Solid phase synthesis of diazepam binding inhibitor fragments 32-38 and 45-50 on a flexible butanediol dimethacrylate crosslinked polystyrene support

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Two short sequences of the neuropeptide Diazeepam Binding Inhibitor (DBI) KQATYGDP (32-38) and GLLDLK (45-50) have been synthesized on a butanediol dimethacrylate (BDDMA) crosslinked polystyrene support by the solid phase technique. The BDDMA crosslinked polystyrene support is prepared by the radical initiated suspension polymerization of the monomers. These supports are found to possess very good mechanical and physical properties. The solvation properties of the new polymer support are found to be superior to the commonly employed styrene-DVB resins. A 2 mole percent crosslinked polymer support is found to be ideal for peptide synthesis. The BDDMA-PS resin is functionalised by chloromethylation. Polymer support with a chlorine capacity 1.8 mmol/g is used for peptide synthesis. The first amino acid is appended to the polymer support by Gisin’s cesium salt method. The syntheses of the target peptides are carried out by the usual solid phase strategy using Boc amino acids. After synthesis the peptides are cleaved from the support using trifluoroacetic acid. The peptides are obtained in high yield and the purity of the peptides are checked by TLC and HPLC.

Results and discussion

Butanediol dimethacrylate (BDDMA) crosslinked polystyrene supports were prepared by the radical initiated suspension polymerisation of the monomers, styrene 1 and BDDMA 2 (Scheme I).

Polymers (3a-3d) with 2%, 4%, 6% and 8% crosslink densities were prepared by varying the monomer mole ratios. The resins were obtained as spherical beads. The polymer beads were characterized by infrared spectroscopy and 13C CP-MAS NMR spectroscopy. IR spectrum shows a sharp band at 1720 cm⁻¹ corresponding to the ester carbonyl group of the crosslinking agent. Solid state 13C CP-MAS NMR spectrum (Figure 1) shows an intense peak at 130.4 ppm corresponding to aromatic polystyrene carbon and a small peak at 148.2 ppm arising from C-3 carbon of the styrene. The backbone methylene carbon of the polymer appears at 42.7 ppm.

A 2% crosslinked polymer with a bead size of 200-400 mesh was chosen for the synthesis of the target peptides. The polymer was functionalised with chloromethyl groups via Friedel-Crafts reaction (Scheme II). By adjusting the reaction conditions resins with varying chlorine capacities were prepared. The functional group capacity was estimated by the pyridine fusion method. The IR spectrum of the
crosslinking and nature of the solvent. The swelling characteristics of the BDDMA-styrene copolymers (3a-3d) were studied in a variety of solvents commonly employed in peptide synthesis. The swelling behaviour of a 2% BDDMA-crosslinked chloromethylpolystyrene resin was compared with that of a 2% DVB-crosslinked chloromethylpolystyrene resin. The results obtained are given in Tables 1a and 1b.

Synthesis of peptides

A 2% crosslinked polymer support with a capacity of 1.8 mmol Cl/g was selected for the peptide synthesis. Using this high capacity resin, we have synthesized three model peptides and two active sequences of the biological polypeptide DBI\textsuperscript{11}. The peptides were assembled on the resin by manual solid phase strategy, using Boc amino acid chemistry. The C-terminal amino acids Boc-Gly, Boc-Lys(-2CI-Z)-OH and Boc-Asp(0BZ)-OH were incorporated as the benzyl ester to the chloromethylated resin by the Gisin's cesium salt method\textsuperscript{15}. The substitution level was determined by Gisin's picric acid method and was

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Swelling studies

The solvation properties of the polymer matrix is very important in SPPS since the reaction is taking place in a heterogeneous medium\textsuperscript{11}. The extent of solvation of the solid phase determines the diffusion of soluble reagents, rates of different reactions taking place and the yield and purity of the synthesized peptides. The extent of swelling of crosslinked polymers is determined by factors like polarity of the backbone, nature of the crosslinking agent, degree-of
Table Ia—Swelling properties of resins 3a-3d

<table>
<thead>
<tr>
<th>Solvent</th>
<th>2% DVB-PS</th>
<th>2% BDDMA-PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane</td>
<td>5.05</td>
<td>7.05</td>
</tr>
<tr>
<td>Chloroform</td>
<td>5.12</td>
<td>7.35</td>
</tr>
<tr>
<td>N-Methylpyrrolidone</td>
<td>4.32</td>
<td>6.56</td>
</tr>
<tr>
<td>N,N-Dimethylformamide</td>
<td>3.44</td>
<td>6.20</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>4.98</td>
<td>8.10</td>
</tr>
<tr>
<td>Toluene</td>
<td>4.63</td>
<td>6.08</td>
</tr>
<tr>
<td>Methanol</td>
<td>2.02</td>
<td>3.15</td>
</tr>
</tbody>
</table>

Table Ib—Comparison of solvation properties of DVB and BDDMA-crosslinked chloromethyl polystyrene resins

<table>
<thead>
<tr>
<th>Solvent</th>
<th>2% DVB-PS</th>
<th>2% BDDMA-PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane</td>
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*Based on weight increase after equilibrating 1g dry resin for 24 hr.

found to be quantitative. Three model peptides (Gly-Leu-Gly, Phe-Gly-Lys and Phe-Thr-Gly-Lys) were assembled by the DCC coupling method. The Boc group was removed by exposing the peptidyl resin to 30% TFA in DCM and subsequent neutralisation with 5% DIEA in DCM. The successive amino acids were then incorporated by the DCC mediated coupling. The extent of the coupling reaction was monitored by the semi-quantitative ninhydrin reaction. For all the coupling steps double coupling was employed to ensure completion of the reaction. A two-fold molar excess of the Boc amino acid was used for the coupling reaction.

For the synthesis of biological peptides DBI 32-38 (Lys-Gln-Ala-Thr-Val-Gly-Asp) and 45-50 (Gly-Leu-Leu-Asp-Leu-Lys), HOBt active ester coupling in NMP was performed. All the aminoacylation reactions were almost complete at the first coupling step. Here also double coupling was employed. The final cleavage of the peptides was effected by neat TFA. The purity of the crude peptide was checked by TLC and HPLC. The TLC of the peptides in the solvent system pyridine:acetic acid:water (50:35:15) showed a single spot. HPLC profiles of the two DBI fragments are shown in Figures 2a and 2b. The results of the amino acid analysis of the DBI fragments are given in Table II. The values agree with the theoretical amino acid composition of the peptides.

This manuscript evaluates the efficiency of the new BDDMA crosslinked polystyrene resin for the stepwise solid phase synthesis of peptides. This copolymer can be easily prepared with good mechanical stability and morphology. Moreover, the BDDMA crosslinked resin exhibits better solvation properties both in polar and non-polar solvents. The support is found to be very efficient for peptide synthesis including biological peptides.

Experimental Section

General: Side chain protected Boc amino acids were purchased from M/s Sigma Chemical Co., USA and the other Boc amino acids were prepared by Schnabel's method. Polyvinyl alcohol, BDDMA, TFA and thioanisole were supplied by Aldrich Chemical Co., USA and styrene by Fluka AG, Switzerland. AR grade solvents were used after distillation and purification. IR spectra were recorded on a Shimadzu IR 470 spectrophotometer using KBr pellets and the $^1$C CP-MAS NMR measurements were carried out on Bruker 300MSL CP-MAS instrument. HPLC was performed using Shimadzu SPD-10A UV-VIS detector HPLC. Amino acid compositions of peptides were determined by amino acid analysis on Shimadzu amino acid analyser system.

Synthesis of BDDMA crosslinked polystyrene support

A mixture of styrene (22.6 mL, 98 mol%), BDDMA (0.88 mL, 2 mol%), toluene (10 mL) and benzoyl peroxide (500 mg) was suspended in a solution of polyvinyl alcohol (PVA) (3.5 g, 7200-100000 mol wt) dissolved in water 350 mL (1% PVA) and kept mechanically stirred at 600 rpm under an inert nitrogen atmosphere at a temperature of 85°C. After 6 hr the beaded resin was filtered and washed with hot water to remove PVA, then it was Soxhletted with acetone followed by methanol to remove all linear polymers and low molecular weight products. Polymers with crosslink densities 4%, 6%, and 8% were also prepared by the above procedure by varying the monomer mole ratios.
Chloromethylation of the styrene-BDDMA copolymer

The dry resin (1 g) was swelled in DCM (10 mL) and refluxed with chloromethylmethylether (6 mL) and freshly prepared ZnCl₂ in THF (0.3 mL) at 50°C for 4 hr with intermittent shaking. The resin was filtered, washed with THF, THF: H₂O (1:1), THF: 2N HCl (1:1) and hot water till free from chloride and finally with methanol. Further purification of the resin was done by soxhlet extraction with THF. To estimate the chlorine capacity the resin (50 mg) was digested with pyridine (4 mL) for 6 hr and free chloride ions thus replaced were estimated by Volhard's titrimetric method. The calculated value was 1.8 mmole Cl/g.

Attachment of first amino acid to the chloromethylated resin

(a) Attachment of Boc-Gly.

Boc-Gly (63 mg, 0.36 mmole) was dissolved in minimum amount of ethanol and pH of the system was adjusted between 7-8 by adding aq. solution of Cs₂CO₃. The solution was then co-evaporated with benzene and dried as the Boc-Gly-O-Cs salt powder and kept in vacuo over P₂O₅. The Cs-salt of Boc-Gly was then dissolved in minimum quantity of NMP and to this solution chloromethylated resin (100 mg, 0.18 mmole Cl) was added. The mixture was kept at 50-60°C for 32 hr with occasional shaking. The resin was filtered, washed with NMP, NMP:H₂O (1:1), methanol and DCM. The Boc amino acid appended resin was dried in vacuo. The amino group capacity was determined by picric acid method and was found to be 1.65 mmole NH₂/g.

(b) Attachment of Boc-Lys(-2Cl-Z)-OH and Boc-Asp(-OBzl)-OH

For appending Boc-Lys(-2Cl-Z)-OH and Boc-Asp(-OBzl)-OH the procedure was similar to that adopted for Boc-Gly. Here, for 100 mg of resin (0.18 mmole Cl) the amount of amino acid used was 0.45 mmole of Boc-Lys(-2Cl-Z)-OH and 0.45 mmol, 145 mg of Boc-Asp(-OBzl)-OH. The amino capacity was estimated to be 1.11 and 1.35 mmole NH₂/g for Lys and Asp respectively.

Peptide synthesis

Synthesis of Leu-Gly-Gly

Boc-Gly resin (100 mg) was placed in a solid phase shaking vessel and the Boc group was removed by treating with 30% TFA in DCM (4 mL × 30 min) followed by neutralization with 5% DIEA in DCM (4 mL, 2 × 5). The resin was then filtered and washed with DCM. The ninhydrin test was performed for the
indication of the free amino group. The second amino acid Boc-Gly (0.33 mmole, 57.8 mg) was then coupled to the deprotected resin by the DCC method. Coupling of the third amino acid Boc-Leu was carried out after deprotection. Double coupling was employed at all stages to ensure 100% completion of reaction. After the TFA cleavage the peptide was obtained as a white powder (24 mg).

**Synthesis of Phe-Gly-Lys and Phe-Thr-Gly-Lys**

The Boc-Lys(-2Cl-Z)-OH resin (100 mg) was taken in a solid phase reaction vessel and the tri- and tetra-peptides were synthesized by the same protocol employed in the synthesis of Gly-Leu-Gly. For each coupling two-fold molar excess amino acid was taken. The amounts of peptide obtained after the cleavage of 100 mg peptidyl resin were 28 and 30 mg respectively.

**Synthesis of Lys-Gln-Ala-Thr-Val-Gly-Asp (DBI 32-38)**

Boc Asp(-OBzl)-OH resin (100 mg) was washed with DCM and the Boc group was removed followed by neutralisation. Here in the synthesis of this peptide, instead of DCC coupling HOBT active ester method was employed. The active ester of the amino acid with HOBT was prepared in NMP using DCC. After the removal of DCU by filtration, the filtrate was used for the coupling. The amount of peptide obtained was 35 mg.


Starting from Boc Lys(-2Cl-Z)-OH resin (100 mg) the amino acids assembled by the same protocol employed in the synthesis of the peptide DBI 32-38. Yield of the peptide obtained was 37 mg.

The protocol for the synthesis consisted of the following steps.

i. washed the resin with DCM (2.5 mL, 5×1 min.)

ii. Boc deprotected using 30% TFA in DCM (4 mL×30 min).

iii. washed with DCM (2.5 mL, 5×1 min).

iv. neutralised using DIEA in DCM (4 mL, 2×5 min).

v. washed with NMP (2.5 mL, 5×1 min).

vi. added active ester of Boc amino acid in NMP, shaken for 45 min.

vii. added DMSO (15% of total volume of NMP) shaken for 15 min.

viii. added DIEA (3.8 meq.), shaken for 5 min.

ix. washed with methanol in DCM 33:67 (2.5 mL, 5×1 min.).

**Cleavage of the peptide from the resin**

The peptidyl resin (100 mg) was suspended in TFA (5 mL) containing thiouanisole (0.5 mL) and kept at room temperature for 24 hr. The resin was filtered off using a sintered funnel and the filtrate was then rotaevaporated to remove TFA. To the residue cold ether (10 mL) was added to precipitate the peptide. The precipitated peptide was washed with ether till the peptide was free of any contaminating agent.

**Amino acid analysis**

The peptide samples (1 mg) were hydrolysed with TFA- 6N HCl (1:2) in evacuated sealed tubes at 110°C for 24 hr. The tube was broken and dried over NaOH and P2O5 in vacuo. The residue was dissolved in citrate buffer and loaded in the analyser.

**Solvation studies**

Solvation studies were carried out using a sintered crucible. The dry polymer (1 g) was placed in a sintered glass crucible (porosity 3) and the resin was equilibrated with the solvent for 24 hr. Excess solvent was removed by applying mild suction and the weight of the crucible with the swollen resin was determined. The process was repeated till a constant weight for the swollen polymer was obtained. A blank experiment was carried out with the empty crucible. Finally the solvation of the polymers were calculated as the volume of the solvent absorbed by unit weight of dry resin (mL/g).

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**References**