylethylamine (DIEA), TFA, 4-(hydroxymethyl) 3-(methoxy) phenoxy butyric acid (HMPB), piperidine were purchased from Sigma-Aldrich Corp., USA. N-Vinylpyrrolidone and 1,6-Hexanediol diacrylate were purchased from E.Merck, Germany. Chloromethylmethyl ether (CMME) was prepared using literature procedure. Solvents (HPLC grade) used were purchased from E. Merck (India) and BDH (India). IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C NMR measurements were conducted on a Bruker 300 MSL instrument operating at 75.47MHz. HPLC was done on a Pharmacia instrument using C-18 reverse phase semi- preparative HPLC column. Amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyser. For this, the peptide was hydrolysed using 6N HCl in a pyrex glass tube fused under N_2 for 15 hours at 130^oC.

PS-NVP-HDODA support

Inhibitors were removed from styrene by washing with 1% NaOH solution (2 x 30 ml) followed by distilled water (3 x 30 ml) and drying over anhydrous calcium chloride. NVP was purified by vacuum distillation. Four-necked reaction vessel equipped with a thermostat, teflon-bladed stirrer, water condenser and nitrogen inlet was used as the reaction vessel. A mixture of styrene (10.54 ml), NVP (0.54 ml), HDODA (0.67 ml) and AIBN(200 mg) were added to a solution containing sodium sulphate(10g), magnesium hydroxide (1g) and disodiumhydrogen phosphate (10 mg) in water (100 ml) by stirring the solution at 1600rpm. Temperature of the solution mixture was maintained at 70^0C under a slow stream of nitrogen. After 6 hours the copolymer was obtained as beads of 200-400μ size. Polymer was washed thoroughly with hot water (to remove the stabiliser), acetone (3 x 50 ml), and methanol (3 x 50 ml). The polymer was further purified by soxhlet extraction with acetone methanol and dried under vacuum. IR (KBr):1724, 1686 cm^{-1} (ester), 690 and 755 cm^{-1} (aromatic).

Chloromethyl PS-NVP-HDODA support

PS-NVP-HDODA support (4g) was swollen in DCM (50 ml). After 1 hour excess DCM was filtered off. The swollen resin was shaken with CMME (24 ml) and 1M $ZnCl_2$ in THF (0.6 ml) for 2 hours at 50°C. The resin was filtered using a sintered glass funnel, washed with THF (4 x 30 ml), THF/water (1:1) (3 x 30 ml), THF (3 x 30 ml), methanol (3 x 30 ml) and then soxhletted with THF and methanol.

Estimation of halogen content in functionalised PS-NVP-HDODA resin (Volhard's method)

Chloromethyl PS-NVP-HDODA support (100 g) was digested with pyridine (3 ml) in a Kjeldahl digestion flask for 3 hours at 100-110°C. It was quantitatively transferred to a 125 ml conical flask using 50% acetic acid (30 ml). Con. HCl was added followed by slow addition of standard $AgNO_3$(0.1N) solution (10 ml) with magnetic stirring. Water (50 ml) was added to the mixture. The excess $AgNO_3$was determined by back titration with standard ammonium thiocyanate solution (0.1N) using ferric alum as indicator till a dark brown colour was obtained. A calibration titration was carried out with standard NaCl solution. From the titre values the halogen capacity of the resin was calculated. Capacity of the resin = 0.24 mmol Cl/g as estimated by Volhard's method.[25] IR (KBr): 1724, 1686 cm^{-1} (ester) and 1256 cm^{-1} (CH_2-Cl).

Aminomethylation

PS-NVP-HDODA (0.24 mmol Cl, 1 g) was made to swell in DMF for 1 hour. Excess DMF was removed. Potassium phthalimide (0.44 g. 2.4 mmol) was dissolved in DMF (1 ml), added to the resin and the mixture was stirred at 120 °C for 12 hours. The resin was filtered and washed with DMF (5 × 15 ml), DCM (5× 15 ml), THF (5 × 15 ml) and ether (5 × 15 ml). It was then dried under vacuum. The dried resin was swollen in distilled ethanol (20 ml) for 1 hour. 5% hydrazine hydrate (0.02 ml) in ethanol was added and the reaction mixture was refluxed at 80 °C for 8 hours. The resin was collected by filtration, washed with hot ethanol (5 × 15 ml), methanol (5 × 15 ml), ether (5 × 15 ml) and dried under vacuum.

e) PS-NVP-HDODA-HMPA support

4-Hydroxymethyl phenoxyacetic acid (1.89 g, 10 mmol), HOBt (2.2 g, 20 mmol) and DCC (2 g, 10 mmol) were dissolved in DCM (10 ml) and shaken for 1 hour. DCU precipitated was filtered off. From the filtrate DCM was removed in vacuum and the HOBt active ester of 4-hydroxymethyl phenoxyacetic acid obtained was dried in vacuum. Aminomethyl resin (5 g, 0.24 mmol NH/g) was swelled in NMP (100 ml) for 1 hour. Excess NMP was removed by filtration. HOBt active ester of 4-hydroxymethylphenoxyacetic acid was added to swelled aminomethyl resin. After 1 hour resin was filtered, washed with NMP (3 × 30 ml), dioxane (3 × 30 ml), dioxane: H_2O (1:1)(3 × 30 ml), MeOH (3 × 30 ml) and dried in vacuum. The resin showed hydroxyl capacity of 0.16 mmol OH/g. IR (KBr):3380 cm^{-1}(OH), 1164 cm^{-1} (ether), 3400 cm^{-1}(NH), 1643 cm^{-1} (NHCO).

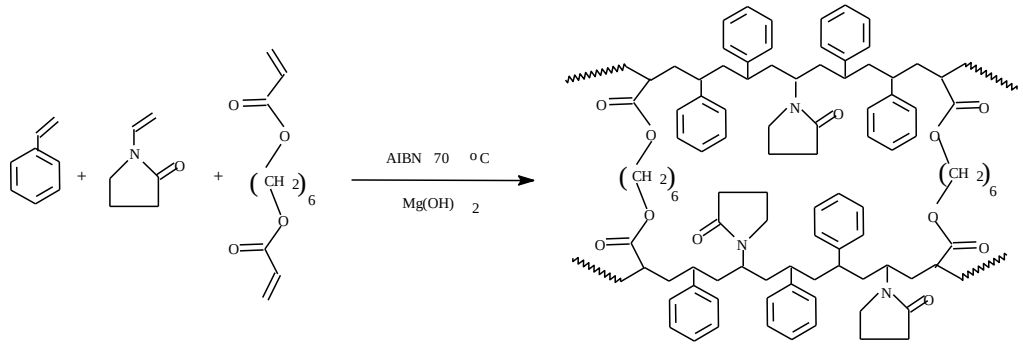
synthesis of peptide using Fmoc- amino acids

Different peptides were synthesised on corresponding C-terminal amino acid attached swollen HMPA resins in DMF using a manual peptide synthesiser. Fmoc protection was removed using 20% piperidine solution in DMF (25 ml x 20 min), followed by washing the resin with DMF (3 x 25 ml). Coupling reactions were carried using the respective amino acids (3.5 meq excess with respect to amino capacity of C-terminal amino acid attached HMPB resin) with a mixture of HBTU (3.5 meq), HOBt (7 meq) and DIEA (3.5 meq) in DMF for 50 minutes. The resin was washed with DMF (3 x 20 ml). Cleavage of Fmoc protection and extent of coupling in each cycle was monitored by Kaiser semi-quantitative ninhydrin test. The following sequence of operations were carried out for the introduction of each amino acid residue: (a) washing with DMF (4 x 25 ml), (b) washing with 20% piperidine in DMF (1 x 25 ml), (c) deprotection with 20% piperidine in DMF (1 x 25 ml x 20 min), and (d) washing with DMF (4 x 25 ml). Acylation was carried out with 3.5 mmol excess of Fmoc-amino acid, HBTU, DIEA and 7 mmol excess of HOBt relative to amino capacity of C-terminal amino acid present in the HMPB resin. After the incorporation of all amino acids, Fmoc protection of N-terminal amino acid in peptide resin was removed using 20% piperidine solution in DMF (25 ml x 20 min). Finally, the peptidyl-resin was washed with DMF (5 x 25 ml), isopropanol (5 x 25 ml), ether (5 x 25 ml) and dried in vacuum.

III. RESULTS AND DISCUSSION

The crosslinked polymer was synthesised by free radical aqueous suspension copolymerisation of the monomers styrene, N-vinylpyrrolidone and 1,6-hexanediol diacrylate. The amount of these monomers was selected according to the mole ratios required to make a definite percentage polymer. Magnesium hydroxide and sodium sulphate were added to the suspension medium . Mechanical stirring was provided to form small uniform droplets of the dispersed monomer mixture suspended in the non solvent phase. The polymerisation

reaction was initiated by adding radical initiator AIBN. It got solubilised in the monomer droplets and promoted the thermally induced polymerisation reaction. The temperature of the medium was raised to 70^{o}C to initiate the polymerisation process and the medium was kept at this temperature till the polymerisation was completed. The bead size distribution of the polymer was found to be affected by the stirring rate, geometry of the reaction vessel and amount of the stabiliser.



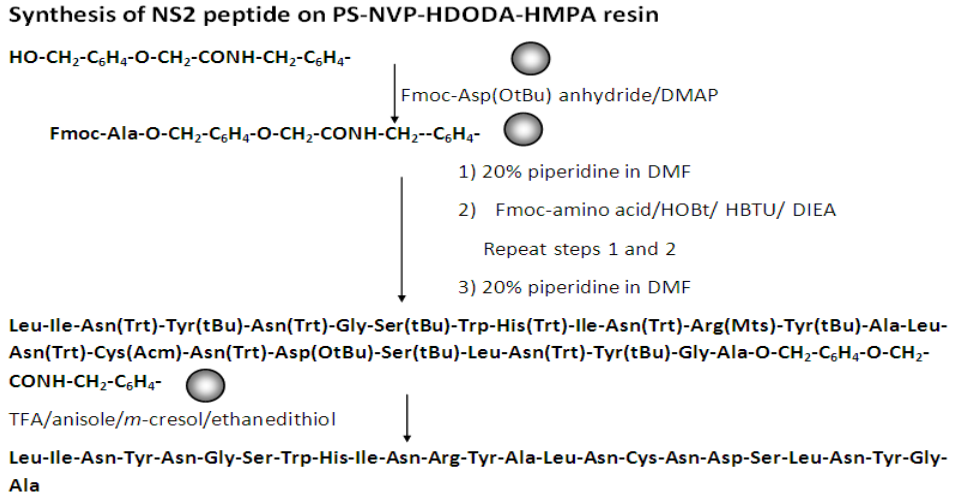
Synthesis of PS-NVP-HDODA polymer

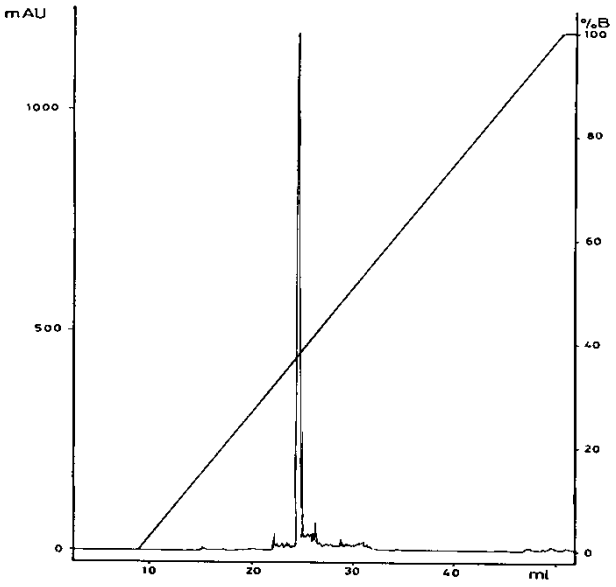
Chloromethylation of the polymer with low concentration of NVP using anhydrous $ZnCl_2$/THF catalyst was very effective for the controlled functionalisation reaction. The extent of chloromethylation depends upon the amount of CMME, concentration of anhydrous $ZnCl_2$, time and temperature of the reaction medium.

Chloromethyl resin was converted to aminomethyl resin by Gabriel's phthalimide reaction using potassium phthalimide followed by hydrazinolysis. The extent of conversion was determined by picric acid method.

PS-NVP-HDODA-HMPA resin was prepared by treating H_2N-CH_2-PS-NVP-HDODA resin with HOBt active ester of HMPA. The linker was converted to its HOBt active ester using HOBt/HBTU/DIEA method and then coupled to aminomethyl resin. This linker helped the cleavage of the resin bound peptide as its carboxylic acid using TFA and acid scavenger in 4 hours.

Twenty five residue NS2 peptide Leu-Ile-Asn-Tyr-Asn-Gly-Ser-Trp-His-Ile-Asn-Arg-Tyr-Ala-Leu-Asn-Cys-Asn-Asp-Ser-Leu-Asn-Tyr-Gly-Ala from the non structural part of HCV polyprotein was synthesised on a PS-NVP-HDODA-HMPA resin. C-terminal amino acid was attached to the support using symmetric anhydride of Fmoc-Ala in presence of DMAP. Complete incorporation of Fmoc-Gly took place after the second attempt. After removing Fmoc protection with 20% piperidine in DMF, respective amino acids were incorporated using HBTU and HOBt in presence of DIEA. All the coupling reactions were monitored by semi-quantitative ninhydrin test. After the synthesis, the target peptide was cleaved from the support using TFA in presence of anisole, *m*-cresol and ethanedithiol as acid scavengers (Crude peptide was obtained in 80% yield. The white powder obtained was dissolved in water, deep freezed and lyophilised. HPLC profile (of the peptide shows only a single peak. Amino acid analysis data also agree with that of target peptide. A, 2.02 (2); G, 2.00 (2); T, 2.68 (3); D, 6.78 (7); L, 3.03 (3) L, 1.67 (2); C, 0.89 (1); R, 1.03 (1); I, 2.01 (2); H, 0.90 (1). Asn hydrolysed to Asp and Trp was destroyed during hydrolysis. High value of Asp was due to the hydrolysis of one Asn to Asp. Low value of Ser was due to its partial hydrolysis.





. **HPLC profile of 25 residue peptide designed from NS2 protein region of HCV A: 0.5% TFA in water, B: 0.5% TFA in acetonitrile, Gradient used:0% to100% B in 40 min; Flow rate: 1 ml/min.**

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