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Gel-Phase Synthesis of Hydrophobic (Thr^{14}) (Thr^{19}) Galanin (1–19) Fragment on a High Capacity Flexible Crosslinked Polystyrene Support

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Abstract: A hydrophobic analogue of human galanin (1–19) fragment has been synthesized using Boc/Bzl tactics to demonstrate the synthetic utility of the flexible crosslinked polystyrene support prepared by the suspension polymerization of styrene and 1,4-butanediol dimethacrylate. The copolymer was chloromethylated to 2.36 mmol Cl/g. The functionalized resin was found to possess all the physicochemical properties similar to Merrifield resin. The free peptide was obtained in high yield and purity as judged by RP-HPLC and characterized by amino acid analysis and ESI-MS.

Keywords: solid-phase peptide synthesis; flexible support; 1,4-butanediol dimethacrylate; hydrophobic peptides; galanin

INTRODUCTION

The chemical synthesis of proteins is a challenging task for peptide chemists, due to the difficulty of obtaining homogeneous peptide fragments for ligation [1]. Although the solid-phase approach of Merrifield has been improved and generalized for the synthesis of a variety of complicated molecules, the homogeneity of the products decreases with increased size of the target sequence. The success of SPPS depends on the nature of the support with regard to its mechanical stability, swelling capacity and hydrophobic–hydrophilic balance in various solvents [2]. Of the various types of support materials developed over the years, polystyrene and polyamide resins have received much attention. Although the polystyrene based supports are favoured by their availability in good physical form, mechanical strength, chemical stability and ease of functionalization, the conventional Merrifield resin [divinyl benzene-crosslinked polystyrene, DVB-PS] suffers from rigidity, hydrophobicity and incompatibility with the growing peptide chains [3]. The highly hydrophobic macromolecular environment of the polymer matrix can induce the adoption of unfavourable conformations by growing peptide chains, which leads to coupling difficulty during chain assembly [4]. Of the various types of supports developed for addressing these problems in SPPS, only polyamide [5] and polyethylene glycol grafted polystyrene resins (tentagel) [6] which are available commercially have received attention.

The properties of a crosslinked polymer depends on the nature and degree of crosslinking, and flexible and polar crosslinking agents can be used to reduce the rigidity and hydrophobicity of the Merrifield resin (DVB-PS). Tetraethyleneglycol diacrylate

Abbreviations: BDDMA, 1,4-butanediol dimethacrylate; BDDMA-PS, 2 mol% butanediol dimethacrylate-crosslinked polystyrene; CMME, chloromethyl methyl ether; DVB, divinylbenzene; DVB-PS, 2 mol% divinylbenzene-crosslinked polystyrene; NMP, N-methylpyrrolidone; RP-HPLC, reversed-phase high-performance liquid chromatography.

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and 1,6-hexanediol diacrylate have already been used for this purpose [7,8]. In the present paper, 1,4-butanediol dimethacrylate was employed to crosslink polystyrene. The copolymer, BDDMA-PS, was chloromethylated and then used to synthesize a hydrophobic analogue of human galanin (1–19) fragment. The present synthesis was carried out to check the efficiency of the BDDMA-PS support for the high capacity synthesis of medium-sized hydrophobic peptides which are otherwise difficult to prepare by either solution phase (insolubility of fragments) or solid-phase (incomplete amino acylations and deprotections) methodologies.

MATERIALS AND METHODS

General

Styrene, BDDMA, 2% DVB-PS (200–400 mesh), TFA, thioanisol, ethanedithiol and *m*-cresol were obtained from Aldrich Chemical Company, USA. All side chain protected amino acids, HOBt and DIEA were purchased from Sigma Chemical Company, USA. All solvents were of commercial grade and purified before use. IR spectra were recorded on a Bruker IFS-55 spectrophotometer in the solid state, using KBr pellets. A pellet was prepared by grinding the polymer with KBr in the ratio 1 : 100 for 5 min under constant pressure. A solid state ^{13}C-CP-MAS-NMR spectrum was recorded on a Bruker 300 MSL instrument at 75.47 MHz. HPLC analysis was conducted on a Shimadzu Model 6A instrument fitted with a UV/Vis spectrophotometric detector. A reverse phase C_{18} column was used for the analysis using the binary solvent system (0.1% TFA containing acetonitrile and water). Detection was made at 220 nm. Amino acid analyses were performed on an LKB 4151 Alpha Plus amino acid analyser using *o*-phthalaldehyde detection. A micromass Quattro II triple quadrupole mass spectrometer was used for recording electrospray ionization mass spectrum.

Preparation of BDDMA-PS

The copolymer was prepared as microporous beads by suspension polymerization under nitrogen atmosphere. 11.22 ml of styrene, 0.44 ml of BDDMA and 500 mg dibenzoyl peroxide were dissolved in 20 ml toluene. The mixture was added to 175 ml of 1% polyvinyl alcohol solution in a reaction vessel equipped with a stirrer, water condenser and nitrogen inlet and kept at 85 °C on a water bath. After 15 h, the precipitated copolymer was filtered, washed with hot water and Soxhlet extracted using toluene, acetone, DCM and finally MeOH. The polymer beads were dried under vacuum at 45 °C. The yield was 9.2 g. The beads were sieved and 200–400 mesh sizes were used for peptide synthesis.

Chloromethylation of BDDMA-PS

The dry resin (1 g) was swelled in DCM for 1 h and then 6 ml CMME, 0.2 ml catalyst ($ZnCl_2$/THF or $SnCl_4$/DCM) were added and kept at 50 °C. The reaction was continued for 24 h and samples were withdrawn at regular intervals to estimate the chlorine substitution level. The resin was filtered and washed with THF (10 ml × 3 × 3 min), THF/4 N HCl (10 ml × 3 × 3 min), THF/water (1 : 1 v/v) (10 ml × 3 × 3 min), water (20 ml × 5 × 3 min) and finally with MeOH. The resin was then soxhlet extracted with THF and dried under vacuum. The chlorine capacity was estimated by Volhard's method [15]; and was 2.36 mmol/g.

Attachment of the C-terminal Threonine Residue to the Support

Boc Threonine was dissolved in a minimum quantity of ethanol and the pH was adjusted to 7.0 with a saturated solution of Cs_2CO_3 and kept for 1 h with stirring. The solvents were rotary evaporated by azeotropic distillation with dry benzene. The white powder of the Cs-salt of Boc threonine thus obtained was dissolved in NMP and chloromethylated resin was added. The reaction mixture was kept at 50 °C for 24 h. The resins were washed with DMF, DMF/H_2O (1 : 1 v/v), DMF, DCM and finally methanol and dried under vacuum. The amino acid substitution level was then determined by elemental analysis and the picric acid method. The amino capacity was 2.19 mmol Thr/g (94%).

Peptide Chain Assembly

Synthesis was performed manually in a silanized reaction vessel, using Boc/Bzl tactics. 100 mg of Boc threonine attached BDDMA-PS resin (2.19 mmol threonine/g) was taken and swelled in DCM for 30 min. 5 ml of 30% TFA/DCM was added and kept for 30 min for the removal of the temporary blocking Boc group. For neutralization, 5 ml of 10% DIEA in DCM (5 min) was used. The resin was washed well with DCM, NMP and then 2.5 mmol excess of the

Table 1 Protocol Adopted for the Chain Assembly of [Thr^{14,19}]-Galanin (1–19) Fragment

Step	Operation (volume × number of times × time)[a]
1.	DCM wash (5 ml × 1; 3 min)
2.	NMP wash (5 ml × 1; 3 min)
3.	30% TFA/DCM (5 ml × 1; 30 min)
4.	DCM wash (5 ml × 2; 3 min)
5.	5% DIEA/DCM (5 ml × 1; 3 min)
6.	5% DIEA/NMP (5 ml × 1; 3 min)
7.	DCM wash (5 ml × 1; 3 min)
8.	NMP wash (5 ml × 1; 3 min)
9.	Kaiser test
10.	Boc aminoacid/DCC HOBt (1 : 1 : 1) in NMP/DMSO (20% v/v) 1 × 60 min
11.	30% methanol/DCM (5 ml × 3 × 5 min)
12.	DCM wash (5 ml × 3 × 5 min)
13.	Kaiser test
14.	Repeat steps 10–13 if test is positive and repeat steps 3–13 if found negative

[a] 100 mg of aminoacyl resin was used for synthesis.

HOBt active ester of the next amino acid (Asparagin) in NMP containing 20% v/v of DMSO, was added and shaken for 60 min. After 60 min, 2.5% v/v of DIEA was added and again shaken for 5 min. The resin was washed with 30% MeOH/DCM mixture three times and then with DCM. The progress of the coupling step was monitored by picking up a single bead from the reaction mixture and performing the Kaiser test. If positive, the next coupling was repeated in the same manner until the test was negative. The same coupling procedure was employed for all the couplings performed. This cycle of operation was repeated for the stepwise incorporation of the remaining amino acids. The protocol adopted for the assembly was given in Table 1. The weight of the peptidyl resin was 605 mg.

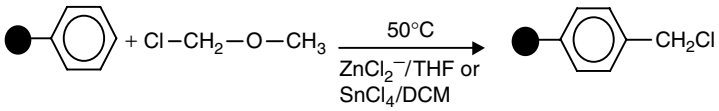
Cleavage of Peptide from the Support

To 50 mg of peptidyl resin in TFA (10 ml), 150 μl of thioanisol, 150 μl of ethanedithiol and 100 μl of *m*-cresol were added and kept at 40 °C for 20 h and filtered. The filtrate was concentrated and cooled. Cool diethyl ether was then added to precipitate the peptide. It was washed repeatedly with ether and dissolved in a 2% acetic acid–water mixture and lyophilized to a fine powder. The yield was 90% (41 mg), calculated on the basis of the first amino acid substitution level.

The purity of the crude sample was checked by analytical HPLC fitted with a reverse phase C_{18} column. TFA (0.1%) containing H_2O (A) and 0.1% TFA containing acetonitrile (B) were used as the solvent system. A gradient of 0 to 60% B for 40 min was maintained at a flow rate of 1 ml/min. Amino acid analysis was performed by hydrolysing the peptidyl resin (2 mg) 6 N HCl: propionic acid mixture (1 : 1 v/v) at 110 °C for 22 h under a nitrogen atmosphere. The peptide was hydrolysed with 6 N HCl at 110 °C for 16 h. Amino acids were detected by post column derivatization with *o*-phthalaldehyde. The ESI-MS was recorded by dissolving the samples in methanol and introducing into an electrospray ionization source though a syringe pump at 0.4 ml/h. The spectra were collected in 4 s scans and printouts were averaged spectra of 4–10 scans.

RESULTS AND DISCUSSION

A very important consideration for the successful solid-phase reaction is the swelling and solvation of the resin bound substrates. The support developed by the suspension polymerization of styrene and 1,4-butanediol dimethacrylate has been found to exhibit an amphiphilic nature, and solvation properties similar to those of the peptide product at 2 mol% crosslinking level [9]. The resin showed the physicochemical properties, topographical structure and solvent compatibility essential for the successful



Scheme 1 Chloromethylation of BDDMA-PS and DVB-PS resins.

synthesis of peptides. The resin was characterized by IR and ^{13}C-CP-MAS-NMR spectroscopy. The incorporation of BDDMA was evident from the sharp peak at 1720 cm^{-1} (ester carbonyl) along with the usual peaks of polystyrene in the IR (KBr) spectrum. ^{13}C-CP-MAS-NMR showed peaks at 130.4 ppm (aromatic polystyrene carbons), 148.3 ppm (*p*-carbon of styrene), 42.7 ppm (methylene carbon of polymer backbone) and 64.0 ppm (methylene carbon of BDDMA).

Chloromethylation using chloromethyl methyl ether was selected for the functionalization of BDDMA-PS resins. Although various Lewis acid catalysts can be used for the reaction, $ZnCl_2$ /THF was reported to be effective for the controlled chloromethylation of DVB-PS resins [10]. Since resins of varying functional group capacities are required for various applications, BDDMA-PS resins were subjected to chloromethylation using $ZnCl_2$/THF and $SnCl_4$/DCM as catalysts (Scheme 1). The reaction was found to depend on the nature and concentration of catalyst, reaction time and temperature. The effect of catalyst and reaction time upon the rate and degree of functionalization is shown in Figure 1. When the reaction was carried out at 50 °C for 3.5 h using $ZnCl_2$/THF, chlorine substitution of 2.36 mmol/g and 0.4 mmol/g was obtained for BDDMA-PS and DVB-PS resins, respectively. The reaction was much faster when $SnCl_4$ /DCM was used as a catalyst and it was found to be not useful for low capacity functionalization. The reaction was carried out in DCM since it allows the maximum swelling to both DVB-PS (7.2 ml/g) and BDDMA-PS (11.4 ml/g) resins. So it is clear that the reaction is easy and fast in BDDMA-PS resins which helps to reduce the unwanted side reactions such as methylene bridging on the polymer matrix. The easy mass transport of reagents in the polymer network due to the swelling also helps to achieve uniform functionalization on the resin [11]. IR (KBr) of chloromethylated resin showed peaks at 690 and 1420 cm^{-1} due to C-Cl stretching. ^{13}C-CP-MAS-NMR spectrum gave a peak at 48.3 ppm corresponding to the ethylene carbon of the chloromethyl group.

The crosslinked polymer becomes a swollen gel in good solvents and then the segmental mobility of the crosslinked polymer chain will be comparable to that of linear polystyrene in solution [12]. This effect can be further increased if flexible crosslinking agents are used. This is clear from the swelling capacities of chloromethylated 1 mol% crosslinked DVB-PS and BDDMA-PS resins (1.25 mmolCl/g) (Figure 2). As the crosslinking was changed from rigid divinyl benzene to flexible and polar 1,4-butanediol dimethacrylate, the swelling capacity was increased to 2–3 fold.

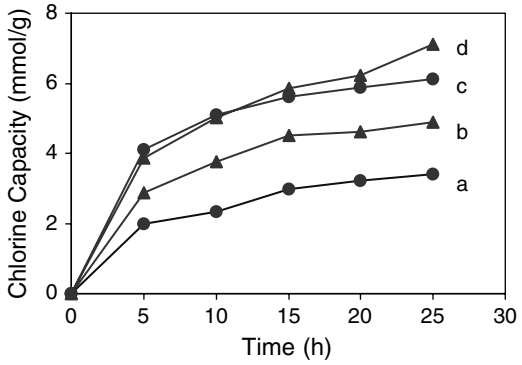


Figure 1 Effect of catalyst upon the chloromethylation of BDDMA-PS. a, DVB-PS and $ZnCl_2$; b, BDDMA-PS and $ZnCl_2$; c, DVB-PS and $SnCl_4$; d, BDDMA-PS and $SnCl_4$.

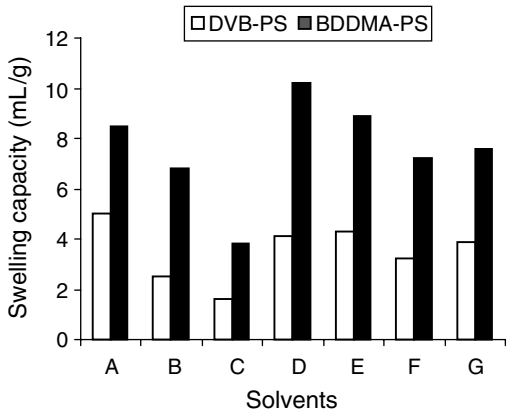


Figure 2 Swelling capacity of chloromethylated DVB-PS and BDDMA-PS resins. A, tetrahydrofuran; B, N,N-dimethylformamide; C, methanol; D, dichloromethane; E, *N*-methyl-pyrrolidone; F, dioxane; G, benzene.

To demonstrate the synthetic utility of BDDMA-PS resins, a hydrophobic analogue of human galanin was selected for synthesis. Galanin is a neuropeptide widely distributed through the central and peripheral nervous system [13]. It plays an important role as a neuromodulator of endocrine and synaptic transmission. In an attempt to derive short peptides as potent therapeutic agents, His^{14} and His^{19} of galanin were replaced by Thr to gather information about the charge, structural requirement and hydrophobicity of the peptides to show biological activity. The sequence synthesized was thus *Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Thr-Ala-Val-Gly-Asn-Thr*.

The peptide was synthesized starting with chloromethylated BDDMA-PS resin (2.36 mmol Cl/g). *C*-terminal Thr was covalently attached to the support using the cesium salt to a substitution level of 2.19 mmol of Thr/g. The subsequent amino acids were attached to the resin using DCC/HOBt in NMP. The Boc group was removed by 30% TFA/DCM followed by neutralization with 5% DIEA in NMP and DCM. Coupling was performed in NMP containing 20% v/v of DMSO. Both NMP and DMSO were reported to be effective for the destabilization of resin bound (Figure 3) β-sheet formation [14] which is a real hurdle to peptide chemists (difficult sequence problems) [4]. Each coupling step was followed by Kaiser and quantitative ninhydrin tests. All the couplings were repeated until a negative Kaiser test was obtained. The number of couplings given for the complete amino acylation of various amino acids during the synthesis is shown in Figure 3. The attachment of Tyr to Leu and Ser to Ala was performed three times to achieve 99.9% acylation. A total amino acylation time of 24 h was needed for the chain assembly. The coupling efficiency of different amino acids as observed by quantitative ninhydrin testing is shown in Figure 4. There was only a small loss of the peptide chains from the support as 98% of the functional sites remained on the resin even after 18 cycles of repeated acid-base treatment. This is clear from the observed weight increment of 552 mg which corresponds to an average coupling efficiency of 99.89%.

When all the amino acids were incorporated, the peptidyl resin was subjected to amino acid analysis. The values are: Thr 2.94 (3.0); Asp 1.86 (2.0); Gly 4.12 (4.0); Val 0.71 (1.0); Ala 1.89 (2.0); Leu 2.72 (3.0); Pro 0.82 (1.0); Tyr 0.51(1.0); Trp 0.78 (1.0); Ser 1.03 (1.0). The amino acid analysis of the peptidyl resin is an indication of the homogeneity of the growing peptide chain. Here the observed values were found to agree well with the theoretical value.

The peptidyl resin was treated with 10% piperidine in DMF at 0 °C for 2 h to deformylate Trp (CHO). The dry resin was suspended in neat TFA in the presence of thioanisole, *m*-cresol and ethanedithiol at 40 °C for 20 h. The crude peptide obtained in 93% (458 mg) yield (based on first amino acid substitution) was found to be 86% pure from the analytical HPLC profile (Figure 5). The crude peptide was purified on a semiprep Pharmacia LKB 5/5 system using the gradient 5 min 10% B, 40 min 70% B and 50 min 100% B where (A) was 0.1% TFA/water and (B) was 0.1% TFA/acetonitrile. The presence of Trp and Tyr residues was confirmed by HPLC profiles detected at 214 and 280 nm. No product was detected at 310 nm [λmx for Trp (CHO)] showing the complete removal of the formyl group. The presence of Trp was established by recording the UV-spectrum which also

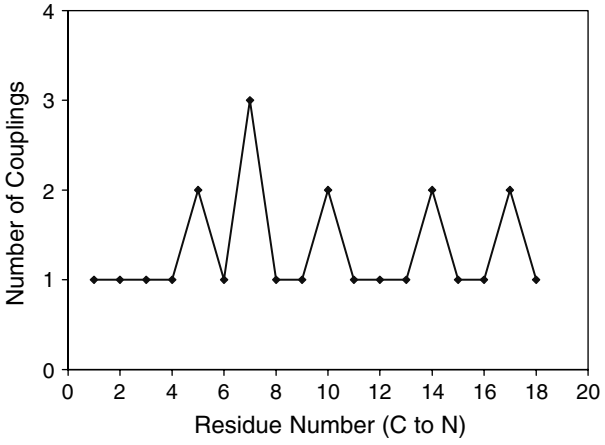


Figure 3 Number of couplings given to each amino acid to achieve a negative Kaiser test.

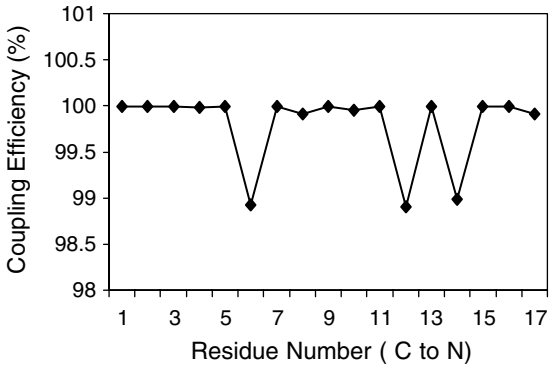


Figure 4 Stepwise coupling yield of various amino acids during the chain assembly.

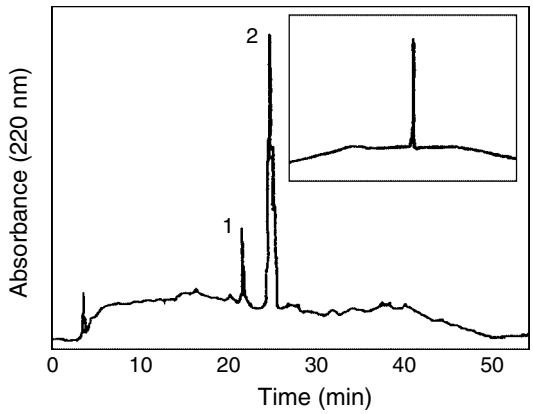


Figure 5 Analytical HPLC profile of crude galanin (1–19) fragment. Analytical HPLC profile of purified (1–19) fragment is given in the inset.

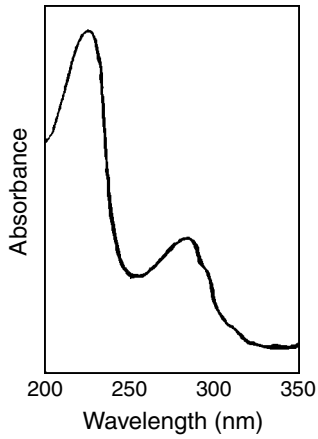


Figure 6 UV spectrum of deformylated galanin (1–19) fragment.

suggested the complete deformylation of Trp residue (Figure 6).

The purified peptide corresponding to the major peak and the small peak observed in the analytical HPLC profile were separately subjected to amino acid analysis (Table 2). It was found that the small peak resulted from the deletion of valine and tyrosine. This is also clear from the amino acid values of the peptidyl resin. Finally the identity of the peptide was confirmed by recording ESI-MS. m/z: 1890.4 $[(M+H)^+ - 100\%]$ $C_{85}H_{128}N_{22}O_{27}$ requires M^+ 1889.7.

Thus, a satisfactory synthesis of hydrophobic [Thr^{14}] [Thr^{19}] galanin (1–19) fragment was achieved in high yield and excellent purity on a high capacity BDDMA-PS support. Hydrophobic peptides on a DVB-PS support often fail to offer the high yield and homogeneity even at low loading levels. Except for a very few reports, synthesis on high capacity resins generally fails due to the possibility of site–site interactions and the aggregation of peptide chains to local clusters within the resin matrix. But the flexible and polar crosslinking agent, BDDMA, offers greater swelling and solvation characteristics which reduce the aggregating tendency of the peptide chain, especially in solvents such as NMP, DMF and DMSO. Hence the reagents can penetrate well into the resin network and the reactions employed can be comparable to the solution phase at molecular level.

Table 2 Amino Acid Composition of [$Thr^{14,19}$]-Galanin (1–19) Fragment

Amino Acid	Actual Value	Peak 1	Peak 2
Thr	3	2.76	2.89
Asp	2	1.42	2.04
Gly	4	3.84	3.98
Val	1	—	0.88
Ala	2	1.01	2.08
Pro	1	1.34	0.92
Leu	3	2.11	3.11
Tyr	1	—	0.76
Ser	1	0.41	0.71
Trp	1	0.32	0.81

CONCLUSION

In summary, BDDMA-PS is an efficient support for the synthesis of medium-sized hydrophobic peptides at high substitution levels. Since it is polystyrene based, it possesses all the characteristic features of DVB-PS systems. When used in conjugation with NMP, DMF or DMSO, it effectively solvates the functional sites and creates a polar micro-environment for the easy progress of polar reactions such as peptide bond formation.

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