Recent Advances in Solid Phase Peptide Synthesis

Peptide chemistry is currently witnessing a tremendous upswing. Recent developments in the biotechnology of new proteins, as well as advances in immunology and the introduction of pharmaceuticals based on inhibitors and antagonists, have led to immense demands for synthetic peptides. The numerous possibilities for research using synthetic peptides in solving biological problems are becoming increasingly better recognized.\textsuperscript{12-19} No other area of organic chemistry seems so dependent on interdisciplinary cooperation as peptide chemistry. The fields of research in modern peptide chemistry include synthesis and analysis, isolation and structure determination, conformational investigations and molecular modelling. Project-oriented studies are being carried out in conjunction with research groups in pharmacology, physiology, immunology, biology and biophysics. More and more peptides with unusual amino acids,\textsuperscript{20-26} modified peptide bonds,\textsuperscript{27-32} linker or spacer molecules\textsuperscript{33,34} and peptide mimetics\textsuperscript{25-37} are being prepared. Highlights of the medicinal chemistry of agonists and antagonists of biologically active peptides and inhibitors have recently been summarised by Hirschmann.\textsuperscript{37a} Significant progress has also been made in immunology at the molecular level regarding recognition mechanisms, using both synthetic peptides and vaccines.\textsuperscript{38} The peptide chemist is continually
being confronted with new challenges at ever shorter intervals.\textsuperscript{39,40} There is a greater demand for new strategies, faster synthesis,\textsuperscript{39} better coupling reagents and protecting groups and especially methods for the simultaneous preparation and analysis of very large numbers of peptides in a short time. Peptide synthesis has proven to be indispensable for the structural elucidation of many recently isolated natural products having a peptide structure such as hormones, neuropeptides, and antibiotics which very often could be isolated in only minute quantities. Investigation of the structure-activity relationships of biologically active peptides also demands the synthesis of many analogues of a given peptide.

The story started with Fisher\textsuperscript{41} and Curtius\textsuperscript{42} at the turn of this century has now been developed into a full-pledged discipline of immense power and sophistication. In the following years formation of peptide bond through acid azides\textsuperscript{43} and acid chlorides\textsuperscript{44} became well established but a general approach for the synthesis of peptides was not yet available. A broadly applicable methodology requires a choice of readily available protecting groups as well. Introduction of the benzyloxy carbonyl group\textsuperscript{45} in 1932 by Bergmann and Zervas laid the ground work for major endeavours in peptide synthesis. The first significant accomplishment of this method was done by du Vigneaud and his associates\textsuperscript{46} by synthesising oxytocin.

The twentieth century has witnessed the development of a number of techniques for the assembly of amino acids to form peptides. The adaptation of mixed anhydrides\textsuperscript{47-50} for peptide bond formation, the introduction of active esters\textsuperscript{51-53} and the discovery of coupling reagents\textsuperscript{54,55} followed each other in rapid succession.

With the development of new reagents and techniques, the synthesis of small peptides has been placed within easy reach by classical approach to
peptide synthesis. However, these procedures are not ideally suited to the synthesis of long chain polypeptides because the technical difficulties with solubility and purification become formidable as the number of amino acid residues increases. The demonstration by Merrifield in 1962 that peptide bond formation could be achieved efficiently when one of the reactants was attached to an insoluble polymeric support has proved to be one of the most important developments in the history of peptide synthesis. It opened the way to the design of rapid, machine-aided, procedures which with due recognition of their advantages and limitations, have now taken their place alongside with more traditional methods of peptide synthesis. The subject has been well reviewed by Erickson and Merrifield and also very valuably by Meienhofer.

The established classical methods of synthesis were too slow and laborious to cope easily with the increasing demands of pharmacology and the approach offered by Merrifield provided a quick and attractive solution. Subsequent improvements of this novel technique and improvements in the segment condensation method in combination with the introduction of the HF cleavage reaction by Sakakibara and coworkers and the application of high performance liquid chromatography by Rivier to the purification of peptides enabled the peptide chemists to synthesise complex peptides and proteins of about 100 amino acids rapidly and efficiently.

Stepwise peptide synthesis on polymer supports is regaining importance due to the recent improvements made in protecting group strategy, anchoring techniques and support properties. The development of efficient separation method, especially preparative high pressure liquid chromatography (HPLC), has led in particular to an important breakthrough in solid phase synthesis. Medium sized peptides of upto 20 amino acid residues can be purified reliably by using the HPLC techniques. Although the
synthesis of higher peptides on polymeric supports still appears to be difficult, it has often been provided valuable preliminary information about the physico-chemical and structural properties of the desired peptide. The combination of stepwise synthesis on a support and subsequent condensation of the segments, either in solution or on polymeric supports, could prove to be the best method for synthesising longer chain peptides. With great accuracy peptide fragments can be synthesised in gel phase or by conventional methods in solution as well.

The first detailed study of the preferred conformations of well characterised low molecular weight peptides was done by Goodman and Schmitt (1959). In the 1960s and early 1970s, the structural analysis of biomolecules was virtually dominated by X-ray crystallography and the foundations of modern structural molecular biology were just beginning to be gradually established. The realisation of the importance of the solution conformation of peptides began in the late 1960s, mainly through the development of high resolution nuclear magnetic resonance spectroscopy, but was essentially limited to non-aqueous solutions of peptides. At just about the same time, circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR) and Raman spectroscopy began to emerge as structural probes of the solution conformation of peptides. As a result of these developments, a spectacular advance in our understanding of solution conformation of peptides was witnessed in the 1970s and 1980s. These experimental observations provided striking support for the discovery of a-helix and B-sheet by Pauling and Corey in the early 1950s.

The application of nuclear magnetic resonance spectroscopy (NMR) in investigating peptide conformation was reviewed by Kessler. Some recent reviews put an insight into the structure determination of proteins by three- and four-dimensional NMR spectroscopy. Bandekar studied IR and Raman spectroscopic results on amide bands in peptides, polypeptides and proteins and
the research group at Copenhagen\textsuperscript{83} described the use of near-infrared (NIR) Fourier-transform (FT) Raman Spectroscopy as a new method for monitoring the secondary structure of the peptide chains during solid phase peptide synthesis. Some more examples of conformational analysis of peptides came from the laboratories of Thornton,\textsuperscript{84} Narita,\textsuperscript{85} Baldwin,\textsuperscript{86} and Ponnuwamy.\textsuperscript{87}

\section{2.1 Chemical synthesis of peptides}

The three most important strategies for the synthesis of peptides are the classical solution phase method, solid phase peptide synthesis (SPPS) and the liquid-phase peptide synthesis.

The classical method has evolved since the beginning of the twentieth century and is characterised by the stepwise synthesis of short segments under homogeneous reaction conditions in solution.\textsuperscript{88,92} The fragments are subsequently coupled via the segment condensation technique. The products are purified from side products and truncated sequences after each synthetic step. For this reason, products obtained via a classical method are distinguished by a high degree of purity and suitable for medical applications. On the other hand, this technique requires skilled chemists, and it is time-consuming. However, the greatest limitation of this approach is the generally low solubility of medium-sized peptides. These problems are of great weight as the chain-length of the peptide increases.

The solid phase method of peptide synthesis differed from general synthetic organic methods, where one of the reactants was reversibly and covalently bound to an insoluble solid polymer support which was then reacted with the reagent to give a resin-bound product. After filtering the latter from the
reaction mixture and after repetition of as many steps as necessary to achieve the synthesis the product was obtained by a suitable cleavage reaction.²,⁹³

The liquid-phase method (LPS) developed by Mutter and Bayer⁹⁴-⁹⁸ combined the advantages of polymer supported technique with those of a synthesis carried out under homogeneous reaction conditions. Unlike the solid phase method, the LPS ensures coupling and deprotection in homogeneous solution. However, reduced operational simplicity and changes in the crystallisation tendency are the two major limitations of liquid-phase peptide synthesis.

In the polymeric reagent method insoluble polymeric amino acid active esters serve as the carboxyl component for the coupling to the soluble amino terminal component. The peptide formed remains in solution from where it can be isolated and purified at each step before proceeding to the next step. An added advantage of this method is the formation of almost racemisation free peptides.

2.2 Solid phase peptide synthesis (SPPS)

The solid phase approach of peptide synthesis was conceived and elaborated by Merrifield beginning in 1959, and it has also been covered comprehensively in many reviews.⁹⁹-¹⁰⁷ The concept of SPPS (Figure 2.1) is to retain chemistry proved in solution (protection scheme, reagents), but adding a covalent attachment step (anchoring) that links the nascent peptide chain to an insoluble polymeric support. Subsequently, the anchored peptide is extended by a series of addition (deprotection/coupling) cycles, which are required to proceed with exquisitely high yields and fidelities.
Figure 2.1. Typical outline of the solid phase peptide synthesis.

1. TFA – Trifluoroacetic acid
2. TEA – Triethylamine
3. DCC – Dicyclohexyl carbodiimide
It is the essence of the solid phase approach that reactions are driven to completion by the use of excess soluble reagents, which can be removed by simple filtration and washing without manipulative losses. Because of the speed and simplicity of the repetitive steps, which are carried out in a single reaction vessel at ambient temperature, the major steps of the solid phase procedure are readily amenable to automation. Once chain elaboration has been accomplished, it is necessary to release protecting groups and to cleave the crude peptide from the support under conditions that are minimally destructive towards sensitive residues in the sequence. Finally, there must follow prudent purification and appropriate characterisation of the synthetic product to verify that the desired structure is indeed the one obtained. In recognition of the maturation and impact of this body of work, Merrifield was honoured with the 1984 Nobel Prize in Chemistry.\textsuperscript{108-111}

The main advantages of solid phase peptide synthesis over classical method of synthesis are:

(a) All the reactions involved in the synthesis can be carried to 100\% completion, so that a homogeneous product is obtained.

(b) All of the laborious steps of purification of intermediates in solution phase could be avoided.

(c) The entire process can be carried out in one container without any transfer of material from one vessel to another.

(d) The system was ideally suited for automatic operation.

(e) The support can be regenerated by a simple, low cost, high yield reaction.

In spite of all these advantages, solid phase method is not devoid of disadvantages. Major limitations of this techniques have been well reviewed.\textsuperscript{112,113} The major short comings of this method are:
(a) Non-compatibility of the support resin with the growing peptide chain.
(b) Non equivalence of functional groups attached to the polymer support.
(c) Racemisation leading to optically impure products.
(d) Formation of error peptides from deletion and truncated sequences.

Later SPPS received new impulses by,

(a) The development of new supports with superior swelling properties permitting an improved solvation of both matrix and growing peptide chain.
(b) The design of novel and more versatile anchoring groups (multidetachable anchors) enhancing the flexibility of the synthetic strategy.
(c) Progress in the field of chromatographic techniques such as preparative and semi-preparative HPLC.

2.3 Improvements in the original solid phase peptide synthesis

Ever since its inception in 1963, the solid phase peptide synthesis has become one of the most important tool in the synthesis of peptides, protein sequences and nucleotides. Although the earlier solid phase chemistry was very useful for making small peptides and even small proteins, it was clear that there was a need for improvement in several areas. The original technique employed by Merrifield has undergone a series of modifications and improvements. Novel improved supports such as 'isocyanide' resin, 114 'Rink' resin,115 5-[4(9-Fmoc) amino methyl 3,5-dimethoxy phenoxy] valeric acid (PAL) resin,116 tertiary alcohol resin,117 carboxyl amide terminal resin (CAT),118 2-chlorotrityl chloride resin,119 polyoxyethylene-polystyrene graft copolymeric support (POE-PS),120
polyacrylate-DVB copolymer, polyamide-kieselgu, support have been introduced. New hydrophilic matrices for the synthesis of small peptides by either batch or continuous flow methods, NPE resin (2-(2-nitrophenyl ethyl) for the synthesis of protected peptides and oligonucleotides and polyethylene glycol-polystyrene resin are some of the recent developments in the field of polymer supported peptide synthesis. Bis-2-acrylamidoprop-1-yl polyethyleneglycol crosslinked dimethyl acrylamide (PEGA) has been introduced as a hydrophilic, biocompatible and flexible solid support in peptide synthesis and recently a new method for preparation of high capacity PEGA resins with well defined loading of functional groups has been described for continuous flow SPPS by Meldal and co-workers.

5,9-(9-fluorenyl methyloxy carbonyl amino xanthen-2-oxy) valeric acid (XAL) has been introduced as an acid labile handle for Fmoc-based peptide amide synthesis. Dimethoxy acido-labile linker (DAL), 4-hydroxymethyl phenoxy acetic acid and 3-methoxy-4-hydroxymethyl phenoxyacetic acid are some of the acid labile peptide resin linkage agents for use in solid phase peptide synthesis. A report from the second Japan peptide symposium dealt with the development of novel acid-labile peptide amide linkers. Progress on handles and supports for solid phase peptide synthesis has been reviewed by Barany and Albericio. Substituted benzhydrol derivatives are also used as linkers in solid phase peptide synthesis.

Recently use of an oxidation labile phenylhydrazide group as a linker for solid phase synthesis was reported. A new type of matrix specific linker was recently introduced by Hauske and Dorff. Cleavage of finished peptide under mild conditions can be achieved by photolysis in cases were photolabile anchoring groups are employed. Development of new photo removable protecting groups like Menpoc (α-methyl nitropiperonyloxy-carbonyl) and
Menvoc (α-methyl nitroveratryloxy carbonyl) were reported in the 13th American peptide symposium.\textsuperscript{137} A new α-nitrobenzyl photolabile linker based on α-methyl-6-nitroveratrylamine was described for the generation of peptides by solid phase approach.\textsuperscript{138} An efficient versatile linker for solid phase peptide synthesis based upon dibenzocyclohepta-1,4-diene system has been developed by McInnes and co-workers.\textsuperscript{139} New carboxyl protecting groups like 2-(1-adamantyl)-propanol-2 esters (Adp) removable under mild acid treatment\textsuperscript{140} and 2-bromoethyl and 2-iodoethyl esters which can be deprotected by Samarium diiodide found place in solid phase peptide synthesis.\textsuperscript{141} Anpe [2-(4-acetyl-2-nitrophenyl)ethyl is a new base-labile carboxyl protecting group introduced by Robles and co-workers.\textsuperscript{142}

Base-sensitive amino groups such as 2-chloro-3-indenylmethylxy carbonyl (CLIMOC) and Benz inden-3-yl methylxy carbonyl (BIMOC) similar to the 9-fluorenyl methyloxy carbonyl (Fmoc) group have been introduced.\textsuperscript{143} Another newly developed base labile α-amino protecting group is 2-(4-nitro phenyl)sulphonylethoxycarbonyl (NSC).\textsuperscript{144} 4-Methylsulphenyl benzylxocarbonyl (Msz) group is introduced as a new class of amino protecting group removable by reductive acidolysis.\textsuperscript{145} Several modifications to the classical tert-butyloxy carbonyl (Boc) group are also introduced.\textsuperscript{146,148} Some newly developed side chain protecting groups for amino acids include, S-phenylacetoamidomethyl (Phacm) for cysteine,\textsuperscript{149} 2-Adamantyloxy carbonyl group (2-Adoc) for the ε-amino group of lysine\textsuperscript{150a} and for the imidazole function of Histidine,\textsuperscript{150b} 2,4-dinitrophenyl (Dnp) group for the protection of hydroxyl function of tyrosine,\textsuperscript{151} p-(methylsulphinyl)benzyl group for serine,\textsuperscript{152} 2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulphonyl group (Pbf) for arginine\textsuperscript{153} and polyethyleneglycol (PEG) bound benzyl and fluorenyl side chain protection for lysine and glutamic acid.\textsuperscript{154}
New Boc deprotecting agents like chlorotrimethyl silane-phenol have been prepared which may replace the conventional ones. Another newly developed reagent for the deprotection of t-butyloxycarbonyl group and N\textsuperscript{a}-benzyloxycarbonyl group is iodotrichlorosilane obtained from silicon tetrachloride and sodium iodide. A new stepwise deprotection methodology using reductive acidolysis is effective to suppress the side reactions at aspartic acid residue. A report from the 22nd European Peptide Symposium (1992) is about the optimised deprotection procedure for peptides containing Arg (mtr), Cys (Acm), Trp and Met residues. N-allyloxycarbonyl (Alloc) protecting group could be efficiently removed using sodium borohydride in the presence of catalytic amount of palladium (0). 21st European Peptide Symposium reported on the use of trimethyl silyl triflate/trifluoro acetic acid/pentamethyl benzene for simultaneous resin cleavage and tert-butyloxycarbonyl (Boc) and benzyl deprotection in solid phase peptide synthesis. Selective removal of N-Boc protecting group in the presence of tert-butyl ester and other acid sensitive groups by dry HCl in ethyl acetate at room temperature is described by Rapoport and co-workers.

Novel activating agents like Benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP), benzotriazololyoxy tri(pyrrolidine) phosphonium hexafluorophosphate (PyBOP), 2-trifluoroacetyl-thiopyridine-1-hydroxy benzotriazole, Bis[4-(2,2-dimethyl 1,3-dioxolyl) methyl]-carbodiimide (BDDC), bromo-tris(pyrrolidino)-phosphonium hexa-fluorophosphate (PyBrop) have been introduced. A mixture of 2-trifluoroacetylthiopyridine with the sodium salt of HOBt (NaOBt) was found to be a highly effective coupling reagent. Enhancement of peptide couplings was recommended by a combination of 4-dimethylaminopyridine (DMAP)-dicyclohexylcarbodiimide (DCC) in the case of hindered amino acid residues. In 1993, Carpino reported the use of 1-hydroxy-7-azabenzotriazole as an efficient peptide coupling...
additive. In the same year Carpino’s group reported the use of Bis (Boc) amino acid fluorides as reactive peptide coupling reagents. 3-Dimethylphosphinothiyl-2(3H)-oxazolone (MPTO), was introduced as a promising new reagent for racemisation free couplings. Fmoc amino acid chloride coupling can be conveniently carried out in the presence of KOBt (potassium salt of 1-hydroxybenzotriazole). A new hybrid that combines the structural features of dicyclohexylcarbodiimide (DCC) and N,N’-diisopropylcarbodiimide (DIC), i.e., N-cyclohexyl-N-isopropyl carbodiimide (CIC) was proposed as an efficient coupling reagent in peptide synthesis. Bis[4-(2,2-dimethyl-1,3-dioxolyl)methyl carbodiimide (BDDC), 2-(benzotriazol-1-yl)oxy-1,3-dimethylimidazolidinium hexafluoro-phosphate (BOI), 2-(IH-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Tris(pyrrolidino) phosphonium reagent, a phosphorous based reagent, are some of the recent additions to the field of coupling reagents. Second Chinese Peptide Symposium reported the use of novel cyclic organophosphorous compounds as coupling reagents in peptide synthesis. 1-β-Naphthalenesulfonyloxybenzotriazole (NSBt) and 6-Nitro-1-β-Naphthalenesulfonyloxybenzotriazole were the two recently introduced activating agents in peptide synthesis. Some of the newly developed activating agents include di-tert-butyl pyrocarbonate in the presence of pyridine and ammonium hydrogen carbonate, tetramethylfluoroformamidinium hexafluorophosphate, for solution and solid phase synthesis, (F$_3$NO$_2$–PyBOP) (1-hydroxy-4-nitro-6-(trifluoromethyl)benzotriazole containing phosphonium salt for in situ coupling of N-methylated amino acids, 1-hydroxy-7-azabenzotriazole (HOAt) and its corresponding uronium and phosphonium salts for the automated synthesis of peptides containing hindered amino acids. ToppipU (2-(2-oxo-1(2H)-pyridyl)-1,1,3,3-bispentamethylene uronium
tetrafluoroborate) and FDP\textsuperscript{186} (pentafluorophenyl diphenylphosphate) are the two new additions to the field of coupling agents in peptide synthesis.

Regarding the cleaving agents a mixture of hexafluoroisopropanol–dichloromethane (1:4 v/v) acts as a fast, effective and convenient reagent for cleaving protected peptide fragments with a minimal amount of racemisation from a 2-chlorotrityl chloride resin,\textsuperscript{187} hydrolysis of polystyrene-bound protected peptides by using dimethyl amino ethanol or triethanolamine, DMF and aqueous sodium hydroxide with acceleration by ultrasound,\textsuperscript{188} use of HF for cleavage and deprotection of peptides synthesised using a Boc/Bzl strategy\textsuperscript{189} were reported recently.

Several investigations are going on to increase the efficiency of peptide synthesis by solid phase approach. Some of the recent publications provide a glance into it. Procedures to improve difficult couplings,\textsuperscript{190} new solvent systems for difficult sequences,\textsuperscript{191,192} optimised solid phase synthesis of large peptides utilising Fmoc-amino acids,\textsuperscript{193} accelerated protocols for solid phase peptide synthesis at elevated temperatures,\textsuperscript{194} new apparatus for synthesis,\textsuperscript{195-198} a general strategy for the synthesis of large peptides by combining the solution phase and solid phase approach\textsuperscript{199} and the development of an apparatus for cleaving peptides from resin supports\textsuperscript{200} were a few of them. Progress in SPPS is well reviewed recently by Kiyoshi, John and Botand.\textsuperscript{201-203} Thus many dramatic advances have occurred in recent years in the field of solid phase peptide synthesis.

The chemical synthesis of longer peptide chains is still a central problem of protein chemistry despite the considerable progress that has been made in the strategy of various synthetic methods and protecting group techniques. The physicochemical incompatibility of the growing peptide chain and the insoluble crosslinked polymeric support has been one of the major problems associated
with the polymer-supported method of peptide synthesis. Development of polymer supports which swell in both polar and non-polar solvents facilitating the different types of organic reactions employed in repetitive stepwise peptide synthesis have therefore been a challenge to organic and polymer chemists for the past two decades. The concept of optimum hydrophobic-hydrophilic balance serves as a guideline for the development of effective supports for peptide synthesis. Structure-reactivity and structure-property correlations in polymeric systems can be made use of in the design of such supports with optimum reactivity characteristics, mechanical stability and other essential requirements of a polymeric support useful for the stepwise synthesis involving a multitude of synthetic operations under widely varying conditions.

Attempts have been made in our laboratory to develop some new polymeric supports based on polyacrylamide and polystyrene for solid phase peptide synthesis. Polystyrene supports include tetraethyleneglycol diacrylate (TTEGDA), triethyleneglycol dimethacrylate (TEGDMA), and 1,6-hexanediol diacrylate crosslinked polystyrene supports. Their stability and solvation characteristics compared to the Merrifield resin (PS-DVB) is better and that resulted the increased use of these resins in solid phase peptide synthesis. Here we used polystyrene crosslinked with 1,6-hexanediol diacrylate as the flexible polymer support, which can be easily prepared and functionalised.

2.4 Synthesis of hydrophobic peptides

Peptides that have polar side chains are hydrophilic in nature and having non polar side chains are hydrophobic in nature. The apparent hydrophobicities of the amino acid side chains vary enormously, depending primarily on whether or not polar groups are present.

The hydrophobicities of the individual amino acid side chains have been measured experimentally in a variety of ways, using the free amino acids, amino
acids with the amino and carboxyl groups blocked, and side-chain analogues with the backbone replaced by a hydrogen atom and using a variety of non polar solvents including ethanol, octanol, dioxane and cyclohexane.\textsuperscript{210}

**Table 2.1.** Relative hydrophilicities and hydrophobicities of amino acid side chains

<table>
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<tr>
<th>Residue</th>
<th>Hydrophilicity (Kcal/mol)\textsuperscript{211}</th>
<th>Hydrophobicity (Kcal/mol)</th>
<th>Side-chain analogues\textsuperscript{211}</th>
<th>Amino acids\textsuperscript{212}</th>
<th>N-acetyl amide\textsuperscript{213}</th>
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Molecules that have polar groups can appear to be more hydrophobic than they really are. The more hydrophobic molecules have the more negative hydrophobicities.

Synthesis of hydrophobic peptides is a difficult process because of the non-polar side chains and because of the coiling nature of the peptides. Peptides that have substantial hydrophobic character also trend to aggregate with increasing concentration.

In the present study, it is proposed to synthesise some of the partial sequences of thioredoxin—a naturally occurring sulphur reducing protein. Thioredoxin contains sequences of varying hydrophobicity-hydrophilicity patterns. Here, most of the sequences synthesised are hydrophobic in nature. These partial sequences were synthesised on a 2% HDODA-crosslinked polystyrene by following the standard solid phase methodology.