IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 15, Issue 3 Ser. III (May –June 2020), PP 61-66
www.Iosrjournals.Org

Synthesis of Peptide Designed From Ns1 Protein Fraction of Hepatitis C Virus Polyprotein on PS-NVP-Hdoda Resin

Dr. SUNIL JACOB
Associate Professor, Catholicate College Pathanamthitta, Kerala

Abstract
*NS1 protein fraction of hepatitis c virus polyprotein on ps-nvp-hdodaresin.**T**he 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 introduced a lightly crosslinked polystyrene- divinyl benzene polymer having a pendant chloromethyl group as the point of attachment, which has come to be known simply as the Merrifield resin. But its rigid nature, inadequate solvation in polar organic solvents, non linear kinetic behaviour due to the non uniform distribution, non accessibility of the functional sites buried within the hydrophobic core of the polymer to substrates and other problems associated with heterogeneous reaction condition leads to the formation of truncated and deletion sequences.[2] Justifiably or not, the physical property of swelling is considered to be of utmost importance in dealing with solid support. The ability of a crosslinked polymer to take up solvents is of paramount importance The Merrifield resin showed good swelling behaviour in non polar solvents but, the hydrophilic peptide chain growing from the support got aggregated in non polar solvents. So, the purity and homogeneity of medium to large size peptide synthesised on this resin is still a challenging problem.[5, 6] Compatibility between the support and the growing peptide can be improved by using polar polyacrylamide type supports.

In our laboratory a series of styrene based new polymeric supports were developed by aqueous radical suspension copolymerisation of the respective monomers introducing hydrophilic flexible crosslinkers to polystyrene. These polymers include tetraethyleneglycoldiacrylatecrosslinked polystyrene (PS-TTEGDA), 1,6-hexanediol diacrylatecrosslinked polystyrene (PS-HDODA), and butanedioldimethacrylatecrosslinked polystyrene (PS-BDODMA).

A new terpolymer was developed by the radical aqueous suspension polymerisation of 1,6-hexanediol diacrylate, N-vinylpyrrolidone (NVP) and styrene(PS-NVP-HDODA). The resin had a very good hydrophobic-hydrophilic balance and showed high mechanical stability, and swelling properties.

II. Materials and Methods

Styrene, 4-(Dimethylamino) pyridine (DMAP), cesium carbonate, Sheppard resins (Novasyn® KA 125), dicyclohexylcarbodiimide (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^{o}C.

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^oC. 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 mmolCl/g as estimated by Volhard's method.[25] IR (KBr): 1724, 1686 cm^{-1} (ester) and 1256 cm^{-1} (CH_2-Cl).

Aminomethylation

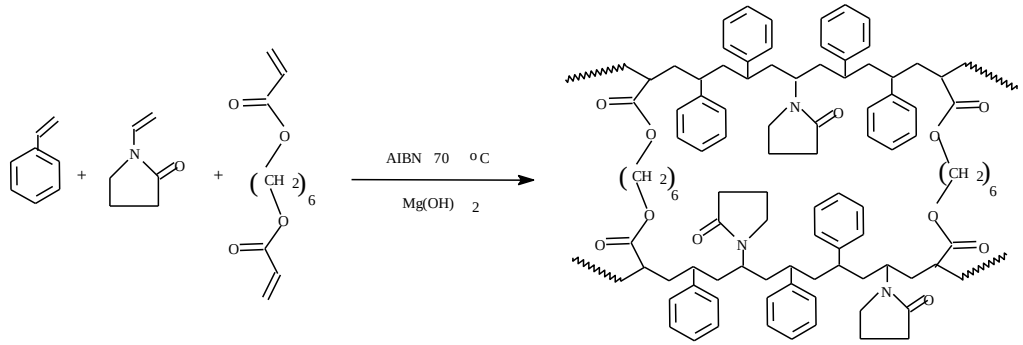
PS-NVP-HDODA (0.24 mmolCl, 1 g) was made to swell in DMF for 1 hour. Excess DMF was removed. Potassium phthalimide (0.44 g. 2.4 mmol) was dissolved in DMF (1 ml), added to the resin and the mixture was stirred at 120 ^{o}Cfor 12 hours. The resin was filtered and washed with DMF (5 × 15 ml), DCM (5× 15 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 ^{o}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.

PS-NVP-HDODA-HMPA support

4-Hydroxymethyl phenoxyaceticacid (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).

III. Results and Discussion

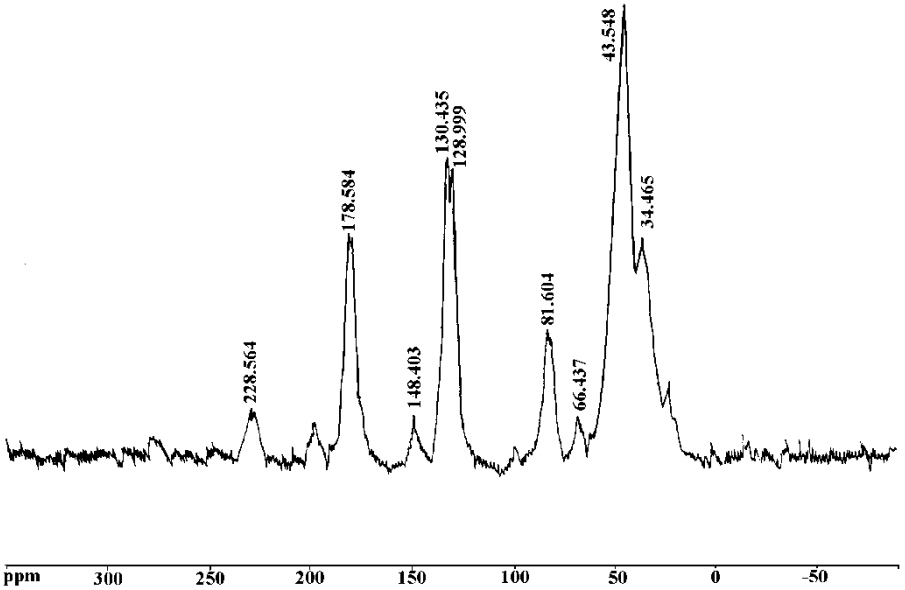
The crosslinked polymer was synthesised by free radical aqueous suspension copolymerisation of the monomers styrene, N-vinylpyrrolidone and 1,6-hexanediol diacrylate. The amount of these monomers was selected according to the mole ratios required to make a definite percentage polymer. Magnesium hydroxide 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^oC 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.



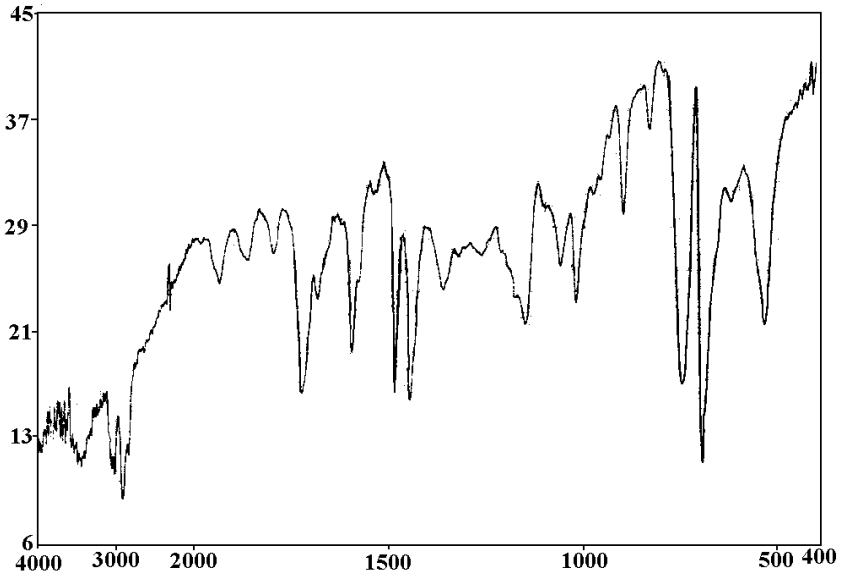
Synthesis of PS-NVP-HDODA polymer

The shape and speed of the paddle together with the shape of the polymerisation vessel influences the formation of droplets where the polymerisation takes place which in turn determine the size and quality of beads. The non solvent phase may be thought of as providing millions of tiny 'spherical moulds' in which the polymer beads formed are constrained by surface tension. Stabilisers helps to reduce the surface tension of the droplets and this prevent droplet aggregation which leads to distorted beads. The indentation force resulting from geometry of the vessel, forces the suspended mixture towards the rotating stirrer blade. This can lead to a homogeneous shearing environment for the monomer droplets that create droplets of uniform size. Reproducible droplet of the monomers with 200-400 μ size was obtained by adjusting the stirring speed between 1500-2000rpm. When the speed increased beyond 3000 rpm polymer yield was found to decrease considerably. This may be due to the excessive shearing of the polymer bead. After each round of polymerisation, it was observed that the next reaction proceeds smoothly only if the reaction vessel was silanised. By the careful adjustment of the above discussed parameters, yield and size of the new PS-NVP-HDODA polymer can be reproduced.

PS-NVP-HDODA polymer was characterised by IR and ^{13}C/ NMR techniques. IR (KBr) spectrum of the powdered polymer showed an intense sharp peak at 1724cm^{-1} corresponding to ester carbonyl of the crosslinker and at 1686 cm^{-1} corresponding to the carbonyl peak of NVP besides the usual peaks of polystyrene. The solid state ^{13}C NMR spectrum showed an intense peak at 130.435ppm corresponding to aromatic polystyrene carbons and a small peak at 148.403 ppm corresponding to C-3 of styrene. The carbonyl carbon of the PVP appears as a peak at 178.584 ppm, methylene carbon of the crosslinker appears as a peak at 66.437 ppm. The peak at 43.548 ppm corresponds to the backbone methylene carbon of the polymer and that at 34.465 ppm was due to the overlapping of the ring with main chain carbon.

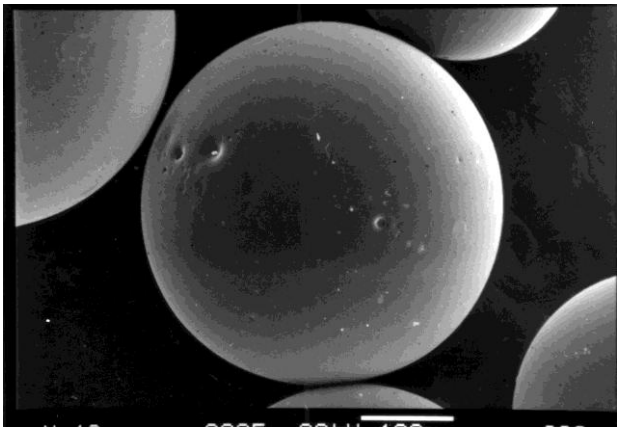


^{13}C/ NMR of PS-NVP-HDODA polymer support

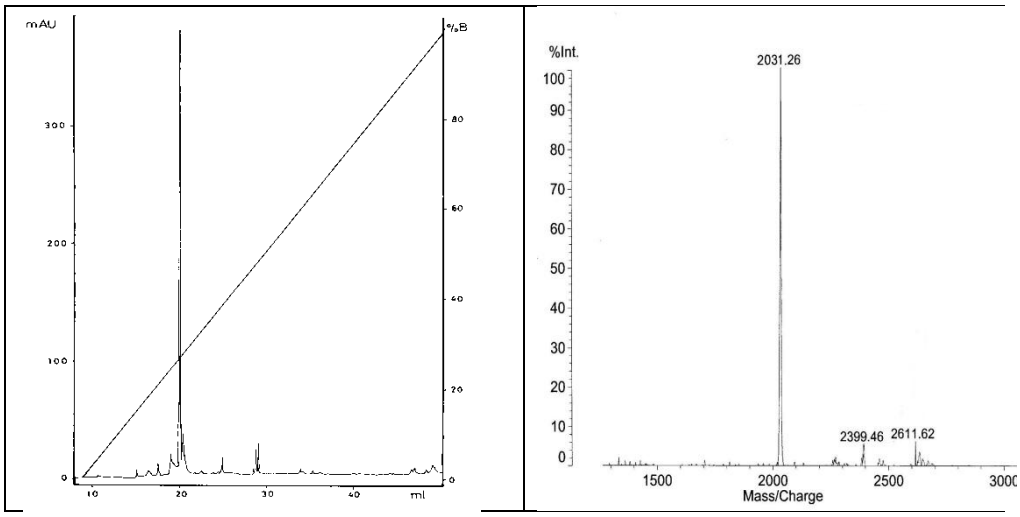


IR (KBr) spectrum of PS-NVP-HDODA polymer support

Morphological character of the polymer was determined by the scanning electron microscope (SEM) analysis . The polymer surface was found to be smooth and spherical. The functionalisation of the polymer with CMME and other reagents did not affect the morphological character of the polymer as revealed by the SEM of the functionalised bead. The smoothness of the polymer surface was found to be unaffected. The morphological character of the polymer was also not affected by the aminomethylation of the polymer using potassium phthalimide.



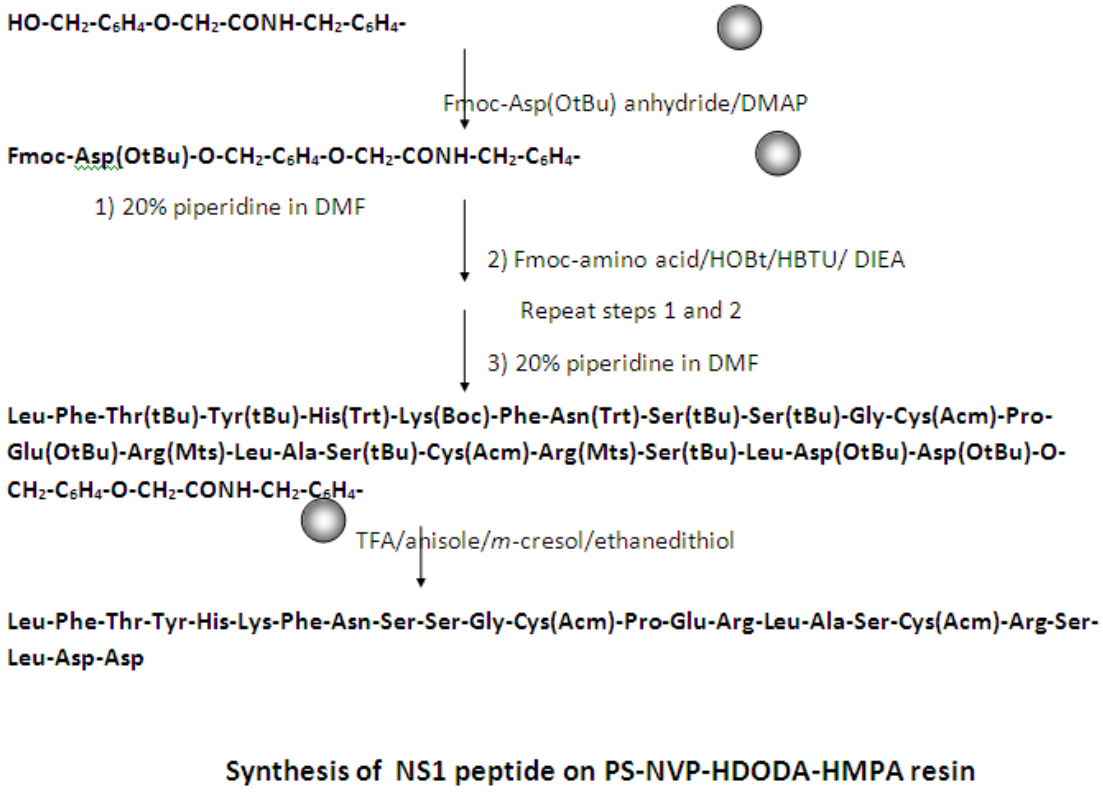
Hepatitis is a disease characterised by inflammation of the liver, producing swelling and in many cases, cirrhosis and hepatocellular carcinoma. Hepatitis C is an RNA virus belonging to the family Flaviviride with a genome of < 9.5 kb, encoding a single polyprotein of 3010 amino acids which are subsequently spliced into ten functional protein units that were coded as: core (C), two envelope (E1 and E2/NS1) and atleast six non structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The variability of the HCV genome posed serious problems for the serological detection of HCV. Ultra sensitive polymerase chain reaction (PCR) was generally used for the detection. But the method is highly expensive and requires well-trained hands. Because of these limitations, a sensitive method to detect the presence of antiviral antibodies in patient sera using synthetic peptide ELISA was developed using a 19 residue fragment of E2/NS1 region of Hepatitis C viral polyprotein H-GSWHINRTALNCNDSLNTA-OH.[7] The synthesis was carried out by the Fmoc strategy. C-terminal Fmoc-Ala–OH was attached to PS-NVP-HDODA-HMPA using MSNT. The Fmoc group was removed using 20% piperidine in DMF. The successive amino acid coupling reactions were carried out using three equivalent excess (with respect to Ala load) of respective Fmoc-amino acid, HOBt, HBTU and DIEA.



(a) **(b)**

(a) HPLC analysis of E2/NS1 peptide. Buffer A: 0.1% TFA in water, Buffer B: 0.08% TFA in 80% MeCN in water. Rate of Flow: 1 ml/min. Gradient: 0-100 % B in 50 min. (b) MALDI-TOF-MS of E2/NS1.

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 acid scavengers such as anisole, *m*-cresol and ethanedithiol (Scheme 4.8). Crude peptide was obtained in 80% yield. The white powder obtained was dissolved in water, deep freezed and lyophilised. The major peak obtained in analytical HPLC corresponds to the target peptide **(**Figure 4.12a**)**. MALDI-TOF-MS m/z 2041.9 Da [$(M+H)^+$, 100%], $C_{86}H_{134}N_{29}O_{30}S_1$ requires M^+ 2039 Da**)**. Amino acid analysis: Gly, 0.98(1); Ser, 1.69(2); His, 0.92(1); Ile, 1.01(1); Asp, 5.01(5); Arg, 0.98(1); Thr, 0.75(1); Leu, 2(2); Cys, 0.98(1); Ala, 2(2). Trp was destroyed during hydrolysis.



Synthesis of NS1 peptide on PS-NVP-HDODA-HMPA resin

References

[1]. Merrifield, R.B. Jl. of Am. Chem. *Soc*. **1964** 246, 1922.
[2]. Stewart, J.M.; Wooly, D.W. Nature(London), **1965,** 206, 619.
[3]. Varkey, J.T.; Pillai, V.N.R. Jl. of Peptide Sci. **1999**, 5, 577.

[4]. Arunan,C.; Pillai,V.N.R.; Protein and Peptide Lett.**1999**,6,391.
[5]. Sheehan, J. C.; Hess G. P.Jl. of Am. Chem. Soc. 77,**1955,** 1067
[6]. Houghten,R.A.; DeGraw, S.T.; Bray, M.K.; Hoffman, S.R.; Frizzel, N.D. Bio Technique. **1986**, 4,522.
[7]. Holm A. and Meldal M. "Peptide 1988" (Eds. Jung. G. and Bayer E.) Walter de Gruyter and Company, Berlin, **1989,**pp.208-210.
[8]. Barany, G.;Abericio, F. in Peptides 1990, Proc. of the 21st Europ. Pept. Symp. Giralt E. Andrew D. (Eds) ESCOM, Leiden, **1991**, 139.
[9]. Bayer, E.; Dengler,.M.; Hemmassi, B. Int. Jl. of Peptide Protein Res. **1985**, 25, 178.
[10]. Barany, G.; Albericio, F.; Sole, N.A.; Griffin, G.W.; Kates, S.A.; Hudson, B. In Peptides, 192 Proc. 22nd Europ. Pept. Symp. Schneider C.H. Eberle, A.W. Eds. ESCOM, Leiden **1993**, 267.
[11]. Pillai, V.N.R.; Renil, M.; Haridasan, V.K. Indian Jl. of Chem. **1991**, 30B, 205.
[12]. Atherton, E.; Cameron, L. R.; Sheppard, R.C. Tetrahedron. **1988**,44, 843.
[13]. Findeis, M.A.; Kciser, E.T. Jl. of Org. Chem. **1989**, 54, 3478.
[14]. Meldal, M. Tetrahedron Lett.,**1992**, 33, 3077.
[15]. Auzanneau, F.I.; Meldal, M.; Bock, K. Jl. of Peptide Sci. **1995**,1, 31.
[16]. Atherton, E.; Brown, E.; Sheppard, R.C.; Rosever, A.J., Chem. Soc. Chem. Commun. **1981**,1151.
[17]. Small, P.W.;Sherrington, D.C. J. Chem. Soc. Chem. Commun. **1989**, 1589.