

JOSE LLOREDA CAMACHO & CO.

ABOGADOS

Calle 72 No. 5-83 Piso 5, Bogotá, D.C. Tel: 6069700 Fax 6069701

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

E. _____ S. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-126531-00006-0000

Fecha: 2012-09-14 16:33:06

Tra. 11 PATENTEIYII

Act. 523 RESPUESTA

Dep. 2020 DIR.NUEVASCR

Eve: 378 FASENACIONALI

Folios: 36

Re: PI.-F.HOFFMANN-LA ROCHE AG.-Solicitud de Patente de
Invención en Fase Nacional PCT para "SÍNTESIS DE UN PÉPTIDO
INSULINOTRÓPICO".-Clase C07K 14/605.-Expediente número
08-126.531.-

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista de 22 de Junio de 2012.

2. De acuerdo con el Concepto Técnico se tiene que:

"Como resultado de la búsqueda de anterioridades de materia relacionada con la de la presente solicitud, se determinó que existe en el estado de la técnica los siguientes documentos que eventualmente afectaría los requisitos de patentabilidad de esta solicitud:

- Lloyd-Williams P. ET AL "CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS". Tethahedron. Vol. 49, No. 48, 1993, Pages 11065-11133.

3. Para responder a los requerimientos del señor Examinador de conformidad con el Artículo 46 de la Decisión Andina 486 de 2000, anexo me permito presentar a ese Despacho copia de los siguientes documentos solicitados:

- ♦ Lloyd-Williams P. ET AL "CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS". Tethahedron. Vol. 49, No. 48, 1993, Pages 11065-11133.

JOSE LLORED A CAMACHO & CO.

Calle 72 No. 5-83 Piso 5, Bogotá, D.C. Tel: 6069700 Fax 6069701

4. En vista de lo anterior, ruego a usted tomar atenta nota y proceder de conformidad.

Señor Superintendente,



ALICIA LLORED A RICAURTE

T.P. 53.215

Dirección para Notificaciones: notificacionespatentes@lloredacamacho.com

TETRAHEDRON REPORT NUMBER 347

CONVERGENT SOLID-PHASE PEPTIDE SYNTHESIS

PAUL LLOYD-WILLIAMS,* FERNANDO ALBERICIO*† and ERNEST GIRALT*

*Department of Organic Chemistry, University of Barcelona, Barcelona 08028, Spain

†Millipore Corporation, Peptide Research Group, 75A Wiggins Avenue, Bedford, MA 01730, U.S.A.

(Received 22 December 1992)

CONTENTS

Introduction	11066
1. Solid-Phase Synthesis of Protected Peptide Segments	11070
1.1. Photolytic cleavage of protected peptides from the solid support	11071
1.1.1. Nitrobenzyl resins	11072
1.1.2. Phenacyl resins	11074
1.2. Acidolytic cleavage of protected peptides from the solid support	11075
1.2.1. The Wang resin	11076
1.2.2. Highly acid-labile resins and linkers	11077
1.2.3. Other acid-labile resins	11079
1.3. Nucleophile- and base-mediated cleavage of protected peptides from the solid support	11080
1.3.1. Cleavage of the peptide-resin anchorage by a β -elimination reaction	11080
1.3.2. The Kaiser oxime resin	11082
1.3.3. Fluoridolysis of the peptide-resin anchorage	11084
1.3.4. Cleavage of glycolamide ester anchorages	11085
1.3.5. Miscellaneous methods	11085
1.3.5.1. Ammonolysis, hydrazinolysis and aminolysis	11085
1.3.5.2. Transesterification and saponification	11087
1.3.5.3. Hydrogenolysis	11089
1.4. Cleavage of protected peptides from allyl-functionalised resins	11089
1.5. Miscellaneous methods for the synthesis of protected peptide segments	11090
1.5.1. Multidetachable resins	11090
1.5.2. Other linkers	11092
2. The Purification of Protected Peptide Segments	11094
2.1. Methods for the purification and characterisation of protected peptides	11094
2.1.1. Classical techniques	11094
2.1.2. Gel-filtration	11094
2.1.3. High-performance liquid chromatography	11095
2.1.4. Other techniques	11095
2.1.5. Characterisation of protected peptide segments	11095
2.2. Approaches to circumvent the problem of insolubility of protected peptides	11095
2.2.1. Structure-solubility relationships of protected peptide segments	11096
2.2.2. Use of special solvents and/or of additives	11097
2.2.3. Use of special protection schemes to enhance solubility or to facilitate purification	11097
3. Solid-Phase Coupling of Protected Peptide Segments	11098
3.1. Choice of solid support	11098
3.2. Coupling strategy	11099
3.3. Incorporation of the first segment onto the resin	11100
3.3.1. Stepwise solid-phase synthesis of the first segment	11100
3.3.2. Attachment of protected peptide segments to the resin	11102
3.4. Segment size	11104
3.5. Coupling methods	11106
3.6. Side reactions	11114

3.7. Monitoring of the coupling reaction	11117
3.8. Examples of convergent solid-phase peptide synthesis	11118
3.8.1. Rat atrial natriuretic factor	11118
3.8.2. Prothymosin- α	11119
3.8.3. β -Amyloid protein	11119
Other Convergent Peptide Synthesis Strategies	11121
Assessment and Prospects for the Future	11123

INTRODUCTION

Over the last ten years there has been a rapid growth in interest in the chemistry of large peptides and proteins.^{1,2} The study of the mechanisms of hormone action, and of enzyme-substrate-, antigen-antibody- and protein-DNA³-interactions has now moved to the forefront of contemporary chemistry. One of the main difficulties facing researchers in these fields is that of obtaining sufficient quantities of the peptide or protein in a pure state. The isolation of proteins from natural sources can be laborious and often provides only tiny quantities of material. Among the contemporary methods for peptide and protein synthesis are the biotechnological techniques which are currently widely used for the provision of protein molecules. One of the most useful methods is the cloning of genes followed by the expression of the naturally occurring peptide or protein in suitable host systems.⁴⁻⁶ Powerful as they are, these techniques have their drawbacks. Once expressed, the isolation of the desired peptide from the fermentation medium can be difficult, and although such methods can be used to provide modified protein structures,⁷⁻⁹ they are not really appropriate for the production of the large number of analogues which are routinely needed for structure-activity relationship studies.

The advances in chemical peptide synthesis over the last 50 years have made the synthesis of large peptides and proteins a realistic possibility. Chemical synthesis is probably the most practical way of providing useful quantities of material, and in addition allows the systematic variation of structure necessary for the development of peptides for therapeutic use.¹⁰ Analogues of the peptide, and modified structures containing specifically-labelled amino acids or non-DNA-encoded amino acids, and also peptide mimetics, are more efficiently made by chemical synthesis. However, for large peptide molecules,¹¹ the synthesis of chemically homogeneous material is a challenging undertaking and success in the endeavour cannot necessarily be guaranteed.

Organic chemists since Emil Fischer¹² have addressed the problems that the chemical synthesis of proteins entail. The syntheses of insulin by Zahn,¹³ Katsoyannis,¹⁴ Kung¹⁵ and Rittel,¹⁶ and of ribonuclease A by Yajima and Fujii,^{17,18} are milestones in the field. Also noteworthy are the attempts of Kenner and associates¹⁹ to synthesise a lysozyme analogue. The synthesis of large peptides in solution, in which the various intermediate compounds are purified and characterised is, in principle, the surest way of obtaining homogeneous material. However, this approach to the synthesis of large peptides is fraught with problems, the most troublesome being that of solubility. Fully protected peptides are often so insoluble even in solvents such as DMF or DMSO, that chemical reactions of such species are effectively rendered impossible. Other important problems associated with the synthesis of peptides in solution are the possible epimerisation at the activated C-terminal amino acid of the peptide segments in the coupling steps and the lack of sufficiently powerful methods for purifying large protected peptides. In spite of the problems, synthesis in solution is one of the major methods of peptide synthesis and continues to find important applications. The large scale synthesis of shorter peptides, the synthesis of peptides composed of unusual or uncommon amino acids,²⁰ the synthesis of cyclic peptides,²¹ of cyclic and of linear depsipeptides,^{22,23} and the semisynthesis of protein molecules²⁴ are all often carried out in solution.

One of the most important contemporary methods for the chemical synthesis of peptides is the

linear solid-phase peptide synthesis (SPPS) developed by Merrifield.²⁵⁻²⁸ This approach, together with the improvement in chromatographic techniques, particularly the development of HPLC, revolutionised peptide synthesis. In SPPS the first amino acid of the sequence, protected at its N^α -terminal and at its side-chain (if necessary) is attached to a solid support or resin by a covalent bond: an ester for peptide acids or an amide for peptide amides. Once the first amino acid is attached, the amino acid sequence of the peptide is built up on the solid support by a series of N^α -deprotection and amino acid coupling steps. When the desired sequence has been synthesised, the peptide is released from the resin (usually by acidolysis) affording the crude peptide which is then purified. The great advantages of the method are its simplicity and the speed with which peptides can be synthesised. Linear SPPS is represented schematically in Fig. 1.

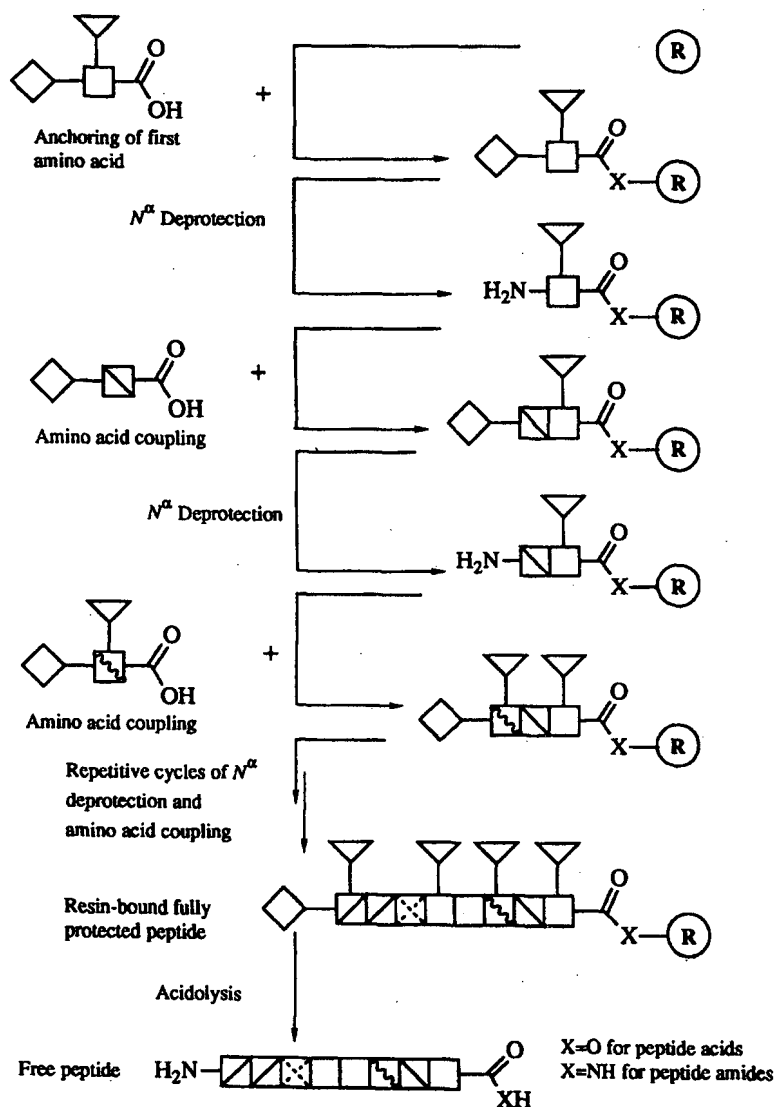


Fig. 1. Schematic representation of linear SPPS. Amino acids are represented by squares, the protected α -amino group by a diamond and the protected side-chain functional groups by triangles. The C-terminal of the peptide is bound to an insoluble solid support designated R for resin.

In SPPS, the peptide is almost always built up from the C-terminal towards the N-terminal (C to N strategy). Although synthesis in the opposite direction (N to C strategy) is possible,²⁹ it is much less popular, not least because the dangers of epimerisation are much more pronounced.

The main problem in linear SPPS, apart from certain sequence-dependent side-reactions during couplings and the final cleavage of the peptide from the solid support, is that of non-quantitative yields in the amino acid coupling steps and in the N^α deprotection reactions. This leads to deletion peptides and to terminated peptide sequences all of which are present in the crude mixture after the final cleavage step of peptidic material from the solid support.³⁰ Since these undesired peptides may very closely resemble the target molecule, high resolution separation methods must be used in order to obtain the desired product in a pure state. In its most extreme form, non-quantitative coupling can lead to so-called 'difficult sequences' in which low coupling yields are observed for a series of amino acids in a sequence.^{31,32} Peptides having these sequences often cannot be satisfactorily synthesised by linear SPPS and either require special protocols or a different type of synthetic strategy. The β -amyloid protein of Alzheimer's disease is such a case. This protein, which consists of 42-amino acid residues, is very insoluble and has been synthesised by conventional linear SPPS.³³⁻³⁵ However it has been difficult to establish with certainty, whether or not the β -amyloid protein produced in these syntheses is free from deletion or termination peptides.^{34,35} Lansbury has recently synthesised this protein in a pure state using a convergent synthetic strategy.³⁶

Linear SPPS is nonetheless, a highly optimised technique and Kent *et al.*³⁷⁻⁴⁰ have reported on a series of linear solid-phase syntheses of large peptide molecules and proteins, demonstrating that large peptides can be made to acceptable standards of purity by the stepwise methodology. Linear SPPS is not however, a general solution to the problem of the synthesis of protein molecules and it may be that other approaches will prove equally, if not more, useful in the long run.

An attractive method for synthesising large peptides would be to build up the desired amino acid sequence by the coupling of purified and fully characterised peptide segments on a solid support. The synthesis of a large peptide built up in this way is inherently more reliable than one synthesised by the linear coupling of the constituent amino acids. The characterisation and purification of each of the peptide segments provide a series of checks on the nature of the material being synthesised, which is absent in the linear approach. In this type of segment coupling synthesis the advantages of the rapidity of SPPS, and those of the purification and characterisation of the intermediates of classical solution synthesis, are combined and the final product is more likely to be homogeneous. An additional advantage is that the purification of the desired peptidic material at the end of the synthesis should be facilitated.⁴¹ Incomplete couplings between peptide segments results in peptides that should be significantly different from each other in terms of their solubilities and chromatographic behaviour, since they will differ by one complete segment of the sequence. In the case of a linear synthesis the difference between the peptides formed after a low coupling yield of one amino acid, in terms of their respective solubilities and chromatographic properties, is often so small as to be negligible, particularly as the size of the peptide chain increases. The potential improvement in the ease of purification of the final mixture is one of the most important advantages of the convergent solid-phase approach to the synthesis of large peptides.

In the late 70s our research group began work on such an approach to peptide synthesis with a long term goal of developing a methodology for the chemical synthesis of large peptide and protein molecules. We have introduced the name *convergent SPPS* to describe this strategy and the procedure is represented schematically in Fig. 2.

Convergent solid-phase peptide synthesis involves the following stages:

1. Solid-phase synthesis of protected peptide segments and the detachment of these from the resin in such a way that the N^α-protecting group, in addition to those of the side-chain functional groups, are retained and that the C-terminal of the segment is either the free carboxylic acid or a derivative of this which is suitable for coupling. Protected peptide segments are usually chosen

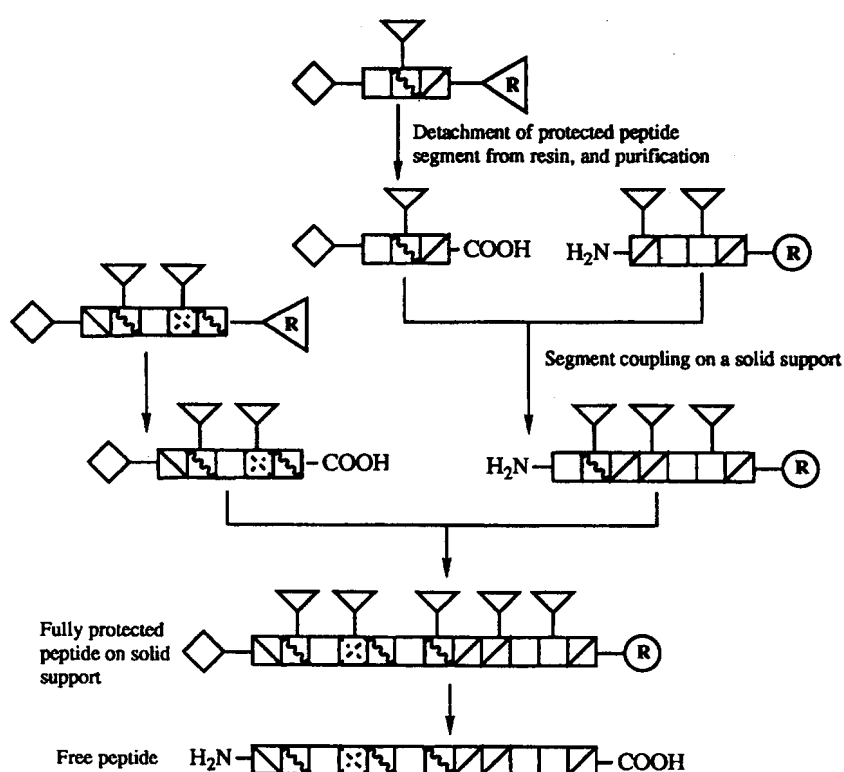


Fig. 2. Schematic representation of convergent SPPS.

so that either Gly or Pro is at the C-terminal in order to avoid epimerisation at the C-terminal amino acid during the segment coupling steps.

2. Purification and characterisation of the protected peptide segments.
3. Coupling of the purified segments on a solid support, in this way building up the amino acid sequence of the desired peptide or protein.
4. Detachment of the completed amino acid sequence from the resin and removal of the side chain protecting groups. This latter process need not necessarily be carried out to completion in one step since the regiospecific formation of disulfide bridges, for example, may demand that thiol functions remain protected after the detachment of the peptide from the resin.
5. Formation of disulfide bridges or other synthetic operations depending on the specific case, and final purification of the desired target molecule.

In this review we consider work in the areas of:

1. Solid-phase synthesis of protected peptide segments;
2. Purification of protected peptide segments;
3. Solid-phase coupling of protected peptide segments;

as related to the goal of the chemical synthesis of large peptide molecules. We do not consider work related to the detachment of free peptides from the solid-support nor the formation of disulfide bridges, since these important issues are also relevant to linear SPPS and have been addressed in detail by other authors.⁴²⁻⁴⁴

1. SOLID-PHASE SYNTHESIS OF PROTECTED PEPTIDE SEGMENTS

The first stage in a CSPPS strategy is the solid-phase synthesis of the various protected peptide segments corresponding to the desired amino acid sequence of the target molecule. Protected, as opposed to free, peptide segments are required because generally speaking, in order to couple them in an unambiguous manner, peptide segments must be protected at all reactive functional groups except those which are required for coupling.

For SPPS, three types of protection are required. The functional groups of the amino acid side-chains must be protected with groups which are stable to the repetitive treatments necessary for removal of the N^α amino protecting group of the growing peptide chain and for repeated amino acid couplings. Such side-chain protecting groups are usually called 'permanent' protecting groups. The protecting group of the N^α amino function is normally referred to as a 'temporary' protecting group. The third type of protection may be considered to be peptide-resin anchorage which protects the C-terminus of the peptide throughout the various synthetic processes required to elaborate the desired sequence, until such time as it is to be detached from the resin.

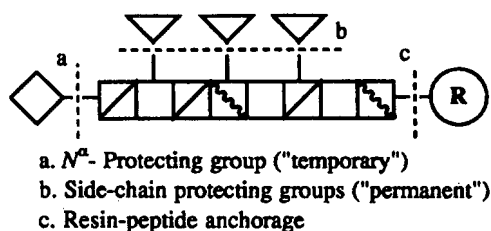
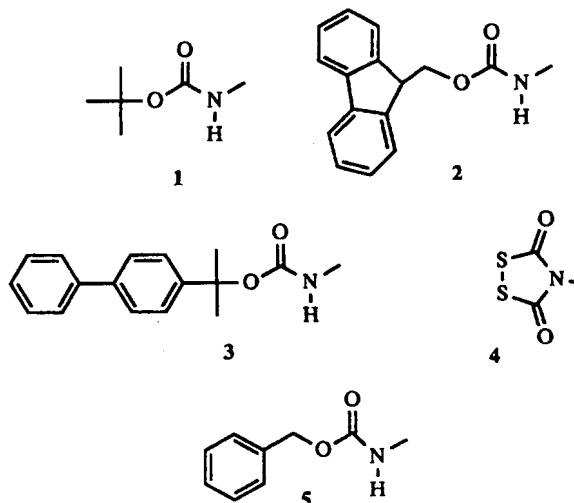


Fig. 3. Protection scheme for solid-phase peptide synthesis.

For the solid-phase synthesis of peptides the N^α protecting group is almost always a urethane derivative. The most important N^α protecting groups are the Boc- and Fmoc- groups (Fig. 3). The Boc group (1) is removed⁴⁵⁻⁴⁷ with moderately strong acid such as 33% TFA in DCM, while the Fmoc group (2) is removed⁴⁸ on treatment with a solution of a secondary amine, usually 20% piperidine in DMF. Two other important but less widely used N^α -protecting groups in solid-phase peptide synthesis are the Bpoc group (3), labile to weak acid,^{49,50} and the Dts group (4) removable by thiolysis.⁵¹ The classical Z group⁵² (5), which can be removed by hydrogenolysis or by acidolysis with strong acid, has not found widespread use in SPPS.



Since the different side-chains of the DNA-encoded amino acids encompass the majority of the common functional groups in organic chemistry, several different types of side-chain protecting groups are required for peptide synthesis. However, these side-chain protecting groups are usually based on the Bzl- or the *t*Bu-group. For amino acids having alcohols or carboxylic acids in the side-chain, these can be protected either as Bzl ethers or as Bzl- or cHex-esters. As an alternative they may be protected as *t*Bu ethers or esters. For other types of functional groups, e.g. the amino group of Lys, the thiol group of Cys, the imidazole of His or the guanidino group of Arg, more specialised protection may be required. Many different protecting groups for peptide synthesis have been described.⁵³ The two most widely-used protection strategies in SPPS are the Boc/Bzl- and the Fmoc/*t*Bu-strategies. In the former the Boc group is used for *N*^α protection and the side-chains of the various amino acids are protected with Bzl- or cHex-based protecting groups. In the latter strategy the Fmoc group is used as *N*^α protection and the side-chains are protected with *t*Bu-based protecting groups.

For the synthesis of protected peptide segments, the peptide must be detached from the resin under conditions which do not provoke the premature deprotection of any of the protecting groups. This requirement differentiates CSPPS from linear SPPS since in the latter the peptide is detached from the solid support by acidolysis of the peptide resin, using strong acid (often liquid HF). In addition to detaching the peptide from the resin this acidolysis usually removes all protecting groups and consequently is not useful for the synthesis of protected peptide segments. In order to synthesise these by the solid-phase method, the cleavage of the peptide-resin bond must be brought about using milder conditions such as saponification or transesterification. Alternatively, the peptide-resin anchorage may be chemically modified in such a way that the peptide-resin bond can be cleaved selectively. This requirement imposes stringent limitations upon the nature of the peptide-resin anchorage, but this aspect of peptide research has proved to be a very fertile area and many different types of anchorage have been described. Stability to all of the conditions necessary to effect synthesis of the peptide is a pre-requisite but at the same time it must be possible to detach the protected segment from the solid support in high yield, under conditions which are sufficiently mild to prevent epimerisation at the C-terminal amino acid and also to allow the 'temporary' and 'permanent' protecting groups to be maintained.

It is important therefore that there be a high degree of compatibility between the various different types of protecting groups such that one type may be removed in the presence of the others. An orthogonal protecting scheme has been defined by Barany and Merrifield⁵¹ as being one based on completely different classes of protecting groups such that each class of groups can be removed in any order and in the presence of all other classes of protecting group. Protecting schemes in peptide synthesis are usually not strictly orthogonal but rather are based upon the difference in rate when the same chemical reaction (usually acidolysis) is used to remove different classes of protecting groups.

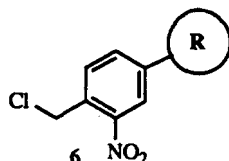
If the Boc/Bzl strategy is to be used for the synthesis of protected peptide segments then ideally the cleavage reaction should not be brought about by acidolysis. Better results will probably be achieved using photolysis or base-mediated cleavage. If the Fmoc/*t*Bu strategy is used then cleavage of the peptide from the resin usually cannot be performed under basic conditions; mild acidolysis is the method of choice here. Other types of cleavage reaction may be used, e.g. thiolysis or allyl group transfer, depending upon the peptide segments to be synthesised and the protection scheme used. We review only those protection strategies and solid-phase anchoring linkages which have found some application in the synthesis of protected peptide segments.

1.1. *Photolytic cleavage of protected peptides from the solid support*

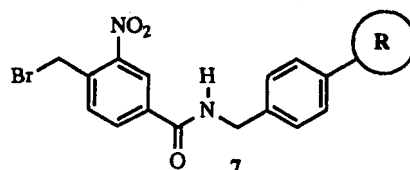
Photolysis⁵⁴ of the peptide-resin bond is a mild, non-invasive technique which in principle is compatible with both of the main protection strategies used in SPPS. These inherent advantages

have been the motivation for the investigation of this technique, by ourselves and by others, for the production of protected peptide segments having a free carboxylic acid at the C-terminus.

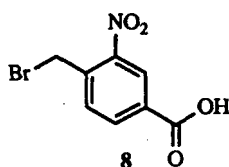
1.1.1. *Nitrobenzyl resins*. Rich and Gurwara⁵⁵ reported on the use of the *ortho*-nitrobenzyl resin (6). Irradiation of a suspension of the peptide-resin in MeOH at 350 nm brought about cleavage of the peptide segments from the solid support.



Aromatic amino acids are not affected by the cleavage conditions and acid-labile protecting groups are also completely stable. The protected tripeptide Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OH was released in approximately 60% yield, but the cleavage yields dropped appreciably for longer protected peptides. Rich⁵⁶ subsequently developed the *o*-nitrobenzyl resin (7) from which protected-peptide free carboxylic acids can be cleaved more efficiently by photolysis.



Resin (7) is formed by the reaction of 4-bromomethyl-3-nitrobenzoic acid (8) at its carboxylic acid terminal with an aminomethyl polystyrene resin.

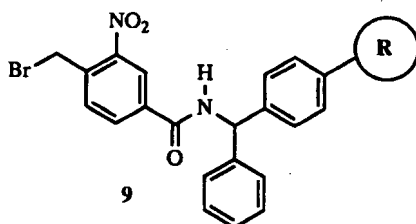


The unit (8) may be regarded as a 'handle' (or 'linkage agent' or 'linker'). Barany and Merrifield have defined⁵⁷ a 'handle' as being a bifunctional molecule which permits the attachment of the first amino acid to the resin in two discrete steps. The distinction between what constitutes a handle and what constitutes a functionalised resin is a somewhat arbitrary one, but the term⁵⁸ is much-used in contemporary SPPS. Broadly speaking, the handle may be used in two ways. The first amino acid of the sequence to be synthesised can be attached by esterification in solution to one of the functional groups (often a benzyl alcohol) of the handle. This unit (now called a 'pre-formed handle'⁵⁹) is then made to react with an amino-functionalised resin, at the other functional groups of the handle (usually a carboxylic acid). In this way the protected amino acid-handle unit is incorporated onto the solid support and the synthesis of the peptide can be carried out. Alternatively, the handle can be attached directly to the resin at one of its functional groups and the first amino acid of the peptide is then esterified onto the free functional group of the handle-resin.

In 1981 we reported⁶⁰ on the use of the Nbb resin (9). Similar to (7) but formed by the attachment of the acid (8) to a BHA resin, it is also photolabile and useful for the synthesis of protected peptide segments. The resins (7) and (9) and similar structures are fully compatible with the Boc/Bzl SPPS strategy and are partially compatible with the Fmoc/*t*Bu strategy.

We have used the Nbb-resin (9) extensively, in work related to the investigation of CSPPS. The first amino acid of the peptide must be incorporated using the cesium salt method,⁶¹ either by esterifying the amino acid onto the handle in solution to give the preformed handle⁵⁹ or by attaching

it to the handle previously incorporated onto the resin. In a synthesis of apamin,⁶² the 1–6 segment of the sequence was synthesised on the resin (9) and detached by photolysis to give the protected peptide carboxylic acid Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OH in yields of 65–70%. After purification, this segment was coupled to the 7–18 peptide bound to a BHA resin, giving the complete apamin sequence. In a different approach,⁶³ the 1–12 protected peptide segment was synthesised on the resin (9) and detached by photolysis in 89% yield to give Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-Glu(OBzl)-Thr(Bzl)-Ala-Leu-Cys(Acm)-Ala-OH, which after purification was coupled to three analogous 13–18 peptides bound to an MBHA resin in order to provide apamin analogues differing in the C-terminal region.



We have also used the resin (9) in the convergent solid-phase synthesis of the sequence of the toxin II from the scorpion *Androctonus australis*.^{64,65} The protected nonapeptide segment Boc-Asn-Ala-Cys(Acm)-Tyr(cHex)-Cys(Acm)-Tyr(cHex)-Lys(Z)-Leu-Pro-OH was detached from the resin by photolysis in 76% yield, and the protected peptides Boc-Val-Lys(Z)-Asp(OtBu)-Gly-OH, Boc-Glu(OBzl)-Ser(Bzl)-Gly-OH and Boc-Asp(OBzl)-His-Val-Arg(Tos)-Thr(Bzl)-Lys(Z)-Gly-OH were cleaved in yields of 72%, 59% and 53% respectively. In an ongoing project we are currently engaged in the total synthesis of the protein uteroglobin⁶⁶ by a CSPPS strategy.^{67,68} Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH, Boc-Leu-Thr(Bzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(Bzl)-Pro-OH and Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH have been detached from the resin in yields of 70%, 74% and 47% respectively. Other protected peptide segments corresponding to the entire uteroglobin sequence have been obtained by photolysis in yields of the order of 70%.⁶⁹

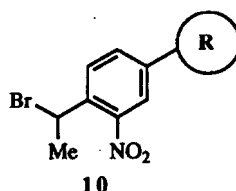
In addition to this work, other investigators have used 4-bromomethyl-3-nitrobenzoic acid as a handle for the synthesis of protected peptide segments. Bayer⁷⁰ has reported on the synthesis of the decapeptide Boc-Glu(OBzl)-Arg(Tos)-Gly-Phe-Phe-Tyr(ClZ)-Thr(Bzl)-Pro-Lys(BrZ)-Ala-OH which corresponds to the 21–30 segment of the insulin B chain, using the handle (8) attached to a polyoxyethylene-polystyrene graft copolymer. Photolysis of a suspension of the resin in MeOH gave the protected peptide in 80% yield. Pillai⁷¹ used a similar resin for the synthesis of thioredoxin partial sequences. Cleavage yields were of the order of 60–70%.

Barany and Albericio⁵⁹ have reported on the use of (8) in the synthesis of [Leu⁵]-enkephalin using a three dimensional orthogonal protection scheme, in which the Dts group (4) (labile to thiolysis) was used for *N*^α protection and the side-chain functionalities were protected as *t*Bu derivatives (labile to acids). The protected peptide corresponding to the sequence of [Leu⁵]-enkephalin was cleaved photolytically in a yield of 62%. This work demonstrates that the resin (7) is stable to thiolysis in addition to its well-known stability to acidic conditions. The same authors⁷² have also described the application of resin (7) to the synthesis of human gastrin-I by solid-phase segment coupling using the Fmoc/*t*Bu strategy. Fmoc-[Glu(OtBu)₃]-Ala-OH was synthesised on, and cleaved from, the resin (7) in 59% yield.

Tam⁷³ has synthesised the tridecapeptide Boc-Asn-Lys(ClZ)-Tyr(BrZ)-Thr(Bzl)-Thr(Bzl)-Glu(OBzl)-Tyr(BrZ)-Ser(Bzl)-Ala-Ser(Bzl)-Val-Lys(ClZ)-Gly-OH of the V_H domain of murine myeloma immunoglobulin M603 using the resin (7) and has investigated the photolytic detachment of the peptide from the resin (yield 54%), its purification and subsequent reattachment to the same resin, in order to continue the process of chain elongation.

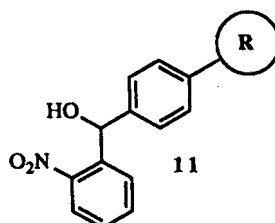
There are several drawbacks that must be considered when using resins incorporating the handle (8) for the synthesis of protected peptide segments. First of all these resins are not fully compatible with the Fmoc/*t*Bu strategy. The peptide-handle bond is not completely stable to treatment with piperidine so that the synthesis of long peptides using the Fmoc/*t*Bu strategy is not advisable. The coupling of the third amino acid of the sequence (regardless of the peptide synthesis strategy used) must be carried out under conditions which minimise the formation of DKPs.⁷⁴ DKP formation is more of a problem when the Fmoc/*t*Bu strategy is used and can only be avoided with this handle by coupling a protected dipeptide, consisting of the second and third amino acids of the sequence, to the first amino acid of the peptide.⁷⁵ When the Boc/Bzl strategy is used DKP formation can be avoided either by using the protocols described by Suzuki⁷⁶ or by ourselves.⁷⁷ These methods involve *in situ* neutralisation of the resin after acidolytic deprotection of the Boc group. In the former method the coupling agent is DCC whereas in the latter BOP is used. A final limitation of the method is that only relatively small quantities (up to 500 mg) of resin may be photolysed at any one time. This latter restriction is thought to be a consequence of the formation of azo and azoxy compounds from the *o*-nitrosobenzaldehyde⁷⁸ produced in the photolysis reaction. These compounds are a deep red colour and act as an internal light filter, thereby reducing the cleavage yields. The effect becomes more pronounced with increasing quantities of resin. We have found that optimum results from the photolysis reaction are achieved by suspending up to 500 mg of peptide resin in a 4:1 mixture of DCM or toluene and TFE. Degassing, followed by irradiation at 360 nm with stirring for 12–16 h, in our hands gives reproducible cleavage yields of up to 85%.

In an effort to overcome the self-quenching of the photolysis reaction of these resins, particularly when larger quantities are used, Pillai⁷⁹ investigated the photolabile resin (10) which was designed to limit the formation of the *o*-nitrosobenzaldehyde byproduct.



Unfortunately, although this resin performed reasonably well in providing the protected peptide segment Boc-Asp(OBzl)-Val-Tyr(Bzl)-Val-Glu(OBzl)-OH upon photolysis at 320–350 nm of 1 g batches of resin in yields of up to 50%, the cleavage yields dropped significantly when peptide resins incorporating more than 5 amino acid residues were photolysed.

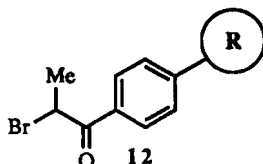
Pillai⁸⁰ has also described the synthesis of model protected peptide segments on the nitrobenzhydryl resin (11).



The protected Merrifield-peptide Boc-Leu-Ala-Gly-Val-OH may be cleaved from the resin (11) by photolysis at 350 nm, in 55–60% yield.

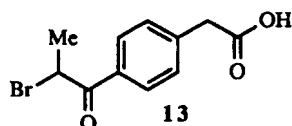
1.1.2. *Phenacyl resins.* Wang⁸¹ investigated the photolysis of the α -methylphenacyl ester⁸² anchoring linkage (12) for the preparation of protected peptide segments using the Boc/Bzl strategy. Photolysis of the peptide-resin at 350 nm yielded the segment Z-Lys(Z)-Phe-Phe-Gly-OH in 70%

yield. The photolytic cleavage yield of the peptide from the resin (12) was superior to that obtained when the same peptide was synthesised using the *o*-nitrobenzyl ester anchoring linkage.



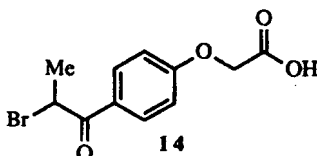
The resin (12) has also been used by Tjoeng⁸³ for the synthesis of Boc-Phe-Thr(Bzl)-Phe-Ser(Bzl)-Asp(OBzl)-Phe-Tyr(Dcb)-Met-Glu(OBzl)-Trp(For)-Val-Arg(Tos)-Gln-Pro-Pro-Gly-OH corresponding to the 27–42 hexadecapeptide of the sequence of the heavy chain from myeloma immunoglobulin M603. The authors report on several side reactions which must be overcome in order to synthesise peptides successfully using this resin. Incorporation of the second amino acid of the sequence can be hampered by the cyclisation of the amino group of the first amino acid (upon base treatment of the resin after removal of the Boc *N*^α protecting group) onto the carbonyl group of the phenacyl resin, thus leading to Schiff base formation. The incorporation of the second and third amino acids may proceed in low yield because of the formation of DKPs. These side reactions can be avoided by coupling previously-prepared peptide segments onto the solid support, although this does limit the usefulness of the resin. These difficulties notwithstanding, the target hexadecapeptide was synthesised successfully on (12) and cleaved by photolysis in 91% yield.

The use of the photolabile handle (13) attached to an aminomethyl polystyrene resin was reported by Tjoeng and Heavner⁸⁴ for the synthesis of the protected peptide segment Z-Arg(Z,Z)-Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OH, part of the sequence of thymopoietin.



The cleavage yields were of the order of 90%. The authors report that the side reactions observed for the resin (12) occurred in negligible amounts when the handle (13) was used.

Bellof and Mutter⁸⁵ have described the use of the phenacyl handle (14).



N^α-Boc-protected [Leu⁵]-enkephalin [Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-OH], synthesised on a resin incorporating (14) was cleaved by photolysis in 71% yield.

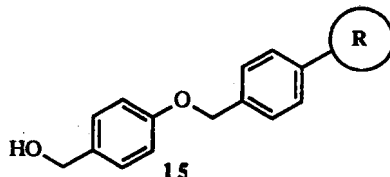
Gauthier⁸⁶ has used the chloro-analogue of the handle (14), attached to a BHA resin, for the synthesis of mammalian glucagon, in an elegant example of CSPPS. The protected peptide segments Boc-Asp(OcHex)-Ser(Bzl)-Arg(Tos)-Arg(Tos)-Ala-Gln-Asp(OcHex)-Phe-OH and Boc-Thr(Bzl)-Ser(Bzl)-Asp(OcHex)-Tyr(Dcb)-Ser(Bzl)-Lys(ClZ)-Tyr(Dcb)-Leu-OH, corresponding to the 15–22 and 7–14 sequences respectively, were synthesised on the solid support, and were detached by photolysis in yields of over 85%. These segments were then used to build up the sequence of glucagon on a BHA resin incorporating the 23–29 sequence of the molecule.

1.2. Acidolytic cleavage of protected peptides from the solid support

In linear SPPS, peptides synthesised on Bzl-type resins are normally detached from the support by acidolysis with strong acid, usually liquid HF. This method is incompatible with the preparation

of protected peptide segments since in both the Boc/Bzl and Fmoc/*t*Bu strategies, most of the protecting groups used are removed by HF treatment. In order to allow the solid-phase synthesis of protected peptide segments, the conditions for acidolytic cleavage must be as mild as possible.

1.2.1. *The Wang resin.* Wang⁸⁷ has reported on the synthesis and use of the *p*-alkoxybenzyl alcohol-resin (15). Peptides synthesised using this resin can be cleaved from the solid support using TFA.



The standard Boc/Bzl- or Fmoc/*t*Bu- strategies for the preparation of protected peptide segments are not fully compatible with the conditions required to bring about cleavage from the resin (15). In the former strategy the TFA treatments required to remove the *N*^α-protecting group would lead to cleavage of the peptide from the resin and in the latter, the side-chain protecting groups will be removed upon treatment of the peptide-resin with TFA. The resin (15) can nevertheless be used for the synthesis of protected peptide segments if modified peptide synthesis strategies are adopted, for example using Bzl-type side-chain protecting groups and *N*^α protecting groups which are stable to TFA. The peptide can be built up on the resin using Bpoc- or Fmoc-*N*^α-protected amino acids, since the mild acidolytic conditions required for the removal of the former group, or the basic conditions required for the removal of the latter, do not normally cause premature cleavage of the peptide from the resin, nor do they cause loss of side-chain protecting groups. If the last amino acid of the sequence is incorporated with the Z or Fmoc *N*^α protecting group then protected peptide segments can be obtained by cleavage of the peptide from the resin with TFA. In this way Wang synthesised Z-Lys(Z)-Phe-Phe-Gly-OH among other segments. Cleavage yields are of the order of 60%. The resin (15) has become one of the most successful resins in SPPS and is used extensively, more usually for the preparation of free peptides using the Fmoc/*t*Bu strategy.

Wang and Kulesha⁸⁸ also used the *p*-alkoxybenzyl alcohol-resin in a synthesis of the active core of ovine pituitary growth hormone. H-Arg(Tos)-Glu(OBzl)-Leu-Glu(OBzl)-Asp(OBzl)-Val-OH was detached from resin (15) by treatment with 50% TFA in DCM, conditions which remove the Bpoc *N*^α protecting group so that the peptide segment is obtained as the *N*-terminal free amine salt. The side-chain protecting groups are maintained, however.

Felix⁸⁹ has reported on the use of the resin (15) for the synthesis of peptide segments with their side chain functional groups unprotected but with Fmoc *N*^α protection. Peptides synthesised using the Fmoc/*t*Bu strategy are cleaved from this resin using TFA which also removes the *t*Bu side-chain protecting groups but leaves the Fmoc group in place.

We⁹⁰ have used resin (15) for the synthesis of protected peptide segments comprising the sequence of LHRH. Fmoc *N*^α protection and TFA-stable side-chain protection was used.⁸⁹ Fmoc-Leu-Arg(Tos)-Pro-Gly-OH and pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Dcb)-Gly-OH were detached from the resin by treatment with TFA. The segments were subsequently purified and coupled on a solid support in a model synthesis of LHRH.

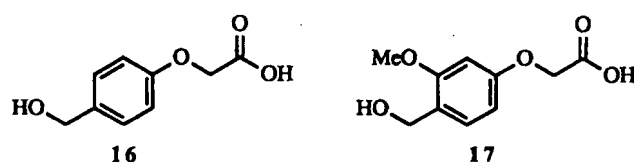
We^{91,92} have also applied this strategy to the synthesis of the peptides Fmoc-Tyr(cHex)-Cys(Acm)-Gln-Trp-Ala-Ser(Bzl)-Pro-Tyr(cHex)-Gly-OH, Fmoc-Glu(OBzl)-Ser(Bzl)-Gly-OH, Fmoc-Tyr(cHex)-Ile-Val-Asp(OBzl)-Asp(OBzl)-Val-Asn-Cys(Acm)-Thr(Bzl)-Tyr(cHex)-Phe-Cys(Acm)-Gly-OH and Fmoc-Arg(Tos)-Asn-Ala-Tyr(cHex)-Cys(Acm)-Asn-Glu(OBzl)-Glu(OBzl)-Cys(Acm)-Thr(Bzl)-Lys(Z)-Leu-Lys(Z)-Gly-OH, corresponding to the sequence of the toxin II of the scorpion *Androctonus australis*. Cleavage yields were over 95% in each case.

A similar approach to the synthesis of protected peptides corresponding to the sequence of human cardiodilatin has been used by Nokihara.⁹³

The formation of DKPs when the Wang resin is used is not normally a serious problem, although when sequences which are especially prone to form DKPs are synthesised, it may be advisable to use shorter deprotection times for the Fmoc group of the second amino acid of the sequence.⁷⁵ The Wang resin (15) is significantly more labile to acid than the conventional Merrifield polystyrene resin, but its acid-lability is still not sufficient for it to be used efficiently for the synthesis of protected peptide segments which have their side-chains protected with *t*Bu groups or which have the Boc group as *N*^α protection.

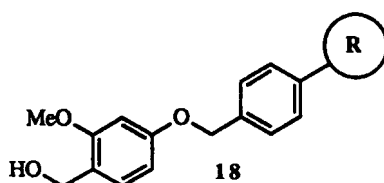
The development of the solid-phase segment condensation strategy as an approach to peptide synthesis has stimulated the search for ever more acid-labile peptide-resin anchorages, resulting in a series of highly acid-labile resins and linkers. These are compatible with the Fmoc/*t*Bu strategy since the use of acidic conditions is avoided throughout the synthesis. Protected peptides can then be detached from such resins by mild acidolysis.

1.2.2. *Highly acid-labile resins and linkers.* Sheppard and Williams^{94,95} first reported on the use of highly acid-labile handles in SPPS. Peptides synthesised using the linker (16) may be detached from the resin by treatment with TFA but this leads to loss of the *t*Bu side-chain protecting groups. If linker (17) is used, however, protected peptides may be detached from the solid support using 1% TFA in DCM, conditions which do not cause the deprotection of the *t*Bu-based protecting groups.



The utility of the handle (17) was demonstrated in the synthesis of the gastrin segments Fmoc-Glu(O*t*Bu)-Ala-Tyr(*t*Bu)-Gly-OH and Fmoc-Leu-[Glu(O*t*Bu)]₄-OH in more than 95% yield.

The resin (18), known as SASRIN has been described by Mergler.^{96,97} Peptides synthesised on this resin can be detached in high yield using 1% TFA in DCM.

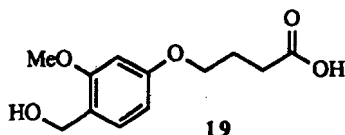


SASRIN is somewhat more acid-labile than the handle (17) so that, although the Fmoc/*t*Bu strategy must be used for peptide synthesis, the acidolytic cleavage conditions are sufficiently mild to allow the Boc group to be maintained. SASRIN has been used to synthesise a range of protected peptide segments in order to demonstrate its usefulness. As illustrative examples, Boc-Asp(O*t*Bu)-Val-Pro-Lys(Boc)-Ser(*t*Bu)-OH corresponding to the 1–5 sequence of kassinin and Z-Asn-Lys(Boc)-Phe-His(Boc)-Thr(*t*Bu)-Phe-Pro-Gln-Thr(*t*Bu)-Ala-Ile-Gly-OH corresponding to the 17–28 sequence of human calcitonin⁹⁷ were cleaved from the resin in 90% crude yield and 43% yield after counter current distribution, respectively.

Jung^{98,99} has used SASRIN for the synthesis of protected peptide segments corresponding to the sequences of the nucleocapsid protein of the HIV virus, and of a model peptide for the VP₁ protein of the foot and mouth disease virus. SASRIN has also been used in the solid-phase synthesis of mono- and di-saccharide-containing glycopeptides.¹⁰⁰

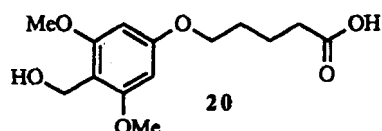
Riniker^{101,102} has developed the HMPB linker (19), which is related to the handle (17) but has two extra CH₂ groups between the carboxyl group and the oxygen atom of the aryl-alkyl ether. These two extra CH₂ units diminish the electron-withdrawing effect of the carboxyl group at the

ether oxygen, allowing it to function as a more powerful electron donator thereby increasing the lability of the handle to acid, resulting in milder detachment conditions.

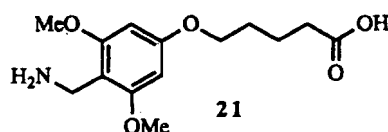


This linker, when attached to a BHA resin provides a highly acid-sensitive solid support for peptide synthesis using the Fmoc/*t*Bu strategy. Three protected peptides corresponding to the 1–10, 11–23 and 24–33 segments of human calcitonin were synthesised using this handle. These segments were detached from the solid support by treatment with 1% TFA in DCM for 6–8 min at room temperature. Riniker¹⁰³ has developed a synthetic approach to the synthesis of larger peptides, called the ‘lipophilic segment coupling strategy’, which is based on the use of the HMPB handle and on the use of *t*Bu-, trityl-, and Boc-groups for side-chain protection. In this approach the protected peptide segments are coupled in solution and the strategy has been applied successfully to the synthesis of human neuropeptide Y.

Albericio and Barany¹⁰⁴ have reported on the use of the HAL handle (20), which is related to the handle (21) known as PAL.

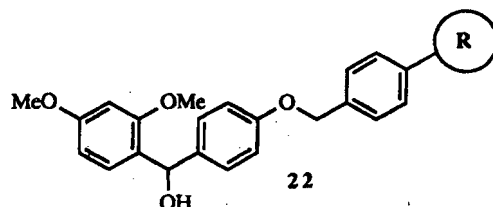


PAL is useful for the synthesis of peptide C-terminal amides^{105,106} under mild conditions using the Fmoc/*t*Bu strategy.



Peptides synthesised on the handle (20) attached to a suitable resin can be cleaved by treatment with 0.1% TFA in DCM for 60 min at 25°C. In order to demonstrate its usefulness, the peptides Fmoc-[Glu(O*t*Bu)]₅-Ala-OH, Fmoc-[Tyr(*t*Bu)]₅-Ala-OH and Fmoc[Lys(Boc)]₅-Ala-OH, related to the sequence of human gastrin-I, were synthesised.

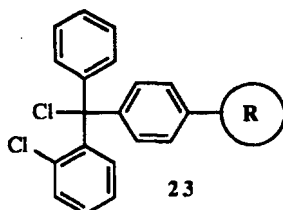
Rink¹⁰⁷ has described the synthesis of the protected nonadecapeptide segment Boc-Asp(O*t*Bu)-Arg(Mtr)-Gly-Phe-Tyr(*t*Bu)-Phe-Ser(*t*Bu)-Arg(Mtr)-Pro-Ala-Ser(*t*Bu)-Arg(Mtr)-Val-Ser(*t*Bu)-Arg(Mtr)-Arg(Mtr)-Ser(*t*Bu)-Arg(Mtr)-Gly-OH among others, on the resin (22) using the Fmoc/*t*Bu strategy. Cleavage of peptides from this resin may be brought about by treatment with 0.2% TFA in DCM for 3–5 min.



A drawback of the resin (22) is that its sensitivity to acids is so pronounced that premature cleavage of peptide from the resin occurs if HOBT is used in the amino acid-coupling steps. In order

to use HOBt with this resin, the coupling solution must be buffered with DIEA. Addition of extra base is also recommended when using symmetrical anhydrides or active esters for amino acid coupling.

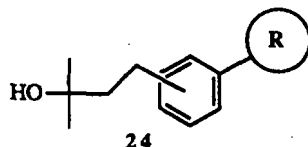
Barlos^{108,109} has developed the 2-chlorotrityl resin (**23**) for use in CSPPS.



This resin is compatible with the Fmoc/*t*Bu strategy and initial work focused on the synthesis of protected peptide segments such as Fmoc-Arg(Mtr)-Pro-Lys(Boc)-Pro-OH, related to the 1-4 sequence of substance P, and of Fmoc-Phe-His(Trt)-Thr(*t*Bu)-Phe-Pro-OH, Fmoc-Leu-Ser(*t*Bu)-Thr(*t*Bu)-Cys(Trt)-Met-Leu-Gly-OH and Fmoc-Cys(Trt)-Gly-Asn-Leu-Ser(*t*Bu)-Thr(*t*Bu)-Cys(Trt)-Met-Leu-Gly-OH, which are related to the sequence of calcitonin. Amino acids can be esterified onto the resin (**23**) in high yield and with negligible racemisation. The formation of DKPs on incorporation of the third amino acid is suppressed due to the significant bulk of the trityl resin.¹¹⁰ Cleavage of peptides from (**23**) can be accomplished in excellent yield by treatment of the peptide-resin with a mixture of AcOH-TFE-DCM, and cleavage times are 1-2 h. Yields are usually greater than 90%. In one of the most important demonstrations of CSPPS to date, Barlos has used the resin (**23**) for the synthesis of prothymosin α , a protein consisting of 109 amino acid residues.¹¹¹

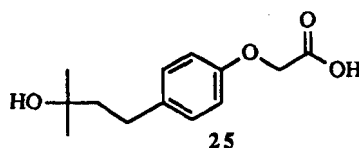
A trityl linker useful in the solid-phase synthesis of protected peptide segments using the Fmoc/*t*Bu strategy has recently been reported by Tesser.^{112,113}

1.2.3. *Other acid-labile resins.* Wang and Merrifield^{114,115} described the use of the *t*-alkyl alcohol resin (**24**), in combination with Z or with phthaloyl-*N* α protecting groups.



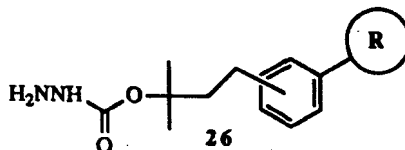
Z-Lys(Z)-Phe-Phe-Gly-OH was cleaved from this support by the action of 50% TFA in DCM. This resin however has not become popular in SPPS.

Carpino¹¹⁶ has synthesised the *t*-alkyl alcohol handle (**25**) and used it for the synthesis of the eleven residue peptide, bradykinin potentiator B.



The peptide was cleaved from the resin by treatment with TFA-thioanisole for 3 h at 25°C.

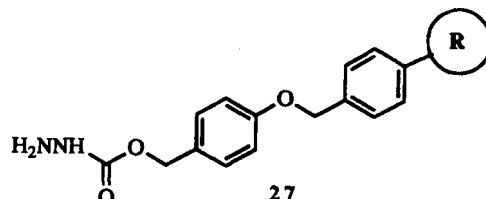
The *t*-alkoxycarbonylhydrazide resin (**26**) was developed by Wang and Merrifield.^{114,115} Acidolysis of protected peptides bound to resin (**26**) gives rise to the protected peptide hydrazides, which may then be purified and converted to the corresponding azides, for coupling to other segments.



The tetrapeptide hydrazide Z-Phe-Val-Ala-Leu-NHNH₂ and the decapeptide hydrazide Z-Ala-Val-Ser(Bzl)-Glu(OBzl)-Ile-Gln-Phe-Met(O)-Asn-Leu-NHNH₂ were synthesised on this resin and detached by treatment with 50% TFA in DCM for 30 min. Yields for the cleavages were 36% and 42% respectively.

The resin (26) has been used by Tesser¹¹⁷ for the synthesis of Msc-Tyr-Gln-Leu-Glu-Asn-Tyr-NHNH₂ comprising the A¹⁴⁻¹⁹ sequence of ovine insulin. The last amino acid of the sequence was incorporated as its Msc N^α protected derivative.¹¹⁸ Cleavage from the resin was brought about by treatment with 50% TFA in DCM, conditions which leave the N^α protecting group intact but which remove the *t*Bu-based side-chain protecting groups. The cleavage yield was 62%.

The resin (27) is the carbazoyloxybenzyl derivative of the Wang resin (15) and was used^{87,119} for the preparation of the protected peptide hydrazides Boc-Gly-Phe-Phe-Tyr(Bzl)-NHNH₂, Z(NO₂)-Gly-Val-Ala-Leu-NHNH₂ and Fmoc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-NHNH₂.

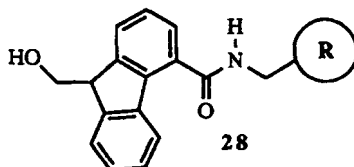


The peptide segments were released from the resin in yields of up to 50%, by treatment with 50% TFA in DCM.

We⁹⁰ have experimented with the resin (27) for the synthesis of Fmoc-Leu-Arg(Tos)-Pro-Gly-NHNH₂ and pGlu-His(Tos)-Trp-Ser-(Bzl)-Tyr(Dcb)-Gly-NHNH₂, peptides which correspond to the sequence of LHRH. We found that the hydrazides were obtained in low yields, mainly because of a significant drop in the level of functionalisation after incorporation of the second amino acid of the sequences. We also found that the hydrazides obtained after cleavage were difficult to purify.

1.3. Nucleophile- and base-mediated cleavage of protected peptides from the solid support

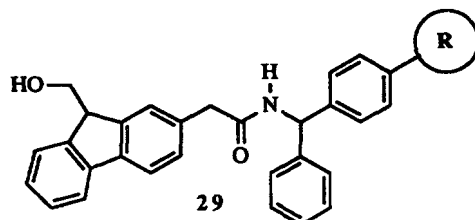
1.3.1. *Cleavage of the peptide-resin anchorage by a β-elimination reaction.* Several fluorene-based handles, which allow cleavage of peptides from the solid support by a β-elimination mechanism, have been described. These handles are only compatible with the Boc/Bzl synthesis strategy. Mutter and Bellhof¹²⁰ have investigated the use of the resin (28) in the synthesis of the model peptide Boc-Leu-Ala-Gly-Val-OH.



The authors report that the peptide-resin anchorage is completely acid-stable under the synthesis conditions, although upon prolonged exposure to DIEA in DMF some cleavage of the peptide from the resin occurs. Since this base is normally used for resin neutralisation after removal of the N^α Boc group, this premature cleavage could become troublesome in the synthesis of longer peptides. In order to avoid cleavage of the peptide during the synthesis, the authors recommend that the DMF be freshly distilled before use and that the DIEA be added to the resin only after the addition of the next activated amino acid of the peptide sequence. Quantitative cleavage of the model peptide from resin (28) was brought about by treatment with 15% piperidine in DMF for 5 min. A different synthetic approach to the resin (28) has been described by Tjoeng.¹²¹

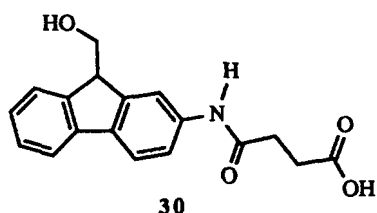
Felix¹²² has reported on the use of the resin (29) for the synthesis of the protected heptapeptide

Boc-Val-Val-Ser(Bzl)-His(Tos)-Phe-Asn-Lys(Z)-OH corresponding to the sequence 1-7 of rat-transforming growth factor α , in an overall yield of 46%.



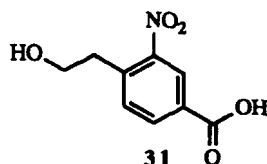
Cleavage from the solid support was brought about by treatment with 15% piperidine in DMF for 5 min. The authors suggest that less premature cleavage from the support should occur with (29) than with (28) because the extra methylene group between the fluorene nucleus and the carbonyl function should render (29) somewhat more stable to base-catalysed cleavage. However, partial premature cleavage of the anchorage is provoked by the free amino group of amino acids such as Gly, Phe or Ser(Bzl) during peptide synthesis. The authors recommend the use of HOBt in the coupling reactions in order to avoid this. Oroszlan and co-workers¹²³ have also used the resin (29) for the synthesis of a series of nonapeptide C-terminal amides required for structure-activity studies of HIV proteinases. The protected peptide segments Boc-Val-Ser(Bzl)-Gln-Asn-Tyr(BrZ)-OH and Boc-Val-Ser(Bzl)-Gln-Asn-Tyr(BrZ)-Pro-OH were synthesised and detached in yields of 83% and 71% respectively. After purification they were coupled to several different MBHA resin-bound peptides, in order to give the nonapeptides required for the study.

We have reported on the use of the handle (30) in peptide synthesis.¹²⁴



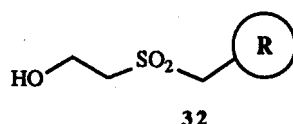
This handle is more stable than supports (28) or (29) with respect to premature cleavage during peptide synthesis. This may be because the link between the fluorene nucleus and the side-chain is an electron-donating *N*-amide group. The handle (30) is completely stable to solutions of DIEA in DMF so that premature loss of peptide chains from the support during synthesis is avoided. However, lability to secondary amines such as piperidine is maintained, allowing protected segments to be released from the resin in high yield. The model peptide Boc-Leu-Ala-Gly-Val-OH and the segment Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH corresponding to the 31-38 sequence of uteroglobin⁶⁶ were synthesised and detached from the resin in high yields using 20% morpholine in DMF over 2 h.

We have also described^{125,126} the NPE handle (31), useful for the solid-phase synthesis of both oligonucleotides and of peptides. Using this handle attached to an MBHA resin, protected peptides may also be detached from the solid support by a base-catalysed β -elimination reaction.



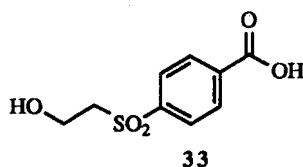
Several model peptides were synthesised using this handle and it was determined that there is essentially no DKP formation during the incorporation of the third amino acid, that a wide range of protecting groups are completely stable to the cleavage conditions, and that cleavage proceeds with almost negligible epimerisation of the C-terminal amino acid. Peptides can be cleaved from the support by treatment with 0.1 M DBU in dioxane or 20% piperidine in DMF, for 2 h at room temperature. Cleavage yields are very high (> 95%) and the peptide crudes are very clean. Initial results with this handle are very encouraging¹²⁷ and it may be that it will become more widely used in the synthesis of protected peptide segments.

Tesser^{128,129} reported the synthesis of several model peptides including Z-Glu(O^{*t*}Bu)-His-Phe-Arg(NO₂)-Trp-Gly-OH, corresponding to the 5–10 sequence of ACTH, on the resin (32).



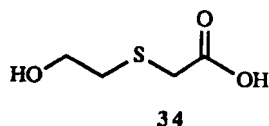
The peptides were detached by base-catalysed β -elimination, brought about by treatment of the resin with a mixture of sodium hydroxide in dioxane-MeOH for 3 min. The cleavage yields were of the order of 66%. Using this procedure the detachment of protected peptides from the resin (32) proceeded more rapidly than from a standard Merrifield resin.

Schwyzler¹³⁰ has reported on the use of the related handle (33) which in principle also allows detachment of the peptide from the resin by β -elimination.



The handle (33) is compatible with the Boc/Bzl synthesis strategy. Cleavage of several protected amino acids from the resin-handle was investigated. The cleavage conditions are mild and rapid; typically treatment of the resin with 0.1 M NaOH or Ba(OH)₂ for several minutes.

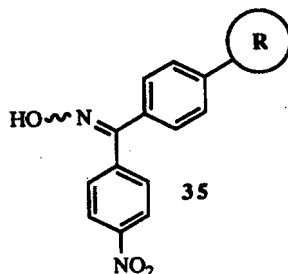
The linker (34) has been described by Katti.¹³¹ The authors report that this linker is compatible with both the Boc/Bzl- and Fmoc/*t*Bu- strategies.



The first amino acid is esterified onto the alcohol terminal of the linker and the thioether is then oxidised to the corresponding sulfone. This preformed handle⁵⁹ is then attached to a suitable amino-functionalised resin and the synthesis continued by standard procedures. In order to demonstrate the usefulness of the handle, [Leu⁵]-enkephalin was synthesised using both the Fmoc/*t*Bu- and Boc/Bzl-strategies. In each case the final cleavage was brought about by treatment of the resin with a solution of aqueous sodium hydroxide in dioxane-MeOH. Cleavage yields were greater than 90%. The handle is not useful for synthesis of peptides which have amino acids which are sensitive to oxidation, such as Met, Cys or Trp, at the C-terminus.

1.3.2. *The Kaiser oxime resin.* The *p*-nitrobenzophenone oxime resin (35) synthesised and developed by Kaiser and his group has been used extensively in studies on CSPPS.^{132,133} The first of a series of communications¹³⁴ exploring the use of polymer-bound oxime esters as supports for the synthesis of protected peptide segments appeared in 1980. The use of oxime esters in peptide

synthesis in solution had previously been investigated by Losse.^{135,136} Peptides synthesised on the oxime resin can be detached by hydrazinolysis or aminolysis using a suitable amino acid ester. This allows the preparation of a range of protected peptides differing at the C-terminus. An alternative procedure for the cleavage reaction is the treatment of the peptide-resin with HO-Pip. This gives rise initially to the HO-Pip ester of the protected peptide segment. Treatment of this ester with Zn in AcOH then gives rise to the corresponding free carboxylic acid. These procedures do not lead to epimerisation at the C-terminal amino acid nor to loss of acid-sensitive protecting groups. The yields for the cleavage reaction are usually high.



The resin (35) is compatible with Boc *N*^α protection and with Bzl-/cHex-based side-chain protection. Kaiser and collaborators have used this resin¹³⁷ for the preparation of the protected peptide segments Z-Leu-Leu-Glu(OBzl)-Ser(Bzl)-Leu-O^tBu, Nps-Leu-Ser(Bzl)-Leu-O^tBu, Nps-Leu-Gln-Ser(Bzl)-Leu-Leu-Ser(Bzl)-Leu-O^tBu, Nps-Leu-Leu-Gln-Trp(For)-Leu-O^tBu and Boc-Lys(ClZ)-Arg(Tos)-Lys(ClZ)-Arg(Tos)-Gln-Gln-NH₂, comprising the sequence of an analogue of melittin.¹³⁸ All peptide segments were cleaved from the oxime resin by aminolysis with H-Leu-O^tBu. Yields for the cleavage reaction were between 46% and 65%.

Resin (35) has also been used for the synthesis of model peptides^{139,140} for apolipoprotein A-I.¹⁴¹ Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH, Boc-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-OH and Boc-Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-OH were synthesised using the oxime resin and cleavage with HO-Pip, in yields of around 80%. These peptide segments were subsequently purified and coupled on the resin (35) to give a peptide consisting of 21 amino acid residues. Detachment from the resin was achieved by aminolysis with H-Ala-OBzl, affording a 22-residue fully-protected peptide which after deprotection gave the desired 22-residue model peptide. The yield for the aminolysis step was 73%. A 44-residue model peptide consisting of two of the 22-residue peptides was also synthesised by segment condensation on the oxime resin.

Kaiser^{142,143} also used (35) for the synthesis, of 'helichrome' a designed heme protein, for which the sequence H-Ala-Glu-Gln-Leu-Leu-Gln-Glu-Ala-Glu-Gln-Leu-Leu-Gln-Glu-Leu-NH₂ was required. Since this sequence is a repeat of two identical subunits, the segment Boc-Ala-Glu(OBzl)-Gln-Leu-Leu-Gln-Glu(OBzl)-OH was synthesised on the oxime resin and divided into two batches. Using one batch, the protected peptide carboxylic acid was obtained by cleavage with HO-Pip followed by reduction with Zn in AcOH. Using the other portion of the peptide-resin, the peptide was detached by aminolysis with H-Leu-NH₂. Segment coupling was carried out in solution.

The most ambitious syntheses carried out by Kaiser using the oxime resin are those of a 60 residue peptide corresponding to the homeo domain of *Antennapedia*¹⁴⁴ and of ribonuclease T₁.¹⁴⁵ The protected peptide segments corresponding to the sequence of these proteins were synthesised using (35) and after detachment and purification were coupled together either on the oxime resin or in solution to give the complete sequences. Final cleavage of the peptide from the resin, removal of the protecting groups and purification then gave the desired proteins. Unfortunately in the case of ribonuclease T₁ the biological activity of the synthetic material was very low.

Kaiser¹⁴⁶ also investigated the combination of a *p*-nitrobenzophenone oxime handle with a

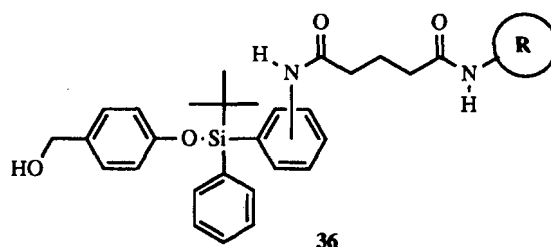
polyamide resin.^{147,148} This alternative to the resin (35) was studied because of difficulties encountered in the synthesis of certain specific sequences and in the coupling of some segments on resin (35). The investigators speculate that the polyamide resin might be superior to (35) because of its more hydrophilic polymeric matrix. It remains unclear whether or not this represents an improvement over the original polystyrene-based resin (35).

Resin (35) has been used by Lansbury¹⁴⁹ for the preparation of protected peptide segments related to the β -amyloid protein of Alzheimer's disease.^{150,151} Boc-Leu-Met-Val-Gly-OH, Boc-Ser(Bzl)-Asn-Lys(ClZ)-Gly-Ala-Ile-Ile-Gly-OH, Boc-Val-Phe-Phe-Ala-Glu(OBzl)-Asp(OcHex)-Val-Gly-OH and Boc-Asp(O ι Bu)-Ala-Glu(OBzl)-Phe-Arg(Mts)-His(Bom)-Asp(OcHex)-Ser(Bzl)-Gly-Tyr(Dcb)-Glu(OBzl)-Val-His(Bom)-His(Bom)-Glu(OBzl)-Lys(ClZ)-Leu-OH were synthesised on (35) using BOP as coupling reagent^{152,153} and were cleaved from the resin by treatment with HO-Pip, followed by reduction with Zn in AcOH. The advantages of CSPPS have been highlighted by Lansbury's total synthesis of the β A4-amyloid protein.³⁶

The resin (35) has also been used by Taylor¹⁵⁴⁻¹⁵⁶ for the synthesis of peptide models required for the study of the functional conformations of biologically active peptides,¹⁵⁷⁻¹⁵⁹ and for the synthesis of the cyclic peptide tyrocidine A. Nishino¹⁶⁰ has used the Kaiser oxime resin for the synthesis of [D-pyrenylalanine^{4,4'}] gramicidin S.

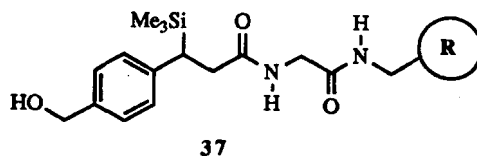
A disadvantage of the Kaiser oxime resin is that it is labile to nucleophiles. This can lead to loss of peptide from the resin upon neutralisation with base after acidolytic removal of the N^{α} Boc group. Also, precautions must be taken against the formation of DKPs in the coupling of the third amino acid, by using *in situ* neutralisation of the resin after removal of the Boc group and by carrying out the coupling with BOP or with related compounds.^{76,77}

1.3.3. *Fluoridolysis of the peptide-resin anchorage.* The rather original idea of using silicon-based peptide-resin anchorages that can be cleaved by treatment with fluoride ion was explored independently by both Barany and Mullen^{161,162} and Ramage.^{163,164} Barany and Mullen have investigated the use of the resin (36) which is compatible with the Fmoc/ ι Bu strategy but not with the Boc/Bzl strategy, since it is not stable to the acidolysis required to remove the N^{α} Boc group.



The model peptide Fmoc-Glu(O ι Bu)-Ala-Tyr(ι Bu)-Gly-OH was synthesised on, and cleaved from the support (36), using tetra-*n*-butylammonium fluoride in DMF, in the presence of thiophenol and DIEA. Although fluoride ion can be used to deprotect the Fmoc group,¹⁶⁵ the cleavage conditions used by the investigators allow the N^{α} -Fmoc protected peptide to be cleaved from the resin in 90% yield.¹⁶²

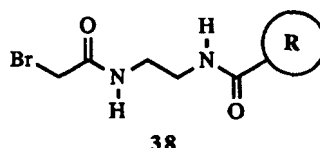
Ramage has investigated the use of the silicon-containing resin (37) for the synthesis of the protected peptide Dpp-Leu-Val-Gly-Phe-Ala-Leu-OH, and also for the synthesis of a protected peptide segment corresponding to the 1-35 sequence of ubiquitin.



In these syntheses the final amino acids of the sequences were incorporated not as the N^α Fmoc derivatives, but rather as the N^α Boc- or alternatively as the N^α Dpp-derivatives.¹⁶⁶ The protected ubiquitin segment was obtained in 48% yield. Again this resin is compatible with the Fmoc/*t*Bu strategy but not with the Boc/Bzl strategy.

Ueki and Amemiya¹⁶⁷ have reported on the cleavage of Boc-Tyr-D-Ala-Gly-Phe-Leu-OH from a Pam resin^{168,169} by the action of tetra-*n*-butylammonium fluoride in DMF. The peptide is cleaved from the resin in a yield of 45%. Unfortunately the BrZ group used for the protection of the Tyr side-chain is not stable to the cleavage conditions thus restricting the usefulness of the procedure.

1.3.4. *Cleavage of glycolamide ester anchorages.* Daunis¹⁷⁰ and Calas^{171,172} used the solid support (38) to prepare protected peptide segments. The cesium salt⁶¹ of the first amino acid (protected at its N^α terminal) is made to react with the bromo-functionalised solid support, giving rise to an anchorage which has a glycolamide ester linkage between the resin and the peptide.



This type of ester is stable to the repeated acidolytic conditions required for the elaboration of peptides using the Boc/Bzl strategy, but may be cleaved readily by nucleophiles. The resin (38) was used¹⁷¹ for the preparation of two peptides related to the sequence of chromosomal histone H4, which were first elaborated in their protected forms and then the Dnp protecting group of His was removed by thiolysis. The peptides were then detached from the resin by methanolysis giving rise to the peptide methyl esters in overall yields of approximately 50%. Since these segments were required with protecting groups at only the *N*- and *C*-terminals, the other side-chain functional groups were removed by hydrogenolysis to afford the desired products Ac-Gly-Ala-Lys-Arg-His-Arg-Lys-Val-OMe and Ac-Gly-Ala-Lys-Leu-Arg-His-Arg-Lys-Val-OMe.

Calas¹⁷² used the same resin for peptide synthesis using the Fmoc/*t*Bu strategy. This demonstrates that although the resin (38) is base-labile, it is stable to the conditions of the Fmoc/*t*Bu strategy, adding to the versatility of the solid support. If N^α -protected peptide segments are desired then the final amino acid of the sequence must be incorporated as its N^α -Boc derivative or some other N^α derivative which is stable to the basic cleavage conditions, usually treatment with aqueous NaOH. Using this procedure Boc-Arg-Lys(Boc)-Asp(O*t*Bu)-Val-Tyr(*t*Bu)-OH was synthesised in good yield.

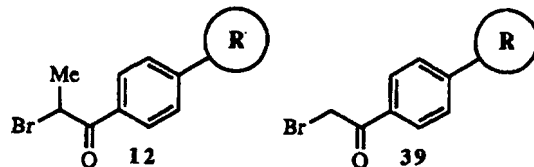
1.3.5. Miscellaneous methods.

1.3.5.1. *Ammonolysis, hydrazinolysis and aminolysis.* In linear SPPS using Merrifield-type resins, the peptides are normally detached by acidolysis with strong acid, giving free peptides. However, protected peptide segments may be detached from such resins by ammonolysis¹⁷³⁻¹⁷⁶ or hydrazinolysis of the *C*-terminal peptide-resin benzyl ester linkage.¹⁷⁷⁻¹⁸¹ This gives rise either to the protected peptide *C*-terminal amides or hydrazides. These latter compounds may be converted into the azides for subsequent coupling reactions.

Wang¹¹⁹ prepared several protected peptide hydrazides by subjecting the peptide-resins to hydrazinolysis. The cleavage yields were of the order of 60%. Wang⁸⁷ also applied the same procedure to the synthesis of the active core of the ovine pituitary growth hormone. The protected peptides were detached from the resin in good yields by hydrazinolysis.

Continuing an important series of contributions to the field, Wang¹⁸² described the first automated synthesis of a protected peptide segment, cleaved from the resin by hydrazinolysis. The sequence used as a model was the peptide hydrazide Boc-Gly-Phe-Phe-Tyr(Bzl)-Thr(Bzl)-NHNH₂.

Mizoguchi^{183,184} investigated the use of the haloacyl polystyrene resins (12) and (39) for the solid-phase synthesis of protected peptide segments.

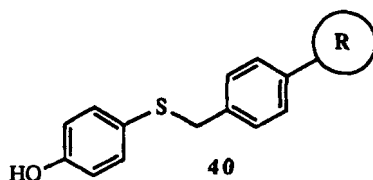


Protected peptides were obtained by ammonolysis of the peptide-resin using NH_3 in MeOH and yields were of the order of 60%. Protected peptides may also be cleaved from these resins by hydrazinolysis.⁸¹

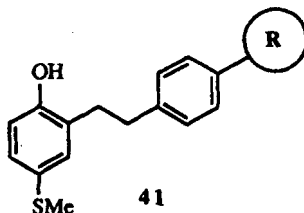
We⁶² have detached the apamin 1–6 sequence from the Nbb resin (9) by hydrazinolysis, obtaining the protected peptide hydrazide Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-NHNH₂ in a yield of 88%, compared with a cleavage yield of 65–70% for the photolysis.

Epton¹⁸⁵ used a phenolic support for the synthesis of several protected peptide hydrazides. The amino acid sequences were synthesised on the support and cleaved by hydrazinolysis. The same type of resin was used for a synthesis of the opioid peptide dynorphin.¹⁸⁶

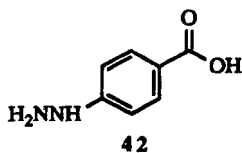
Marshall and Liener¹⁸⁷ reported on the support (40), based on the methylthiomethyl group.¹⁸⁸



The sequence Z-Arg(NO₂)-Ser(Bzl)-Val-Glu(OBzl)-O-resin was synthesised and treatment of the peptide-resin with hydrogen peroxide converted the sulfide to the corresponding sulfone. Aminolysis with H-Gly-OEt gave rise to the fully protected pentapeptide Z-Arg(NO₂)-Ser(Bzl)-Val-Glu(OBzl)-Gly-OEt. Although the amide bond is not affected by the treatment with hydrogen peroxide, Met, Trp and Cys would suffer oxidation and the method is not compatible with the use of these amino acids. The support (40) and the related (41) were used by Flanigan and Marshall¹⁸⁹ for the synthesis of cyclic peptides.



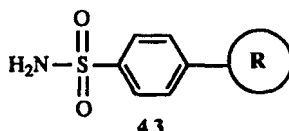
Wieland¹⁹⁰ has described the use of the handle (42) in the solid-phase synthesis of peptides.



The first amino acid is attached to the handle at the hydrazide functional group. This preformed handle⁵⁹ is then attached to an appropriate amino-functionalised resin and the peptide chain is

elongated. The peptide-resin is treated with NBS, oxidising the hydrazide to the corresponding azo compound, which then undergoes facile hydrolysis giving the desired peptide. Detachment from this resin is also possible by aminolysis with amino acid esters or by ammonolysis. This latter procedure of course gives rise to the C-terminal amides.

Kenner also reported on the use of this type of 'safety-catch' principle in SPPS.¹⁹¹ The resin (43) is stable to the normal conditions of SPPS and the peptide-resin bond is also stable to basic hydrolysis. However, *N*-methylation with diazomethane leads to an *N*-methylated peptidyl-sulfonamide resin from which protected peptides can be cleaved by mild basic hydrolysis or hydrazinolysis.



1.3.5.2. *Transesterification and saponification.* Transesterification of polystyrene-based resins as a means of detaching protected peptides from the solid support has been quite extensively investigated.¹⁹²⁻¹⁹⁶ This process produces protected peptide esters which can be transformed into the azides, via the corresponding hydrazides, for subsequent coupling to other segments. Alternatively the peptide esters may be saponified giving the free carboxylic acids which can then be coupled. This approach was used by the Merck, Sharpe and Dohme group¹⁹⁷ in their convergent solid-phase synthesis of rat atrial natriuretic factor, a peptide consisting of 26 amino acids. The protected peptide segments Boc-Ala-Gln-Ser(Bzl)-Gly-OH, Boc-Arg(NO₂)-Ile-Gly-OH and Boc-Arg(NO₂)-Arg(NO₂)-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-Phe-Gly-Gly-OH were synthesised on a polystyrene resin and detached by transesterification with MeOH containing Et₃N. The methyl esters were then saponified to give the protected peptide carboxylic acids. The protected peptide-methyl esters of the first two segments were obtained in yields of 78% and 97% respectively. The third segment was obtained as the protected peptide carboxylic acid after saponification of the crude peptide methyl ester, in a yield of 49%. After purification the segments were coupled on a polystyrene resin.

Barton¹⁹⁸ has developed a useful transesterification procedure using DMAE. Peptides synthesised on a chloromethyl resin were cleaved from the support by treatment with DMAE-DMF (1:1). Quantitative cleavage of the peptide from the resin was achieved in 70 h at room temperature. This procedure gives rise to the protected-peptide DMAE esters which can be hydrolysed to the corresponding free carboxylic acids under mild conditions by treatment with H₂O in DMF. Barton's work also indicates that the reaction rates for the transesterification reaction also depend upon the nature of the C-terminal amino acid, lower reaction rates being observed for Pro and other sterically hindered amino acids such as Val or Ile.

Merrifield¹⁹⁹ reported on the cleavage of protected peptides from Bzl resins by saponification. Spatola²⁰⁰ has described the cleavage of protected peptide segments from Bzl resins using K₂CO₃ in the presence of the phase-transfer reagent tetra-*n*-butylammonium hydrogen sulfate. Protected peptide segments were cleaved from the resin in high (70-100%) yields. However some removal of the Bzl ester protecting groups of Asp and Glu was also observed.

We^{201,202} have used the Nbb resin (9) in work related to the synthesis of peptides with repetitive sequences corresponding to the maize glutelin-2 protein, and have detached the protected peptides by treatment with NaOH in dioxan-MeOH (yield 84%) and NaOH-DMAE-H₂O (yield 88%). The photolysis yield was 66%.

Tam²⁰³ described the preparation of protected peptide segments on the oxyacyl resins (12) and (39). The peptide Boc-Gly-Gly-Leu-Val-Gln-Pro-Gly-OH was obtained by treatment of the peptide-resin with thiophenoxide ion (yield 22%), with aqueous NaOH (yield 57%) and by photolysis (yield 48-82%). Use of KCN/18-crown-6 in DMF led to a significant improvement in yield (up to 97%),

but certain side-chain protecting groups e.g. the Dnp group in Boc-His(Dnp)-OH are unstable to the cleavage conditions.

Mizoguchi^{183,184} cleaved the protected peptide Boc-Leu-Leu-Leu-Tyr(Bzl)-OH from the resins (12) and (39) in moderate yield by saponification with NaOH.

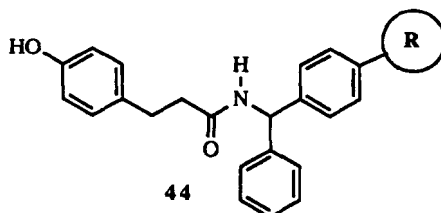
Birr^{204,205} has described the convergent solid-phase synthesis of the mast cell degranulating peptide from bee venom using the resin (12). The protected peptide segments Ddz-Ile-Lys(Z)-Cys(Acm)-Asn-Cys(*t*Bu)-Lys(Z)-Arg(Tos)-OH, Ddz-His(Boc)-Val-Ile-Lys(Z)-OH and Ddz-Pro-His-Ile-Cys(Acm)-OH were synthesized on the solid support and detached by treatment of the peptide resin with 0.1 M benzyltrimethylammonium hydroxide in MeOH-dioxan (1:1). Cleavage yields ranged from 30% to 60%. After purification the protected peptide segments were coupled on the same resin. Birr²⁰⁶ has also described the synthesis of the insulin A chain, and of five protected peptide segments corresponding to the sequence of the insulin B chain using resin (12). The protected peptides were cleaved from the solid support using a saponification procedure designed to maximise yields and minimise epimerisation at the protected peptide C-terminal.²⁰⁷

Peptides synthesised using the handle (14) may also be cleaved by saponification of the peptide-resin with alkali in aqueous dioxan, or by treatment with benzyltrimethylammonium hydroxide.⁸⁵ Alternatively, the peptide methyl ester may be formed by methanolysis in the presence of MeNH₂ and the peptide hydrazide by treatment of the peptide resin with hydrazine hydrate. All of these cleavage reactions proceed in high yield as exemplified by the synthesis of Boc-[Leu⁵]-enkephalin.

Phenyl esters are more susceptible to nucleophilic attack than are Bzl esters, and are correspondingly more stable to acidolysis. Several investigations of their use in solid-phase peptide synthesis are documented.²⁰⁸⁻²¹¹ Inukai²¹² has prepared protected peptide segments related to the sequence of oxytocin using a phenol resin and *t*-amyloxycarbonyl *N*^α protection. The peptides were cleaved from the resin by the action of 25% NaOH in MeOH or by ammonolysis or hyrazinolysis. Losse and Neubert²¹³ used a phenol resin²¹⁴ to synthesise H-Val-Orn(Tos)-Leu-D-Phe-Pro-Val-Orn(Tos)-Leu-D-Phe-Pro-OH, a precursor to gramicidin S, using a strategy based upon the use of the furfuryloxycarbonyl-*N*^α temporary protecting group.

Kenner²¹⁵⁻²¹⁹ extensively investigated the use of phenyl esters in peptide synthesis and noted that the hydrolysis of such esters, either in solid-phase or in solution synthesis, were accelerated significantly by the presence of peroxide ion. Kenner used a phenol resin for the preparation of [Met⁵]-enkephalin and isosteric analogues,²¹⁸ although in this case since peroxide ion led to oxidation of the Met residue, the protected peptide was detached from the resin by transesterification with DMAE in DMF¹⁹⁸ followed by hydrolysis.

In perhaps the most important application of a phenol resin in CSPPS, the resin (44) was used by Rivaille²²⁰ for the synthesis of LHRH and protected peptides related to human calcitonin. Cleavage of the segments was brought about either by saponification with NaOH in DMF or by transesterification with DMAE followed by hydrolysis.¹⁹⁸



After purification the segments were coupled together on the resin (44) to give the LHRH sequence which was then removed from the solid support in 58% yield, by ammonolysis. Subsequent removal of the protecting groups by treatment with HF and final purification gave LHRH with full biological activity.

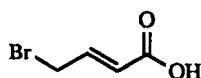
Although transesterification and saponification can give good results for the detachment of some

protected peptide segments from solid supports, these techniques are not compatible with protected peptides containing Asp or Glu residues since the protecting groups of these would also either be transesterified or removed in the saponification step. The methods therefore are of limited utility.

1.3.5.3. Hydrogenolysis. Although catalytic hydrogenation is one of the mildest chemical methods for the removal of Bzl-based protecting groups in organic synthesis, this technique is not widely used in SPPS. Catalytic hydrogenation as a method for the preparation of protected peptide segments is not compatible with the use of the Z group nor of Bzl-based side-chain protecting groups. Schlatter²²¹ has described the hydrogenolytic removal of resin-bound Boc-Tyr-Gly-Gly-Phe-Val-OH from a polystyrene resin, using H₂ gas in the presence of palladium(II) acetate. The yield of Boc-protected-[Val⁵]-enkephalin obtained was 71% and the method is compatible with Boc N^α protection and with *t*Bu- side chain protecting groups. Jones²²² used a similar procedure to cleave Boc-protected-[Val⁵]-enkephalin from a polystyrene resin in 88% yield. Kahn and Sivanandaiah²²³ have described the solid-phase synthesis of bradykinin using catalytic transfer hydrogenolysis to detach the peptide from the solid support. Ljungqvist and Folkers²²⁴ reported on the synthesis of Boc-Val-Ile-Gln-Gly-Val-Gly-Val-OH, Boc-Ile-Leu-Ala-Val-OH and Boc-Phe-Gln-Arg(Tos)-Ile-OH corresponding to the sequence of human leukocyte interferon, using hydrogenolysis of the peptide-resin as a means of detaching the segments from the support. Cleavage yields varied between 3 and 100% depending upon the conditions used, the highest yields being obtained upon hydrogenolysis with H₂ gas in the presence of Pd, in DMF. Hydrogenolysis of protected peptide segments from the *o*-nitrobenzoyl PEG support has been reported.²²⁵ Anwer and Spatola^{226,227} have described the hydrogenolytic cleavage of the pentadecapeptide Boc-Asp-Ala-Gly-Glu-Asp-Gln-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH, an ACTH-25-39 analogue, from a polystyrene resin, using ammonium formate catalytic transfer hydrogenation.

1.4. Cleavage of protected peptides from allyl-functionalised resins

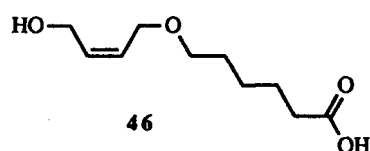
Allyl protecting groups²²⁸ have been used in organic chemistry since 1950 but their use did not gain widespread acceptance until Corey and Suggs discovered²²⁹ that such groups could be removed under mild conditions by using transition metal complexes. The first report of an allyl handle in solid-phase peptide synthesis is that of Kunz and Dombo,²³⁰ who used 4-bromocrotonic acid (**45**) as a handle in the synthesis of several model protected peptides such as Z-Tyr(Bzl)-Gly-Gly-Phe-Leu-OH, Boc-Leu-Ala-OH and a model glycopeptide.²³¹



45

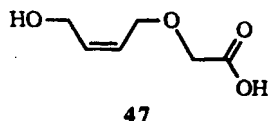
The handle (**45**) is made to react at the carboxylic acid terminal with a suitable, amino-functionalised resin, forming an amide bond. The first amino acid of the peptide is attached to the handle using the cesium salt method,⁶¹ and peptide synthesis is then carried out using normal SPPS protocols. The conditions required for the cleavage of peptides from the resin are very mild, usually 0.1 equivalent of the catalyst (Ph₃P)₄Pd and a several-fold excess, relative to the degree of allyl substitution of the resin, of a nucleophile such as HOBT, morpholine or *N,N*-dimethylbarbituric acid. In principle this allyl transfer cleavage reaction is compatible with both the Boc/Bzl and Fmoc/*t*Bu-strategies. Cleavage yields are high and if the Fmoc/*t*Bu strategy is used then the allyl handle provides an orthogonal protection scheme for the synthesis of protected peptide segments.

Blankemeyer-Menge and Frank²³² have used the allyl handle (**46**) on a cellulose support for the synthesis of protected peptides using the Fmoc/*t*Bu strategy. Representative examples of peptides synthesised are Fmoc-[Glu(O*t*Bu)]₅-OH, Fmoc-Trp-Met-Gln-Arg(Mtr)-Cys(*t*Bu)-OH, and Fmoc-His(Bom)-Cys(S*t*Bu)-Gln-Arg(Mtr)-Lys(Boc)-OH.



Peptides were cleaved from the solid support using 2 equivalents of $(\text{Ph}_3\text{P})_4\text{Pd}$ and 3 equivalents of HOBT relative to the level of allyl substitution of the resin.

Guibé²³³ has used the handle (47) for the synthesis of pGlu-His-Ser(Bzl)-Tyr(Dcb)-OH, a model peptide related to the sequence of LHRH.



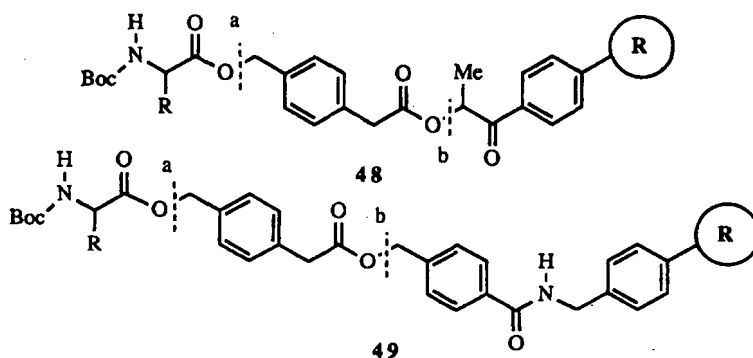
In order to detach the peptide from the allyl resin the authors used a hydrostannolytic cleavage method²³⁴ in which the peptide resin is treated with PdCl_2 and $n\text{Bu}_3\text{SnH}$ in the presence of a proton donor. Again the conditions are mild and the cleavage yields are high. Guibé²³⁵ has recently described the Pd-catalysed allyl transfer reaction using silylated amines, a mild and selective procedure which may find use in the cleavage of protected peptides from allyl-type resins.

We have investigated the use of the handles (45) and (47) for the synthesis of segments related to the sequence of uteroglobin.²³⁶ Our results indicate that in order to achieve acceptably high cleavage yields using these handles it is important to have a sufficiently polar reaction medium. Cleavage of peptides from both resin-handles was achieved in high yield (> 90%) by carrying out the reaction in a mixture of 2:2:1 THF-DMSO-0.5 M HCl, using $(\text{Ph}_3\text{P})_4\text{Pd}$ as catalyst and morpholine as nucleophile. These conditions however are not completely compatible with the use of the Fmoc/*t*Bu strategy, since the use of morpholine leads to some loss of the Fmoc group.

The use of allyl-type resins for the synthesis of protected peptide segments is potentially very useful because of the very mild detachment conditions and because in principle the cleavage conditions are compatible with both of the major solid-phase peptide synthesis strategies.

1.5. Miscellaneous methods for the synthesis of protected peptide segments

1.5.1. *Multidetachable resins.* The idea of using multidetachable resins, introduced by Tam,^{237,238} is a novel, more flexible approach to the use of handles in SPPS. Such multidetachable resins may be cleaved at more than one site giving rise to different products. The resins (48), Boc-aminoacyl-2-[4-(oxymethyl)phenylacetoxyl]-propionyl-resin (known as Pop resin) and (49) Boc-aminoacyl-4-[4-(oxymethyl) phenylacetoxymethyl]-3-nitrobenzamidomethyl-resin (known as Pon resin) are examples of multidetachable resins. In both (48) and (49) the benzyl ester may be cleaved at bond 'a'



either by acidolysis or by hydrogenolysis whereas the ester linkage at bond 'b' may be cleaved either by photolysis or by a variety of nucleophiles. The two cleavage points can therefore be orthogonal and these resins can, in principle, be used for the synthesis of free or of protected peptides.

Depending upon the reagents and conditions used for the cleavage reaction, three different products can be obtained. Cleavage with strong acids or by hydrogenolysis give the protected peptide C-terminal free carboxylic acid by cleavage at bond 'a'. Photolysis or treatment with thiophenoxide ion give the protected peptide OMPA ester by cleavage at bond 'b'. If reagents such as tetra-*n*-butylammonium cyanide in DMF or benzyltrimethylammonium hydroxide are used then initial cleavage at bond 'b' is followed by a rapid decarboxylative 1,6-elimination, cleaving bond 'a' to give the protected peptide C-terminal free acid, as shown in Fig. 4.

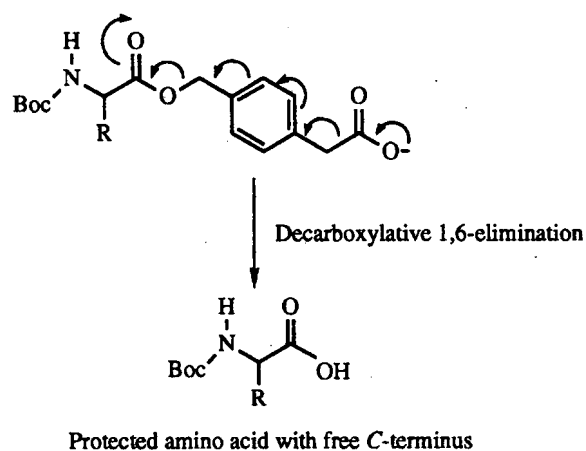


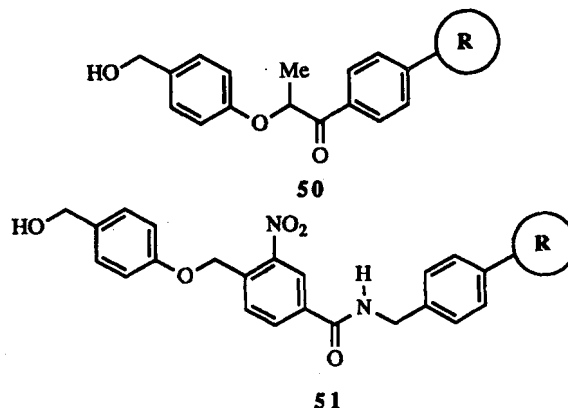
Fig. 4.

The resins (48) and (49) do not appear to be prone to the usual side-reactions associated with the use of phenacyl resins. Boc-Leu-Ala-Gly-Val-OMPA and Boc-[Leu⁵]-enkephalin-OMPA were synthesised using the resin (48) and were detached by photolysis.

The resin (48) was used by Tam⁷³ for the synthesis of the tridecapeptide Boc-Asn-Lys(CIZ)-Tyr(BrZ)-Thr(Bzl)-Thr(Bzl)-Glu(OBzl)-Tyr(BrZ)-Ser(Bzl)-Ala-Ser(Bzl)-Val-Lys(CIZ)-Gly-OH (56-68) of the V_H domain of mouse myeloma immunoglobulin M603. The same tridecapeptide was also synthesised on the resin (7). In both cases the peptide was detached by photolysis. For the resin (7) this yielded the protected peptide C-terminal free carboxylic acid directly whereas in the case of (48) the OMPA ester of the segment was produced, which was then hydrolysed giving the free carboxylic acid. After purification the tridecapeptide was reattached to resin (48) for subsequent elongation. The authors²³⁹ also report on the synthesis of six protected peptide segments of up to 16 amino acid residues in length, corresponding to the 1-68 sequence of the M603 murine immunoglobulin V_H domain. The cleavage of the fully protected peptide segments from the resin (48) was brought about by a novel base-catalysed β -elimination reaction, upon treatment of the peptide-resin with TMG, affording the peptide segments in high yield after 1 h reaction time. The same approach²⁴⁰ using the resin (48) was also used to synthesise a 53 residue protected peptide corresponding to the 16-68 sequence of the M603 V_H domain. The protected undecapeptide Boc-Gly-Ser(Bzl)-Leu-Arg(Tos)-Leu-Ser(Bzl)-Cys(MeBzl)-Ala-Thr(Bzl)-Ser(Bzl)-Gly-OH, corresponding to the 16-26 sequence was synthesised using resin (48), and cleaved from the solid support by treatment of the peptide-resin with TMG as described by Tam.²³⁹ After purification this peptide was coupled to the N-terminal of the protected 27-68 sequence, also bound to resin (48), giving the

16–68 sequence of the peptide. This peptide was then detached from the resin by treatment with liquid HF.

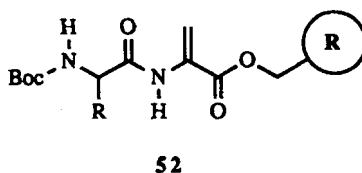
Tam²⁴¹ subsequently reported on the multidetachable resins (50) and (51) which can also be cleaved under a variety of conditions, e.g. photolysis, acidolysis or with nucleophiles, depending on which bond cleavage takes place.



Resin (50) can be used for the synthesis of protected peptide segments with a free carboxylic acid at the C-terminal or for the synthesis of protected peptide segments with the C-terminal protected in the form of a *p*-methoxybenzyl ester, which can then be used for the coupling of peptide segments in solution. These resins are compatible with the Boc/Bzl protecting strategy and with the use of the Bpoc *N*^α protecting group.

The use of multidetachable resins for the synthesis of peptides is a sophisticated and elegant approach which merits further investigation as a possible strategy for the synthesis of large peptides by a convergent solid-phase approach.

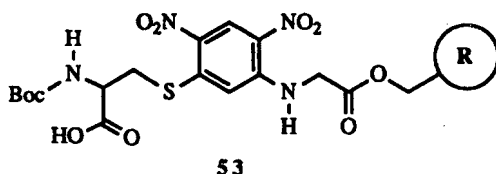
1.5.2. *Other linkers.* The use of α,β -unsaturated amino acids as handles for the synthesis of peptide amides was investigated by Gross.^{242,243} The link between the growing peptide chain and the resin is an α,β -unsaturated amino acid such as Dha giving rise to a support of the type (52) in which an *N*^α Boc-protected amino acid has been attached to the handle-resin.



The support is stable to the conditions required for repetitive removal of the Boc group and for repeated amino acid couplings. Once the desired peptide has been synthesised, the protected peptide amide can be removed from the resin by hydrolysis with 1 M HCl in glacial acetic acid in the presence of 3 equivalents of H₂O. This methodology was applied to the synthesis of oxytocin. The protected peptide amide Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH₂ was detached from the resin by the above-mentioned acidolytic conditions in 68% yield. The side-chain protecting groups were removed by treatment of the peptide amide with Na in liquid NH₃ to give crude oxytocin, which was purified by gel-filtration.

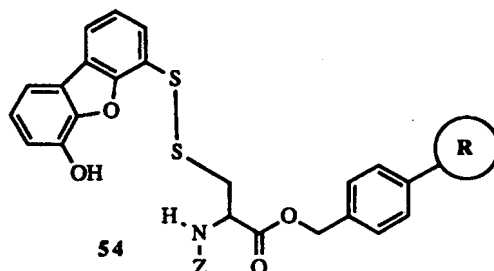
A similar approach has been used for the synthesis of oxytocin analogues incorporating the unusual amino acid L-thiazolidine-4-carboxylic acid.²⁴⁴

Walter²⁴⁵ has described the dinitrophenylene-bridging method in which a Cys residue is linked to the resin through its side-chain to a dinitrophenyl bridge as shown in (53).



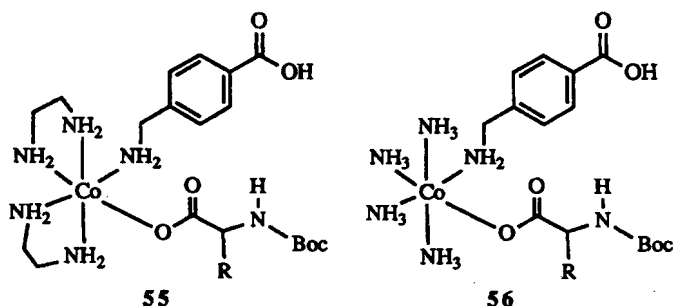
Peptide synthesis can now take place either by attachment of amino acids to the free C-terminal of Cys or after *N*^α-Boc removal by addition to the *N*-terminal. Such bidirectional methods may be useful for the synthesis of cyclic peptides. Protected peptide segments can then be obtained by thiolysis of the peptide resin, cleaving the Cys-resin bond but allowing other side-chain protecting groups to be maintained. A limitation of the method is that it is only useful for Cys-containing protected peptide segments. Subsequently Walter²⁴⁶ extended the method by developing analogous bridging systems for use with His-, Gln- or Glu-containing peptides.

In a related approach, Kemp²⁴⁷⁻²⁴⁹ used the support (54) in which a dibenzofuran spacer group, on which the peptide is synthesised at the phenolic function, is bonded to a Cys residue attached to the resin.



Cleavage of the disulfide bond can be brought about by treatment of the resin with *n*Bu₃P in dioxan-H₂O at 25°C. This cleavage gives rise to the protected peptide mercaptodibenzofuran ester which can then be purified and reattached to the solid support for further elongation, or which can be saponified to give the protected peptide free carboxylic acid. The method has been applied to the synthesis of the fully protected peptide Z-Ile-Glu(OBzl)-Ala-Leu-Asp(OBzl)-Lys(ClZ)-Tyr(Dcb)-Ala-O-mercaptodibenzofuran.

Isied^{250,251} has reported on the cobalt(III)-derived handles (55) and (56).



The handles may be attached to a suitable resin at the carboxylic acid terminal. Both handles are compatible with both the Boc/Bzl- and Fmoc/*t*Bu- strategies for peptide synthesis. The handles (55) and (56) were used in a synthesis of [Leu⁵]-enkephalin which was detached from the resin by thiolysis using mercaptoethanol in DMF in a yield of over 90%. The handle (55) was also used for the synthesis of the protected peptide segment Boc-Asp(OcHex)-Arg(Mts)-Gly-Asp(OcHex)-Ala-Pro-Lys(ClZ)-Gly-OH. This peptide was cleaved from the resin by thiolysis in approximately 65% yield.

Recently, an enzyme-scissile linker for solid-phase peptide synthesis has been described by

Elmore.²⁵² Protected peptides synthesised on this type of solid support may be cleaved by the action of calf spleen phosphodiesterase.

2. THE PURIFICATION OF PROTECTED PEPTIDE SEGMENTS

The difficulties encountered in the purification of protected peptide segments constitute perhaps the most serious problem in CSPPS. Protected peptides are often only sparingly soluble in most of the commonly-used solvent systems in organic chemistry and the poor solubility of these intermediates is a recurring theme in the classical literature of peptide synthesis.²⁵³ This poor solubility complicates the purification procedure, making it difficult to obtain chemically homogeneous material, and can lead to low chemical yields for the formation of peptide bonds between such species.

2.1. *Methods for the purification and characterisation of protected peptides*

Unfortunately, no universally-applicable technique exists for the purification of protected peptide segments and several different approaches must be tried for each individual peptide. Often the best results are obtained by applying a series of purification procedures to the same protected peptide segment. Some of the techniques which may be applied to the purification of free peptides are not useful in the purification of protected peptides. For example, techniques such as gel- or capillary-electrophoresis or metal chelate affinity chromatography²⁵⁴ are not normally useful for the purification of protected peptides. Other chromatographic techniques such as gel filtration or ion-exchange chromatography are normally carried out using aqueous eluents, when they are applied to the purification of free peptides. Protected peptides are usually not soluble in such solvent systems and the application of these techniques to their purification is possible only after drastic modification of the operating conditions.

Here we give a brief and selective overview of some of the most widely used techniques for the purification of protected peptides.

2.1.1. *Classical techniques.*²⁵⁵ For those protected peptides which are soluble in the commonly-used organic solvents, the classical purification techniques such as recrystallisation from single or mixed solvent systems and classical- or 'flash'-column chromatography²⁵⁶ using silica-gel as a stationary phase and eluants such as chloroform and alcohols, with or without additional carboxylic acids, may be used.²⁵⁷ More often than not, however, this is not the case and recourse must be made to polar aprotic solvents such as DMSO, DMF, NMP or even HMPA,²⁵⁸ although the carcinogenicity of the last solvent restricts its use. Recrystallisation from, and normal-phase chromatography in, such solvents is generally impractical, and other techniques must be used.

2.1.2. *Gel-filtration.* One of the most important and generally-applicable purification methods for peptide molecules is gel-filtration.^{259,260} The separation of molecules by this technique is governed by molecular size—larger molecules elute more rapidly than smaller ones, which are retarded by the stationary phase to a greater extent.^{261–263} The application of this method to the purification of protected peptide molecules was reported by Nyström and Sjövall^{264–266} using CHCl_3 -MeOH or EtOH- H_2O solvent systems for column elution. Subsequent reports on the use of gel-filtration for the purification of protected peptide segments²⁶⁷ showed that there was good correlation between the R_f values of the compounds and the logarithm of their molecular weights when the eluant was DMF. A similar but less well-defined trend was discernible when dioxan was used as eluant. The use of DMF for the purification of large fully-protected peptide segments by gel filtration has been reported.^{259,268}

Kenner^{269–272} studied more extensively the use of gel-filtration as a method for the purification of protected peptide segments. The use of HMPA and NMP for the purification of protected peptide segments having molecular weights of up to almost 8000 was investigated and HMPA containing 5% H_2O was found to be superior to DMF or DMSO for dissolving large protected peptides. The

use of TFE and of 2M urea in DMF was also investigated. 50% Aqueous HCOOH²⁷³ has been used for the purification of protected peptide segments by gel filtration.

Gel filtration is now used routinely in the purification of both free and protected peptides and its use is extensively documented.²⁷⁴ However, the resolving power of the method is somewhat limited and although gel filtration is an efficient technique for the removal of low molecular weight impurities from a crude mixture of peptides after a synthesis, it is normally not sufficient on its own for separating the target peptide from closely related peptidic impurities.

2.1.3. High-performance liquid chromatography. The importance of HPLC to the peptide field cannot be overemphasised.^{275,276} The development and refinement of this technique has exerted a profound influence upon peptide analysis, characterisation and synthesis.²⁷⁷⁻²⁸⁰ Normal-phase HPLC using silica-gel as stationary phase and CHCl₃-EtOH mixtures as eluants has been used to purify some protected peptide segments on the multigram scale,^{206,281,282} and a similar technique has been used for the separation of hydrophobic oligopeptides.²⁸³ Reversed-phase HPLC has been used very extensively for the analysis of free peptides,²⁸⁴⁻²⁹² but only rarely have there been reports on the use of this technique for the analysis of protected peptides.^{293,294} These latter are usually significantly more hydrophobic than unprotected peptides and special approaches for their purification by reversed-phase HPLC have been reported.^{295,296}

There have been several attempts to correlate HPLC retention times with amino acid sequence for free peptides;²⁹⁷⁻³⁰⁰ however, as yet there appears to be no consensus on the predictive capabilities of such studies. The effects of pH,³⁰¹ of ion-pairing reagents^{302,303} and the use of detergents as eluants for hydrophobic peptides³⁰⁴ have been studied. The use of size-exclusion high-pressure liquid chromatography for the purification of protected peptides, using DMF as the mobile phase, has been investigated.³⁰⁵ In spite of the huge amount of work in the area, the analysis and purification of peptides by HPLC must still be carried out on a case-by-case basis. For protected peptides poor solubility is the main problem and can lead to precipitation of the peptidic material on the column.

We have reported^{67,68} on the purification of protected peptide segments by MPLC using solvent systems containing DMF,³⁰⁶ in order to prevent precipitation of the peptide on the column, and similar procedures have been reported by others.

2.1.4. Other techniques. Other techniques have been applied to the purification of peptides although as yet no one method has become generally applicable, and there are only isolated examples of the purification of protected peptide segments. Although ion exchange chromatography is not a widely-used method for purification of protected peptide segments, reports of its use are to be found.³⁰⁷ There are examples of the use of counter current distribution for the purification of free-^{308,309} and protected-peptides.³¹⁰⁻³¹³ Newer techniques such as reverse-phase flash chromatography³¹⁴ and perfusion chromatography^{315,316} may become more important in the future.

2.1.5. Characterisation of protected peptide segments. The characterisation of the protected peptide segments after purification is an important aspect of the convergent solid-phase synthesis strategy. Characterisation of large protected peptides is not always straightforward. Amino acid analysis is used very extensively for the analysis of both free and protected peptides. In the latter case, however, it gives no information on whether or not protecting groups are still present. NMR spectroscopy is one of the most important and widely-used techniques for structural elucidation in organic chemistry. However, for large protected peptides the interpretation of NMR spectra, especially of one-dimensional ¹H NMR spectra, can be complicated. Mass spectrometry, especially in the FAB mode,^{317,318} is a very useful technique for the structure elucidation of this type of compound and can provide detailed information on the structure of the purified protected peptide. Extensive use should be made of all three techniques in the characterisation of each protected peptide segment.

2.2. Approaches to circumvent the problem of insolubility of protected peptides

Since the purification of protected peptide segments is often complicated by their poor solubility,

the investigation of the solubility properties of protected peptides and of methods for enhancing their solubility is becoming more and more important. In many cases, the insolubility of protected peptides is thought to be due to intermolecular association and current investigation is focusing on the nature of these associations and how they might be overcome or reduced, either by using special solvents or additives or by introducing structural changes into the protected segment itself. These structural changes may be brought about by redesigning the protected peptide, that is to say, by synthesising a longer or shorter segment in an attempt to increase its solubility, or by incorporating special protecting groups or using protection strategies which might enhance its solubility.

2.2.1. *Structure-solubility relationships of protected peptide segments.* There is very little information on the conformational analysis of protected peptide segments in solution and the extrapolation of the data which are available for free peptides should be undertaken only with caution. Conformational studies of free peptides are normally carried out in aqueous solution but protected peptides are usually insoluble in water. Protected peptides have protecting groups on the side-chains and at the N^α -terminal which may profoundly affect the conformational behaviour with respect to that of the free peptide.

It is now becoming accepted that in many cases the insolubility of protected peptide segments is due to self-association of the peptide chains, thereby forming β -sheet like structures. Infrared spectroscopy and to a lesser extent circular dichroism are the main techniques which have been used to study the conformational behaviour of protected peptide segments. For protected peptides in solution, the decrease in solubility is paralleled by a transition from a conformationally unordered, solvated species to a partially or fully ordered, intermolecularly hydrogen-bonded species, eventually forming a regular β -structure.³¹⁹⁻³²¹

Even less information is available on the conformational behaviour of protected peptide segments attached to polymeric resins. Association of protected peptide chains on the solid support was first postulated by Atherton and Sheppard.³²² Unfortunately, few techniques are suitable for studying such phenomena. Gel-phase ^{13}C NMR³²³ has been successfully applied to the study of certain cases^{324,325} and Ludwick³²⁶ has reported on a thorough study using solid-state deuterium NMR. ^{13}C NMR studies of the conformation of peptides bound to solubilising polymers during stepwise synthesis have been carried out by Jung.³²⁷

So-called 'difficult sequences' are thought to be due to the formation of β -sheet-like structures which leads to poor solvation of the resin-bound protected peptide chain and thence to low coupling yields.³²⁸ The use of special protocols for improving the coupling yields in SPPS during certain 'difficult sequences', using DMF,³²⁹ TFE,³³⁰ HFIP,³³¹ or alternatively using chaotropic salts,^{332,333} has been reported. Similar self-association of protected peptide chains was reported by Toniolo³³⁴ for PEG-bound peptides. [See section (2.2.3.).]

Weygrand^{335,336} has demonstrated that when the peptide bonds of several model peptides are protected with the Dmob group, in this way forming tertiary peptide bonds, there is an appreciable increase in the solubility of the protected peptides in organic solvents. The use of the Dmob group for protection of the amide bond and for improving the solubility of protected peptides has been reinvestigated by Tesser.³³⁷ Toniolo^{338,339} and Mutter³⁴⁰ have shown that the introduction of Pro into a peptide sequence disrupts the formation of β -sheets and leads to an increase in solubility. Investigations by Narita³⁴¹⁻³⁴⁵ in which tertiary peptide bonds have been introduced into protected peptide segments at suitable intervals, either by inserting Pro into the sequence or by using Dmob-protected Leu residues, showed that considerable increases in solubility may be brought about. Similar protocols for the enhancement of the solubility of protected peptides have been described by Bartl,^{346,347} and Haack and Mutter^{348,349} have used Ser-derived oxazolidines as secondary structure-disrupting, solubilising building blocks in peptide synthesis.

Subsequent work by Narita³⁵⁰⁻³⁵² has shown that significant solubility improvements may be achieved for protected peptide segments by incorporating Aib residues into the peptide chain. Although this has limited applicability in peptide synthesis, it does reinforce the idea that poor

solubility of protected peptide segments is due to β -sheet formation, since the α -helix promoting and β -sheet disrupting properties of Aib are well-known.³⁵³ Narita has proposed a method for predicting the solubility of protected peptide segments³⁵⁴ and has suggested that the design of synthetic routes to peptides might be based on this solubility prediction method.³⁵⁵⁻³⁵⁷

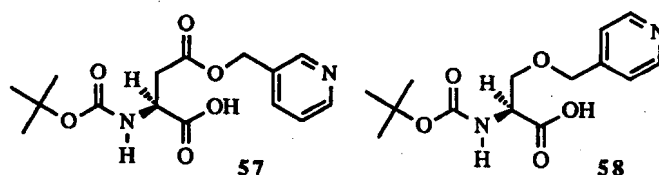
It therefore appears that, as for the enhancement of coupling yields in 'difficult sequences', processes which disrupt the formation of β -sheets should in principle enhance the solubility of protected peptides. It may be that the strategy of designing protected peptide segments in such a way that Pro is at the C-terminal may have to be reconsidered. Up to now this has been done in order to minimise epimerisation in the segment couplings. It is possible that the improvement in solubility that might be gained by designing peptide segments such that Pro occupies a central position in the segment outweighs this consideration.

2.2.2. *Use of special solvents and/or of additives.* Attention has focused on the use of solvents³⁵⁸ which disrupt the β -sheet like structures thought to be responsible for the poor solubility of protected peptide segments. Narita³⁵⁹ has reported on the use of HFIP for solubilising protected peptides. Interestingly, it appears that peptides are almost as soluble in a 5-10% solution of HFIP in DCM as in pure HFIP. The use of HFIP as a cosolvent with DMF in the tryptic synthesis of peptides has also been reported.³⁶⁰ Mixed solvent systems³⁶¹⁻³⁶⁵ such as HMPA-DMSO, HFIP-EtOH-DCM, and HFIP-DMF have also been used for the solubilisation of poorly soluble peptides.

Seebach³⁶⁶ has shown that a dramatic increase in the solubility of peptides in non-polar solvents such as THF is observed on the addition of inorganic salts. This method for the solubilisation of poorly soluble protected peptide segments has been used successfully by Lansbury,³⁶⁷ and is one of the most interesting and potentially useful new developments in this area of research.

2.2.3. *Use of special protection schemes to enhance solubility or to facilitate purification.* It has been suggested that if a certain protected peptide segment is only poorly soluble, then a simple remedy is to change the synthetic strategy such that a different, longer or shorter, but more soluble segment is used.³⁶⁸ This however is not always a practical proposition and efforts have been devoted to developing protection schemes which might enhance the solubility of the protected peptide segments which the initial synthetic strategy demands. The liquid-phase peptide synthesis of Bayer and Mutter^{369,370} is a special case of a solubilising protecting group, and peptides which are normally insoluble in organic solvents may be solubilised by attaching them to a soluble PEG polymer.³⁷¹

We have explored³⁷² the use of the polar picolyl group, used extensively by Young,³⁷³ for the protection of the side chains of Ser, Thr, Asp and Glu. The 3-picolyl group was used for the protection of Asp (57) and of Glu, and the 4-picolyl group for the protection of Ser (58) and also of Thr.

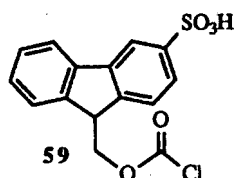


Peptides protected in such a fashion are more polar than their Bzl-protected analogues, have lower HPLC retention times and are more soluble in acetic acid. The use of such picolyl-protected peptides opens up the possibility of using cation exchange chromatography for their purification. The affinity-based N^α protecting group described by Ramage and Raphy³⁷⁴ might also be useful for the purification of protected peptides.

We have also reported³⁷⁵ on the reversible protection of Lys residues in order to facilitate the purification of protected peptides. Peptide segments which contain Lys residues can be protected at the N^α -amino group with the Fmoc group. If the peptide segment has been synthesised using the Boc/Bzl strategy then the Fmoc groups can be selectively removed to afford a peptide which is more

polar and in principle more amenable to purification. After purification, the Lys *N*^ε amino groups can be reprotected by trifluoroacetylation. A related approach to the purification of protected peptides has been described by Suzuki.³⁷⁶

The dihydroxyborylbenzyloxycarbonyl,³⁷⁷ 2-(triphenylphosphonio)ethoxycarbonyl,³⁷⁸ isonicotinylloxycarbonyl,³⁷⁹ and the 2-(2-pyridyl)ethoxycarbonyl groups^{380,381} have all been reported to have solubility-enhancing properties. However none of them has achieved widespread use in peptide synthesis. The ferrocenylmethyl group has been reported to improve the solubility of protected peptides, by disruption of β -sheet formation, when used as a protecting group for the amide bond,³⁸² and the Sulfmoc group (59) was designed by Merrifield to be useful in the purification of peptides.³⁸³



As yet neither of these have gained widespread acceptance. More promising perhaps, as an aid to the solubilisation of protected peptides for their purification is the use of protecting groups incorporating PEG units as first suggested by Mutter³⁸⁴ and Bayer³⁸⁵ and also currently under investigation in our own laboratory.³⁸⁶ A similar philosophy underlies the work of Ball and Mascagni³⁸⁷⁻³⁸⁹ for the design of removable chromatographic probes based on the Fmoc group, for the purification of large peptides.

3. SOLID-PHASE COUPLING OF PROTECTED PEPTIDE SEGMENTS

Weygrand^{390,391} and Omenn and Anfinsen³⁹² were among the first to carry out model studies for the solid-phase coupling of protected peptide segments. The coupling of protected segments on a solid support may be carried out using segments synthesised in solution or using those synthesised by solid-phase techniques. In order to couple protected peptide segments successfully on a solid support³⁹³ several factors must be taken into account and we discuss the more important points.

3.1. Choice of solid support

The nature of the solid support can profoundly influence the outcome of segment coupling reactions. The effect of the solid support on amino acid coupling yields has been studied by Losse.^{394,395} We³⁹⁶ have studied the model segment coupling of the protected 60-67 segment of uteroglobin, Fmoc-Thr(Bzl)-Glu(OBzl)-Lys(Z)-Ile-Val-Lys(Z)-Ser(Bzl)-Pro-OH onto the resin-bound 68-70 sequence, H-Leu-Cys(Acm)-Met-O-polymer, using five different polymeric solid supports, polystyrene-1%-divinylbenzene (1), macroporous-polystyrene (2), Kel-F-g-styrene (3), polyacrylamide (4) and controlled-pore glass (5). Couplings were performed in DMF at room temperature, using 1.25 or 2.5-fold excesses of the segment, of DCC and of HOBT. The results are summarised in Table 1.

These results indicate that microporous polystyrene and polyacrylamide are the best solid supports for segment condensation reactions, giving higher yields and requiring shorter reaction times than the other polymeric supports.

Most examples of the solid-phase coupling of protected peptide segments have been carried out using polystyrene resins, although Sheppard^{397,398} has carried out model studies for segment couplings on polyacrylamide resins and isolated examples using other polymer supports are to be found.

In addition to the resin type, the degree of functionalisation is also important. We believe that

Table 1. Effect of solid-support on segment coupling yields.

Support	Equivalents of peptide	Yield %				
		2h	7h	24h	48h	72h
(1)	2.5	31	100	-	-	-
(1)	1.25	8	24	56	-	80
(2)	2.5	6	12	22	34	37
(2)	1.25	2	4	-	8	17
(3)	2.5	24	49	92	100	-
(3)	1.25	3	9	15	23	35
(4)	2.5	24	87	100	-	-
(4)	1.25	4	13	22	32	44
(5)	2.5	18	35	41	46	66
(5)	1.25	7	21	31	53	64

the optimum level of resin functionalisation for segment coupling is probably between 0.2 and 0.4 meq/g. Levels which are much higher or much lower should be avoided in this type of work.

3.2. Coupling strategy

In addition to the normal *C* to *N* elongation strategy used in SPPS, examples of the opposite *N* to *C* elongation strategy for the coupling of segments have been described. Felix³⁹⁹ has reported on the coupling of resin-bound peptide azides with protected peptide segments in an *N* to *C* coupling strategy. Segment coupling yields were of the order of 60%. Matsueda⁴⁰⁰ described the synthesis of LHRH by segment condensation on a solid support, using both *C* to *N*- and *N* to *C*-elongation strategies. The segment coupling reactions were carried out using the oxidation-reduction method.⁴⁰¹ In the first *C* to *N* strategy, a 1.5 fold excess of the protected peptide segment Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OH was coupled onto a tetrapeptide bound to an hydroxymethyl resin, H-Leu-Arg(NO₂)-Pro-Gly-O-resin, using a three-fold excess of Ph₃P and of DPD. After removal of the Boc protecting group of the heptapeptide resin, this was then coupled using a three-fold excess of the segment Boc-His(Tos)-Trp-OH, using an equimolar amount of Ph₃P and of DPD. The protected nonapeptide was cleaved from the resin by ammonolysis to give the *C*-terminal amide. Treatment with HF, purification and coupling to pGlu, gave synthetic LHRH which was identical with a natural sample.

In the second *N* to *C* strategy, H-His-Trp-Ser(*t*Bu)-Tyr-Gly-O-*t*Bu was coupled to resin-Glu-OH, using a three-fold excess of the protected peptide segment, of Ph₃P and of DPD. After deprotection of the resin-bound *t*Bu ester, the protected segment H-Leu-Arg(NO₂)-Pro-Gly-NH₂ was coupled with the resin-hexapeptide, again using a three-fold excess of the protected peptide segment, of Ph₃P, and of DPD. After detachment from the resin, conversion of the Gln residue to pGlu and purification, fully active LHRH was again obtained.

The same group^{402,403} synthesised ACTH using the same type of *N* to *C* strategy, as shown in Fig. 5. The protected peptide segments were synthesised in solution.

In each segment coupling reaction a threefold excess of the protected peptide segment and a thirty-fold excess of Ph₃P, of DPD and of 2-mercaptopyridine in DCM were used. Reaction times were 6 h at -15°C and overnight at room temperature. No detectable epimerisation occurred when the peptide segments were coupled in this way. After HF treatment of the peptide resin and purification, fully active ACTH was obtained.

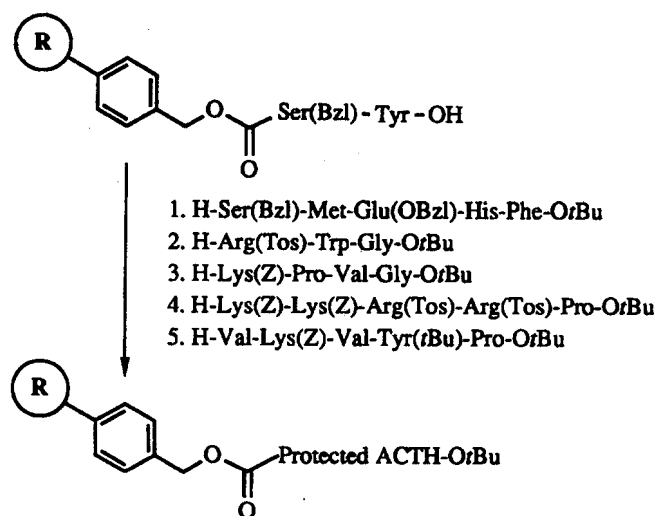


Fig. 5.

Although for stepwise SPPS the *N* to *C* strategy suffers from the disadvantage that the risks of epimerisation of the resin-bound peptide in the amino acid coupling steps are much greater, for CSPPS using either the *C* to *N* or the *N* to *C* strategies, epimerisation of the activated *C*-terminal amino acid is a possibility in both cases. One of the potential advantages of the *N* to *C* strategy for segment coupling is that since the activated *C*-terminal of the peptide is attached to the resin, it should be possible, in principle, to recycle the excess of protected peptide in solution. However, in spite of the successful syntheses described above, the *N* to *C* elongation strategy has not established itself and is rarely used nowadays. Almost all solid phase peptide synthesis is carried out using the *C* to *N* strategy.

3.3. Incorporation of the first segment onto the resin

The first segment can be incorporated onto the resin either by carrying out a stepwise solid-phase synthesis of the desired *C*-terminal sequence or alternatively by incorporating a previously synthesised, purified and characterised peptide segment. This latter process, in principle, gives rise to purer final products, but unfortunately the coupling of protected peptide segments onto polymeric resins does not always proceed in high yield. The incorporation of the first segment is often carried out in such a way as to reduce the substitution level of the resin.^{145,404} If the first segment is to be synthesised in a stepwise fashion, then the first amino acid is incorporated using less than the amount required to react with all of the active sites on the resin. After the coupling of this first amino acid the resin is acetylated to block the remaining reactive sites and the synthesis is continued under normal conditions. If the first segment is to be incorporated as a previously synthesised segment, then similarly the coupling reaction is carried out with less than 1 equivalent of the peptide segment with respect to the degree of functionalisation of the resin. The coupling reaction is allowed to proceed to completion and the resin is then acetylated to block all of the remaining reactive sites. It is thought that by following these procedures, only the most reactive resin-bound functional groups react with the amino acid or peptide segment.

We consider first the stepwise solid-phase synthesis of the *C*-terminal segment.

3.3.1. *Stepwise solid-phase synthesis of the first segment.* The advantage of synthesising the first *C*-terminal segment in a stepwise manner on the solid support is that the segment can be synthesised rapidly. The disadvantage is that it cannot be rigorously purified. If the segment is quite long then the possibility of having deletion or terminated peptides at the *C*-terminus is increased. If the

segment is short, then since modern methods of solid-phase peptide synthesis are so efficient, the C-terminal peptide can be synthesised in an essentially pure state.

Ljungqvist and Folkers^{224,405} synthesised a fragment of human leukocyte interferon using a combination of stepwise SPPS and of segment condensation. The sequence in question is shown below.

Ac-Ile¹¹⁶-Leu-Ala-Val-Arg¹²⁰-Lys-Tyr-Phe-Gln-Arg¹²⁵-Ile-

Thr-Leu-Tyr-Leu¹³⁰-Lys-Glu-Lys-Lys-Tyr¹³⁵-Ser-Pro-NH₂

The C-terminal decapeptide 127–137 sequence was synthesised on a BHA resin and after unmasking of the resin-bound amino group with TFA followed by neutralisation, the protected tetrapeptide Boc-Phe-Gln-Arg(Tos)-Ile-OH was coupled. A five-fold excess of protected peptide was used, with equivalent amounts of DCC and HOBt. The Boc-123-137-resin-peptide thus produced was elongated by stepwise couplings to give the Boc-120-137-peptide-resin. After deprotection of the Boc group, the protected tetrapeptide segment Boc-Ile-Leu-Ala-Val-OH was coupled using a five-fold excess of peptide segment and of DCC/HOBt. Amino acid analysis of the peptide-resin throughout the synthesis indicated that the segment condensations had proceeded smoothly. Deprotection of the Boc group followed by acetylation, HF treatment and purification gave the pure peptide.

Lelievre⁴⁰⁶ has described the convergent solid-phase synthesis of retro-gramicidin A-D-Ala-gramicidin A, a peptide designed to mimic a gramicidin A dimer, and to form ion channels in lipid bilayer membranes similar to those formed by gramicidin A. The synthesis of this molecule is an interesting example of the advantage of a convergent synthesis over a linear one. A linear solid-phase synthesis might lead to the formation of small amounts of gramicidin A which would obscure the observation of the desired physicochemical activity. The convergent synthesis used, shown in Fig. 6, makes it impossible for gramicidin A to be present.

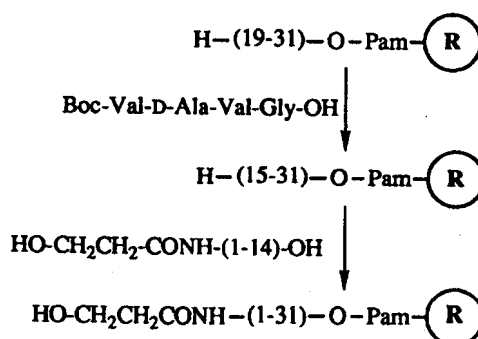


Fig. 6.

The 19–31 C-terminal segment of the molecule was synthesised in a stepwise fashion on a Pam resin.^{168,169} The N-Boc-protected 15–18 segment was synthesised in solution and coupled to the resin-bound 19–31 segment using a 2.5 fold excess of segment, of DCC and of HOBt in DMF. The coupling time was 2 h. In order to complete the synthesis the 1–14 segment was synthesised on a Pam^{168,169} resin and detached by transesterification with DMAE.¹⁹⁸ This segment was then coupled to the resin-bound 15–31 sequence, using a 1.5 fold excess of the 1–14 segment, DCC and HOBt. A second coupling was performed under identical conditions and the yield was estimated to be 53% over the two couplings, by the increase in weight of the peptide resin. The desired molecule was obtained after HF detachment from the resin and purification.

acid analyses of the peptide-resin. HF treatment gave crude LHRH which was purified giving material identical with an authentic sample.

The protected tridecapeptide Boc-Asn-Lys(ClZ)-Tyr(BrZ)-Thr(Bzl)-Thr(Bzl)-Glu(OBzl)-Tyr(BrZ)-Ser(Bzl)-Ala-Ser(Bzl)-Val-Lys(ClZ)-Gly-OH corresponding to the 56-68 sequence of the M603 murine immunoglobulin domain was synthesised by Tam^{73,241} on the resins (7) and (48). After photolytic detachment and purification, the pure segment was reattached to different types of resin. Reattachment of the protected tridecapeptide to 2-bromopropionyl-resin (12) was carried out in 80% yield using the KF method.^{407,408} The yield for the incorporation of the same segment onto an aminomethyl resin using the same method was only 45% and the incorporation onto the 4-bromomethyl-3-nitrobenzamidomethyl-resin (7) using identical conditions was 24%. This illustrates that the yields for the attachment of protected peptide segments to solid supports depends quite critically upon the type of resin used.

With the 56-68 sequence reattached to the resin, a 53 residue protected peptide corresponding to the 16-68 sequence of the heavy chain of the V_H domain of mouse myeloma immunoglobulin M603 was built up^{241,409} by a segment condensation strategy, as shown in Fig. 9.

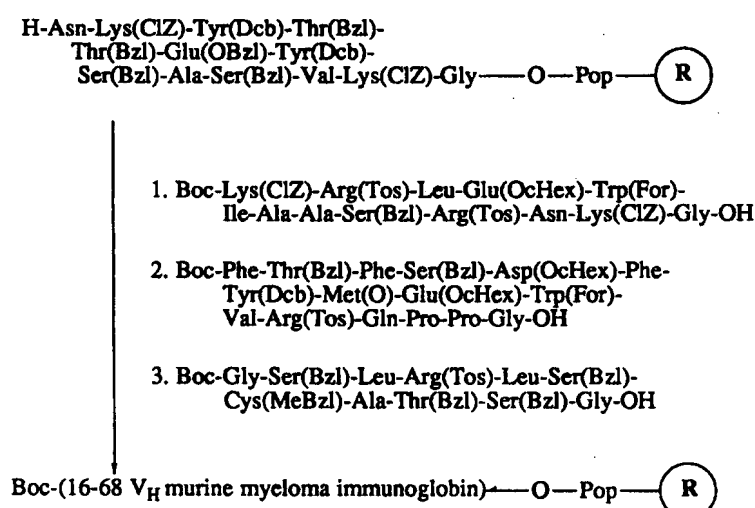


Fig. 9.

The segment couplings were carried out using DCC and HOBt and the coupling yields for the first two segments were 85% and 75% respectively. The resin was acetylated after each segment coupling step. The third protected peptide segment was incorporated in a yield of 50% giving the desired 16-68 sequence.

Kaiser^{139,140} coupled the protected peptide segment, Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH to the oxime resin (35) using 1.4 equivalents of the peptide in DMF and an equimolar amount of DCC and HOBt. After 17 h at room temperature, a further 0.53 equivalents of peptide, DCC and HOBt were added and the coupling reaction allowed to proceed for a further 17 h. The yield of segment incorporation on the resin (35) was 70%. This peptide-resin was then used for the synthesis of model peptides for apolipoprotein A-1.

In Kaiser's synthesis¹⁴⁵ of ribonuclease T₁, the protected peptide segment Boc-Tyr(Dcb)-Gln-Leu-His(Bom)-Glu(OBzl)-Asp(OBzl)-Gly-Glu(OBzl)-Thr(Bzl)-OH corresponding to the 24-32 sequence was attached to H-Val-O-oxime resin in DMF using DCC and EACNOx.⁴¹⁰ The coupling reaction was allowed to proceed overnight after which time amino acid analysis indicated that the reaction had proceeded in 48% yield. In the same synthesis the 48-53 segment, Boc-Tyr(Dcb)-Asn-

Asn-Tyr(Dcb)-Glu(OBzl)-Gly-OH, was attached to the resin using DCC and EACNOx. After shaking for 24 h, the yield for the incorporation of this segment was 82%. Both of these incorporations were carried out with less than one equivalent of the peptide segment relative to the degree of resin functionalisation. The peptide-resin was acetylated after incorporation in each case.

3.4. Segment size

When designing a convergent solid-phase synthesis strategy for a particular peptide, one of the main factors which has a direct bearing upon the size of the segments to be coupled is the frequency of Pro and of Gly residues in the amino acid sequence of the target peptide. If the criterion of choosing only those protected peptide segments having Pro or Gly at the C-terminal is strictly adhered to, then inevitably some segments will be rather long. It is our experience that, as a 'rule of thumb', protected peptide segments of more than twelve amino acids in length are so insoluble that severe problems are encountered both in their purification and coupling. Low solid-phase coupling yields for protected peptide segments having from 14 up to 22 residues have been noted by Rink.⁴¹¹ Nevertheless, Ball and Mascagni³¹⁶ amongst others have successfully coupled longer protected peptide segments on a solid support. Segment size is an important consideration when designing a convergent solid-phase synthesis and the risks of working with an insoluble segment with Gly or Pro at the C-terminal must be balanced against those of working with a shorter more soluble segment in which, since neither Pro nor Gly are at the C-terminal, the dangers of epimerisation in the segment coupling step are greater.

The protection scheme of a given peptide segment can affect its solubility, such that a peptide segment of a given length which is only poorly soluble, and gives only low or moderate coupling yields, might be rendered more soluble by changing its protection scheme, assuming this can be done within the overall context of the synthesis.

Sato⁴¹² has described the synthesis of gramicidin S by a stepwise SPPS approach and has compared the results of this synthesis with two separate segment coupling approaches, one in which dipeptide segments were coupled and the other in which tripeptide segments were coupled. Both of these latter approaches are summarised in Fig. 10. All three syntheses were carried out on a standard Merrifield polystyrene resin.

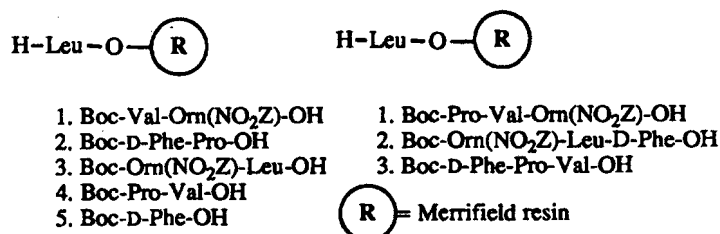


Fig. 10.

In each of the segment coupling strategies the di- or tri-peptide segments were coupled on the solid support using a four-fold excess of each peptide segment and of DCC and HOBt. Coupling yields in each case were between 92% and quantitative after 24 h shaking at room temperature. The overall yields based on amino acid analysis for each of the three syntheses were 68%, 83% and 86% respectively, showing that the segment coupling strategies were superior in this respect to the linear approach. There was little difference between the dipeptide and the tripeptide segment syntheses. No epimerisation was detected in the segment couplings. In each of the syntheses, the peptide chain was subsequently elaborated to give fully active gramicidin S.

A segment condensation strategy on a solid support was investigated by Camble and Petter for the synthesis of the 1-47 sequence of urogastrone,⁴¹³ shown in Fig. 11.

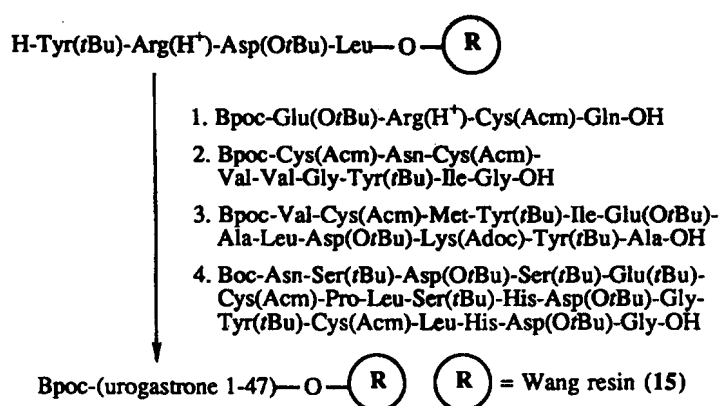


Fig. 11.

The 44–47 C-terminal tetrapeptide was built up in a stepwise fashion on the Wang resin (15). Protected peptide segments corresponding to the 40–43, 31–39 and 19–30 sequences were coupled using a variety of different conditions. Highest segment coupling yields (79–95%) were obtained using DCC and HOBt in DMF at room temperature for 22 or 46 h. Unfortunately, it proved very difficult to couple the relatively large 1–18 segment even though the previous segment coupling reactions proceeded smoothly in acceptable yields. Coupling yields for this segment were very poor even though a variety of coupling conditions were attempted. This illustrates a quite common state of affairs in CSPPS: a protected peptide segment which has been successfully synthesised and purified cannot be made to couple in acceptable yields.

In our synthesis of the sequence of the toxin II of the scorpion *Androctonus australis*,^{64,65,91,92,414} it was found that the 5–17 protected segment, Fmoc-Tyr(cHex)-Ile-Val-Asp(OBzl)-Asp(OBzl)-Val-Asn-Cys(Acm)-Thr(Bzl)-Tyr(cHex)-Phe-Cys(Acm)-Gly-OH could not be coupled to the 18–64 peptide resin in acceptable yields. The segments Fmoc-Asn-Cys(Acm)-Thr(Bzl)-Tyr(cHex)-Phe-Cys(Acm)-OH corresponding to the 11–16 sequence and Boc-Tyr(cHex)-Ile-Val-Asp(OcHex)-Asp(OcHex)-Val-OH corresponding to the 5–10 sequence were synthesised. Coupling of these shorter segments proceeded in very high yields.

Di Bello⁴¹⁵ has reported on the synthesis of bombesin by a CSPPS approach. Two segment condensation strategies were used as shown in Fig. 12.

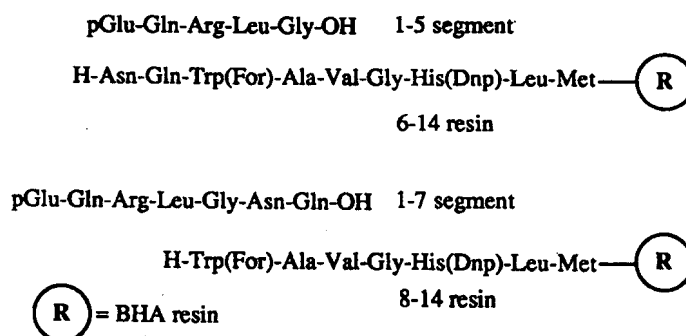


Fig. 12.

In the first strategy, the 1–5 segment was synthesised by standard solid-phase methods on a BHA resin, detached by treatment with HF and purified by gel filtration. For segment coupling

onto the 6–14 BHA resin-bound protected nonapeptide, a five-fold excess of the peptide segment was used with equimolar amounts of DCC and HOBt. Couplings were performed in DMF and were allowed to continue for approximately 12 h. A second coupling was then performed under identical conditions. In the second strategy, the 1–7 segment was synthesised in a similar manner and coupled to the 8–14 protected heptapeptide resin, again using a five-fold excess of peptide segment, DCC and HOBt. A second coupling was then performed under similar conditions but was allowed to continue for 48 h at room temperature. Both synthetic approaches gave similar results, biologically active material being obtained in both cases.

The solid phase coupling of a partially protected peptide has been described by Di Bello.⁴¹⁶ Tfa-Lys(Tfa)-Glu(OMe)-Thr-Ala-Ala-Ala-Lys(Tfa)-Phe-Glu(OMe)-Arg-OH was prepared by enzymatic cleavage of the native S-peptide of bovine pancreatic ribonuclease, and was coupled onto H-Gln-His(Z)-Met-Asp(OBzl)-Ser(OBzl)-O-resin, using a 1.1-fold excess of segment and the active ester method, in 30% yield as calculated by amino acid analysis. The coupling reaction was carried out in pyridine–DMF (1:1) since the peptide segment was only poorly soluble in most solvents.

Kaiser¹⁴⁵ noted that the coupling of large protected peptides on the oxime resin (35) proceeded in poor yields. In order to avoid this problem in the synthesis of ribonuclease T₁, smaller peptide segments only were coupled on the solid support and couplings between large segments were carried out in solution.

In the synthesis of the β A4 protein of Alzheimer's disease, attempts to couple the 1–25 protected peptide segment to the protected resin-bound 26–42 sequence were unsuccessful. Lansbury³⁶ successfully synthesised the protein by coupling a shorter 1–17 segment to the resin-bound 18–42 protected peptide. (See Section 3.8, Examples of CSPPS, below).

3.5. Coupling methods

The formation of an amide bond between a resin-bound peptide and a protected peptide segment is more demanding than for a single amino acid for several reasons. It is normally not economical to use large excesses of the peptide segment to drive the coupling reaction to completion and solubility problems may mean that only a relatively dilute solution of the segment can be used. Even if reasonable excesses of segment can be used, and solubility is not a problem, the coupling reaction may require many hours or even several days. It is important therefore to treat each segment coupling on a case by case basis and to attempt to optimise the coupling reaction by investigating the different variables such as coupling reagents, reaction time, concentration of the soluble components, temperature, and efficiency of the agitation of the resin.

Coupling methods for protected peptide segments have evolved in an analogous manner to those used for the coupling of single amino acids. Early procedures include the azide and oxidation–reduction protocols which were superseded by the use of DCC, or DCC in conjunction with HOSu or HOBt. Contemporary methods for effecting the solid-phase coupling of peptide segments are based on the use of phosphonium or uronium salts in the presence of HOBt.

The classical azide procedure⁴¹⁷ has been used for the solid-phase coupling of protected peptide segments. Visser and Kerling⁴¹⁸ synthesised an analogue of the Boc-protected *N*-terminal tri-decapeptide of bovine pancreatic ribonuclease, H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Cit-Gln-His-Leu-OH, by segment coupling of the protected *N*-terminal octapeptide azide with the resin-bound *C*-terminal pentapeptide. A 2.5 fold excess of the peptide azide was used and the coupling yield was essentially quantitative after 6 days. Cleavage from the resin and purification by gel-filtration gave the desired product.

Weber and André^{419,420} synthesised an analogue of ovine insulin A chain by a convergent strategy as shown in Fig. 13.

The 17–20 protected peptide segment was coupled as the azide to an Ala-containing polystyrene resin, using a two-fold excess of azide. The coupling yield was 75%. The 11–16 segment was then coupled using a two-fold excess of segment and of DCC and HOBt, in DMF. The coupling yield

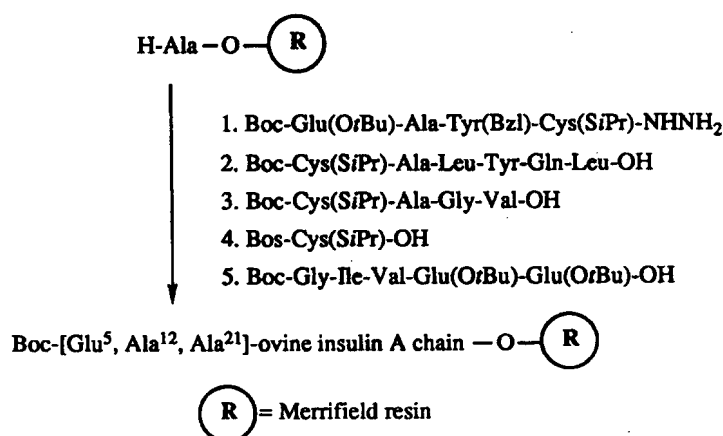


Fig. 13.

was 80–85%. The 7–10 segment was coupled in the same way in a yield of 85–90%. Boc-Cys(SiPr)-OH was coupled in 96% yield and the 1–5 segment was coupled as for the other segments, in a yield of 70–75%. The fully-protected peptide–resin was then subsequently transformed into the ovine insulin A chain analogue. Combination of this analogue of the A chain with natural B chain gave insulin activities comparable with those obtained with the natural A chain.

The solid-phase coupling of the cyclic peptide segment (60) onto a MBHA-resin-bound protected peptide, as shown in Fig. 14, has been described by Noda.⁴²¹

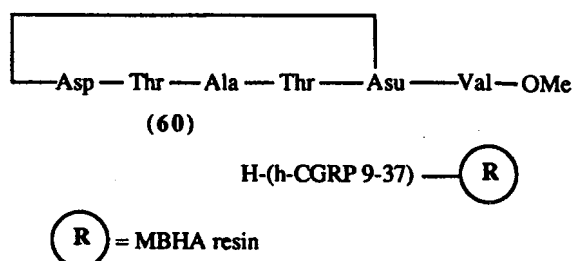


Fig. 14.

The segment (60) was treated with hydrazine, converted into the azide and coupled to the peptide–resin. Amino acid analysis of the peptide resin hydrolysate after coupling indicated that the coupling yield was very high.

The DCC/HOBt coupling protocol has been used extensively for the solid phase coupling of protected peptide segments. This combination of coupling reagents has been used in several examples of peptides having repetitive sequences. In the syntheses of poly-(L-prolyl-L-prolylglycyl) oligomers,⁴²² of the cyclodepsipeptide valinomycin,^{423,424} and its analogue [Lys¹]-valinomycin,⁴²⁵ as well as in our synthesis of repeating peptides related to the glutelin-2 protein of maize,^{201,202} the solid-phase couplings of the repeating units were all carried out using DCC and HOBt. Neubert and Jakubke⁴²⁶ prepared a heptapeptide corresponding to the 28–34 sequence of porcine proinsulin by segment condensation on a solid support using DCC/HOBt as the coupling agents.

In our synthesis of apamin,⁶² the 7–18 sequence was synthesised in a stepwise manner on a BHA resin. The 1–6 segment Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-OH was then coupled onto this resin-bound sequence (Fig. 15).

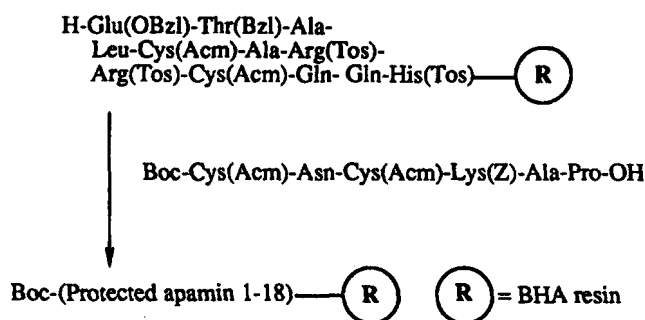
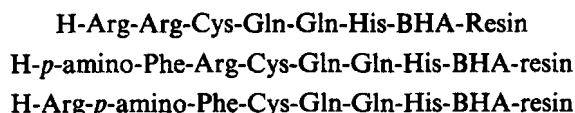


Fig. 15.

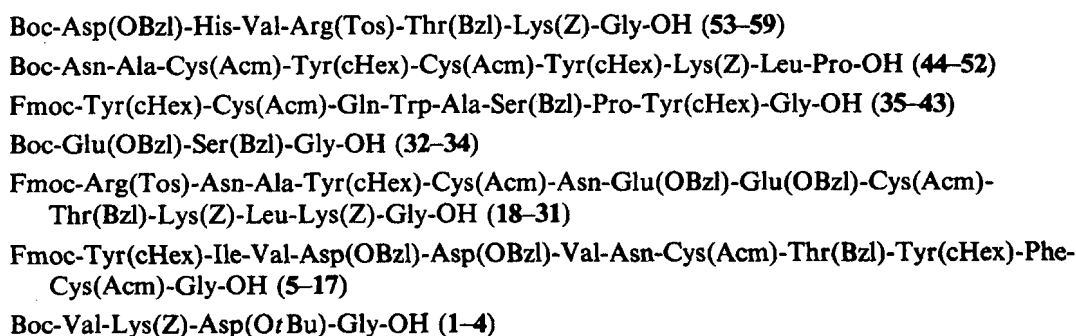
The coupling reaction was carried out using 1.25 equivalents of the 1–6 peptide segment, of DCC and of HOBt, in DMF. A single coupling reaction of 48 h duration at room temperature was performed after which time amino acid analysis of the peptide resin indicated essentially quantitative coupling. Poorer results were obtained in the coupling of the protected 1–6 segment azide (coupling yield of 67% after a double coupling protocol). HF treatment, followed by deprotection of the Cys residues, formation of the disulfide bridges and final purification, gave biologically active apamin.

In another approach,⁶³ the 1–12 protected peptide segment Boc-Cys(Acm)-Asn-Cys(Acm)-Lys(Z)-Ala-Pro-Glu(OBzl)-Thr(Bzl)-Ala-Leu-Cys(Acm)-Ala-OH was coupled to the three different analogous resin-bound 13–18 segments shown below.



These three 13–18 sequences were synthesised in a stepwise manner on a BHA resin. In the case of natural apamin the coupling proceeded in only 77% yield using a two-fold excess of peptide segment, DCC and HOBt, whereas for the apamin analogues, the coupling yields were 94% and 96% respectively, even though only 1.5 equivalents of segment, DCC and HOBt were used. This may be due to the concentration of reagents being greater in the latter two cases. The coupling reactions were carried out in DMF at room temperature over 48 h.

We have also used the DCC/HOBt coupling method extensively in our synthesis of the complete sequence of the toxin II of the scorpion *Androctonus australis*,^{64,65,92,414} a peptide consisting of 64 amino acids. The following protected peptide segments were synthesised and purified:



The protected peptide segments were then coupled onto the resin-bound 60–64 sequence H-Gly-Arg(Tos)-Cys(Acm)-His-MBHA-resin. The degree of functionalisation of the resin was approximately 0.2 meq/g, and the results of the coupling reactions are shown in Table 2.

Table 2. Segment coupling reactions in the synthesis of toxin II from *Androctonus australis*.

Peptide	Equivalents	Amino Component	Time (hrs)	Yield %
53-59	2.1	60-64 resin	20	96
44-52	1.3	53-64 resin	40	91
	0.7		24	quant.
35-43	1.6	44-64 resin	16	90
	0.6		16	quant.
32-34	1.8	35-64 resin	28	94
	1.6		16	quant
18-31	2.8	32-64 resin	48	80

We used the same general procedure for the coupling of all the protected peptide segments. The segments (1.3–2.8 equivalents) were dissolved in the minimum volume of DMF and were added to the peptide resin together with an equimolar amount of HOBT. After 2 min stirring, DCC (1.3-2.8 equivalents) was added and the mixture was stirred for 2 h at 0°C and then at room temperature for the duration of the coupling (See Table 2). Aliquot portions of the peptide resin were removed periodically and the extent of coupling was determined by amino acid analysis of the acid hydrolysates. If the yield appeared to be less than 95%, the coupling was allowed to continue and if necessary was repeated (Table 1). The 5–17 protected segment could not be coupled to the 18–64 peptide resin in acceptable yield. (See Section 3.4, Segment Size).

Yajima⁴²⁷ reported on the synthesis of a decapeptide corresponding to the 5–14 sequence of ACTH. The protected segment Z-Glu(OBzl)-His-Phe-Arg(NO₂)-Trp-Gly-OH was coupled onto the resin bound peptide H-Lys(Z)-Pro-Val-Gly-O-resin using a four-fold excess of the acylating protected peptide segment and of DCC. The reaction time was 72 h at room temperature and the yield for the coupling was between 40 and 45%. Yajima⁴²⁸ has also investigated the EEDQ-mediated coupling of the protected peptide segment Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH onto H-Asp(OBzl)-O-resin. Quantitative coupling yields were obtained using a three-fold excess of the acylating component and a six-fold excess of EEDQ in DMF for 48 h.

Yajima^{404,429} also described the synthesis of BPTI. Protected peptide segments spanning the entire sequence of 58 amino acids were synthesised in solution by classical techniques and then coupled on the solid support (Fig. 16).

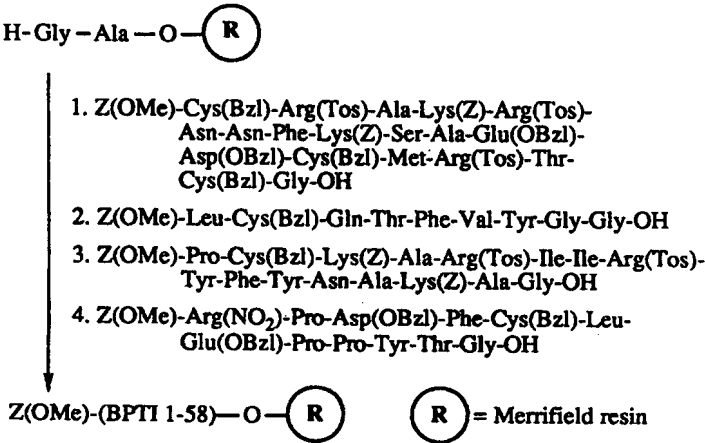


Fig. 16.

The protected peptide segments were coupled onto H-Gly-Ala-O-resin using DCC and HOSu. For the first segment a 1.5 fold excess was used for the coupling, but for later segments in the sequence the number of equivalents was increased to four in order to ensure sufficiently high coupling yields. Coupling times were 4–6 days at room temperature and yields were between 71% and quantitative, depending upon the segment coupled. Only for the last segment of the sequence was a quantitative coupling yield obtained. Yajima noted that the 29–37 protected nonapeptide and 13–28 protected hexadecapeptide segments were very insoluble in DMF and suggests that the coupling yields (68% and 78%, respectively) may be relatively low for this reason.

Noda⁴³⁰ has described the coupling between Boc-Lys(Z)-Asn-Gly-OH and H-Ile-Glu(OBzl)-Ile-O-resin, using DCC and HOSu. The yield was judged to be 90% from the increase in weight of the peptide resin, after a reaction time of 8 h. The hexapeptide so formed corresponds to the 47–52 sequence of the cobrotoxin from *Naja naja atra*.

Kullmann and Gutte⁴³¹ synthesised an open chain asymmetrical cystine peptide corresponding to the sequence A¹⁸⁻²¹-B¹⁹⁻²⁶ of bovine insulin, by the condensation of the protected cystine peptide A¹⁸⁻²¹-B¹⁹⁻²⁰ onto the protected B²¹⁻²⁶ resin, as shown in Fig. 17.

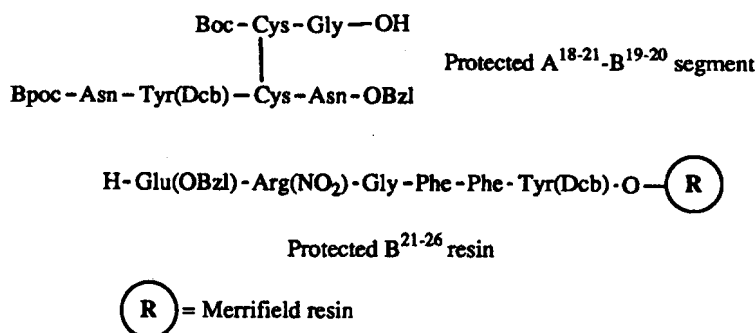


Fig. 17.

The coupling between the two segments was carried out in DMF, using a 1.25 fold excess of the protected A¹⁸⁻²¹-B¹⁹⁻²⁰ segment with an equimolar amount of DCC in the presence of 2 equivalents of HOSu. The coupling yield was 70% after 75 h at room temperature.

This work was subsequently extended to the synthesis⁴³² of the asymmetrical insulin intermediate A¹⁻²¹-B¹⁸⁻²⁶, and is summarised in Fig. 18.

The couplings were carried out in DMF using one equivalent of DCC and two equivalents of HOSu relative to each protected peptide segment. For the coupling of segment 1 above, a 4.2 fold excess of protected peptide was used and the yield was 95%. The residue A,¹⁵ Bpoc-Gln-OH (2 in the above scheme) was incorporated using a double coupling with a 4.2 fold excess of amino acid in a yield of 99%. For segment 3 again a double coupling was performed and each time a 4.2-fold excess was used. The coupling yield for this segment was 78%. For segment 4 a double coupling using a two-fold excess of protected peptide was carried out. The coupling yield was 69%. Segment 5 was coupled using a double coupling of a 3.5-fold excess in a yield of 75%. Boc-Val-OH was then coupled to the B chain in 96% yield. After each coupling step the peptide resin was acetylated. The disulfide bridge is stable to all of the conditions used to elaborate this peptide.

Karlsson and Ragnarsson^{433,434} described the synthesis of bradykinin. Three protected peptide segments, Z-Arg(NO₂)-Pro-OH, Z(OMe)-Pro-Gly-Phe-OH and Z(OMe)-Ser(Bzl)-Pro-Phe-OH spanning the sequence were synthesised in solution and coupled onto H-Arg(NO₂)-O-polystyrene resin. A 2.5-fold excess of each segment, of DCC and HOSu was used and coupling times were of

Coupling reactions were carried out using a two to three fold excess of peptide segment and using either DCC or EEDQ as the coupling agent, in the presence of HOObt. Reaction times varied from 21 h to 63 h at 32°C and yields were between 96 and 99%. Subsequent synthetic transformations gave biologically active, crystalline glucagon.

Kung-Tsung Wang⁴³⁷ has synthesised a fully-active snake venom cardiotoxin consisting of 60 amino acids and incorporating four disulfide bridges (Fig. 20).

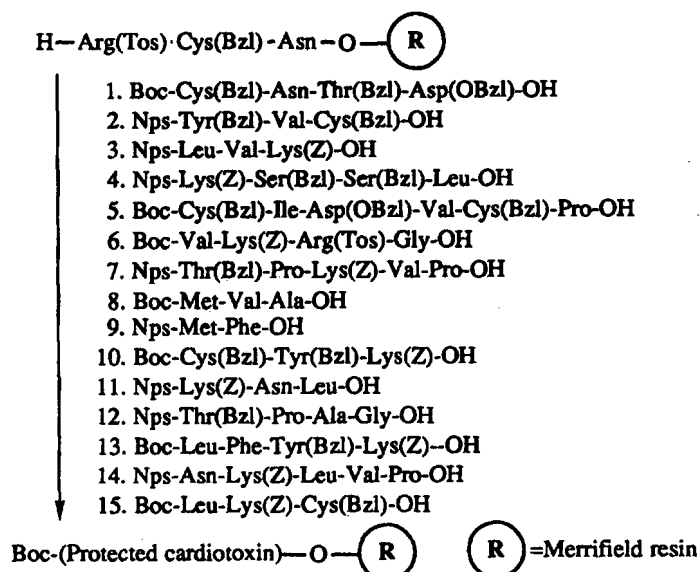


Fig. 20.

Each protected peptide segment was coupled using the DCC/HOBt system. A three-fold excess of each segment was used and coupling times were at least two and sometimes three days. If after this time the ninhydrin test was positive, the coupling was repeated. After each segment coupling the resin was acetylated. The average coupling yield for the segment condensations was 90%. The peptide chain was cleaved from the solid support by treatment with HF. After formation of the disulfide bridges and purification, fully-active cardiotoxin was obtained. Subsequently Wang⁴³⁸ synthesised a cardiotoxin of similar structure using the same strategy, again obtaining fully-active material.

Sivanandaiah⁴³⁹ synthesised an octadecapeptide amide related to the C-terminal of ACTH (Fig. 21).

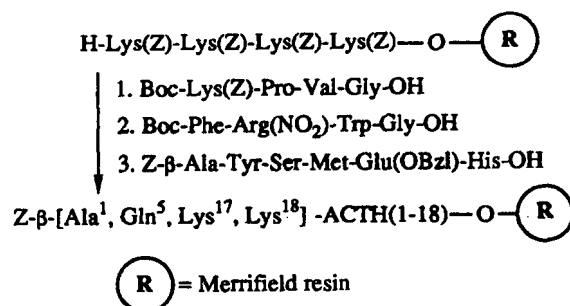


Fig. 21.

Starting from the tetrapeptide resin, chain elongation was carried out by successive coupling of the protected peptide segments. Segment couplings were carried out in DMF using a three-fold excess of segment, DCC and HOBt. Coupling times for each of the segments were 10, 24 and 30 h respectively. The qualitative ninhydrin test was negative after each coupling. The peptide was removed from the resin by ammonolysis, furnishing the desired C-terminal amide and also converting the Glu(OBzl)⁵ residue into the desired Gln⁵ residue. Transfer hydrogenation then gave the desired free peptide amine which was found to be as biologically active as natural ACTH.

Birr²⁰⁶ used the DCC/HOBt system for the coupling of protected peptide segments in the synthesis of the A and B chains of human insulin, and also investigated the use of CDI in the presence of HOBt for similar coupling reactions. In general segment couplings carried out using the CDI/HOBt were complete in shorter reaction times than with DCC/HOBt.⁴⁴⁰

In Kaiser's synthesis of model peptides for apolipoprotein A-1,¹³⁹⁻¹⁴¹ the protected peptide segments, Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH, Boc-Lys(Z)-Glu(OBzl)-Leu-Leu-Glu(OBzl)-Lys(Z)-Leu-OH and Boc-Pro-Lys(Z)-Leu-Glu(OBzl)-Glu(OBzl)-Leu-OH were coupled on the oxime resin to give a peptide consisting of 21 amino acid residues. The protected peptide was then detached from the resin by aminolysis with H-Ala-OBzl affording a 22-residue protected peptide segment which after deprotection gave the 22-residue model peptide. A 44-residue model peptide consisting of two of the 22-residue model peptides was also synthesised by segment condensation on the oxime resin, as shown in Fig. 22.

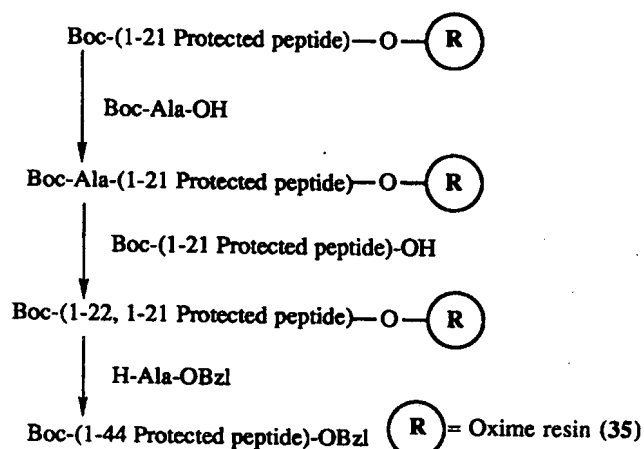


Fig. 22.

When attempts were made to couple the 1-22 segment carboxylic acid with a resin bound 1-22 segment having Pro as the N-terminal amine component, the coupling yields were very low. As an alternative, the 1-21 protected segment was synthesised on resin (35) and using a portion of this peptide-resin, the protected peptide was detached from the solid support by treatment with HO-Pip followed by hydrolysis. Using the other portion of the peptide-resin, Boc-Ala-OH was coupled to the N-terminal of the 1-21 segment. After Boc-deprotection and neutralisation of the resin, the N^α-Boc protected 1-21 segment carboxylic acid was coupled to the peptide-resin. The coupling reaction was carried out in DMF, using a 1.2-fold excess of peptide segment, DCC and HOBt and after 24 h, 77% incorporation of the segment had taken place. The complete 1-44 protected peptide was detached from the resin by aminolysis with H-Ala-OBzl, and after removal of the protecting groups and purification the desired model peptide was obtained.

Coupling reagents based on phosphonium and uronium salts are now becoming more and more frequently used, both for the coupling of single amino acids in linear SPPS and for segment coupling in CSPPS. In Rivaille's synthesis of LHRH,²²⁰ the protected peptide segment pGlu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-OH was coupled onto the resin-bound peptide H-Leu-Arg(NO₂)-Pro-Gly-O-resin using BOP reagent and NMM in DMF. The coupling time was 36 h at room temperature. The peptide was cleaved from the resin by ammonolysis in DMF and after removal of the protecting groups and purification, biologically active LHRH was obtained.

The combination of BOP, HOBt and DIEA has been used for the coupling of protected peptide segments by Chang⁴⁴¹ in the synthesis of cyclic (Asu^{1,7})-eel calcitonin. The same combination has also been used by Nokihara⁹³ for the solid-phase coupling of segments.

Jung^{98,99} has used a combination of TBTU, HOBt and DIEA in order to couple the various protected peptide segments in the syntheses of the nucleocapsid protein of the HIV virus and of a model peptide for the VP₁ protein of the foot and mouth disease virus. Coupling yields were generally 95% or better, using a three-fold excess of peptide segment and coupling times of the order of twelve hours.

Apart from the coupling reagents used, other factors which must be taken into account are the temperature at which the coupling reaction is carried out and the efficiency of the agitation of the resin. Most segment coupling reactions are performed at room temperature although it may be that gentle heating can in some cases be beneficial. Since in the solid-phase method the reaction system is not homogeneous, efficient agitation is necessary in order to ensure good coupling yields. Oroslan¹²³ has reported that ultrasound can improve the yields in solid-phase segment couplings. A series of such coupling reactions were carried out in order to synthesise a range of nonapeptide C-terminal amides, required for structure-activity studies of HIV proteinases. The protected peptide segments Boc-Val-Ser(Bzl)-Gln-Asn-Tyr(BrZ)-OH and Boc-Val-Ser(Bzl)-Gln-Asn-Tyr(BrZ)-Pro-OH were coupled to several different resin-bound peptides, synthesised on an MBHA resin. The coupling reactions were carried out in DMF, using DCC and HOBt. The reactions were sonicated for 2 h after which time coupling was complete, as judged by the qualitative ninhydrin test. The authors note that when magnetic stirring was employed, the reaction time was longer and the yield of peptide was reduced. The desired free peptides were cleaved from the resin by treatment with liquid HF.

3.6. Side reactions

Perhaps the most important side reaction in segment coupling on a solid support is epimerisation at the activated C-terminal amino acid of the segment in the coupling reaction. When this amino acid is Gly, epimerisation is not possible. When this amino acid is Pro, epimerisation is thought to be minimal under normal coupling conditions but for all other amino acids epimerisation may occur to a greater or lesser extent in the coupling reactions.⁴⁴² It is important therefore to couple peptide segments which do not have Gly or Pro at the C-terminal in such a way that epimerisation is minimised. It is also important to use analytical methods which are sufficiently sensitive to detect the presence of diastereomeric peptides in quite small quantities, in order to be able to quantify the amount of epimerisation.⁴⁴³⁻⁴⁴⁵

Work by Di Bello⁴⁴⁶ indicates that the coupling of protected peptide segments having Arg at the C-terminal onto resin bound peptide segments proceeds without detectable epimerisation when DCC and HOBt are used as the coupling agents.

Kaufmann and Dölling⁴⁴⁷ synthesised the N-terminal nonapeptide of ovine insulin. The protected peptide segments were synthesised in solution, and coupled on a solid support (Fig. 23).

The authors report that the best results in the segment couplings were obtained using the DCC/pentafluorophenol complex or the oxidation-reduction protocol.⁴⁰¹ A three-fold excess of peptide segment and of coupling reagent was used and the reaction times were 65 h at room temperature in each case. The desired product was obtained in 47% overall yield with the oxidation-reduction

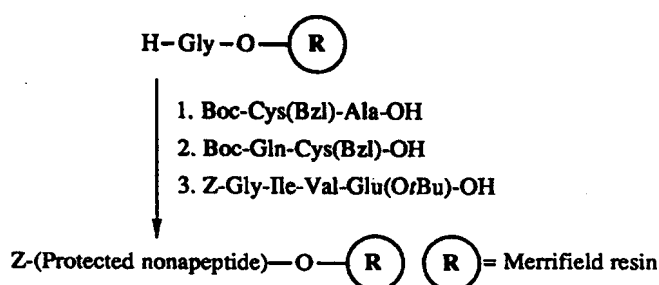


Fig. 23.

protocol. When DCC/HOBt or EEDQ were used, appreciable epimerisation was observed in the segment couplings and the yields were also lower.

Kaiser^{139,140} noted that when the peptide segment Boc-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-OH was attached to the resin (35) using DCC and HOBt, about 10% epimerisation at the C-terminal amino acid occurred. The yield of segment incorporation on the oxime resin was 70%. Carrying out the coupling reaction with EACNOx⁴¹⁰ instead of HOBt both improved the coupling yield to 93% and reduced the amount of epimerisation to 3% (Fig. 24).

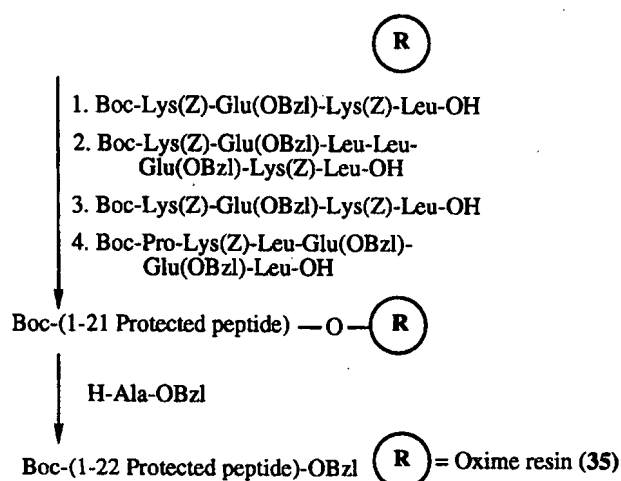


Fig. 24.

The peptide-resin was then successively acylated with the segments 2, 3 and 4 in a similar manner using a 1.2–1.5-fold excess of the acylating segment, and an equivalent amount of DCC and HOBt or EACNOx. Coupling yields of 87%, 100%, and 92% were obtained using the latter additive. These yields were generally higher than when HOBt was used. Aminolysis of the peptide resin with H-Ala-OBzl took place in 63% yield, affording the fully protected peptide segment. The protecting groups were removed by acidolysis to give the free model peptide.

Kaiser's synthesis of ribonuclease T₁ produced material which had very low biological activity. It was suggested that epimerisation of the amino acid residues might account in part for this low activity, even though it seems that epimerisation in the segment coupling steps on the resin is not appreciable.¹⁴⁵

Albericio and Barany⁷² have described the synthesis of human gastrin-I by a CSPPS approach using the Fmoc/*t*Bu strategy. Since Gastrin-I is a peptide C-terminal amide, the synthesis was carried out using the PAL handle (21). This support allows the peptide to be detached under mild conditions at the end of the synthesis, in the form of its C-terminal amide. The protected segment Fmoc-[Glu(OrBu)₅]-Ala-OH was synthesised using the photolabile resin (7), purified by gel filtration, and was then attached to the C-terminal hexapeptide H-Tyr(*t*Bu)-Gly-Trp-Met-Asp(OrBu)-Phe assembled on the PAL support, using a two-fold excess of protected peptide segment in DMF and equimolar quantities of BOP and of HOBT. The reaction was allowed to continue 24 h at 25°C and the coupling yield was more than 98% as judged by amino acid analysis and by Edman degradation. The N-terminal pentapeptide pGlu-Gly-Pro-Trp-Leu-OH was synthesised on the *p*-alkoxybenzyl alcohol-resin (15) and detached by acidolysis. After purification by gel filtration, this segment was then coupled to the peptide resin in a similar manner, in more than 89% yield as indicated by amino acid analysis (Fig. 25).

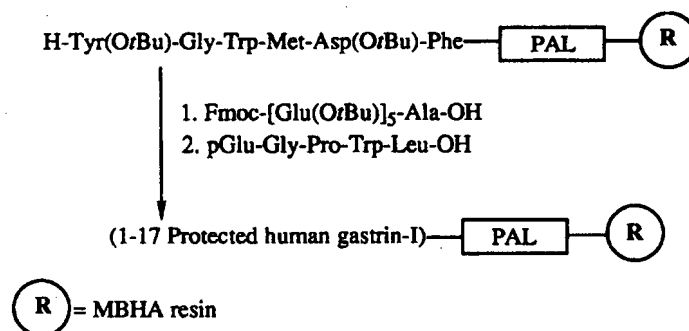


Fig. 25.

The amount of epimerisation in the coupling of the protected peptide segments was estimated to be 4% and 11% respectively. Subsequent synthetic manipulations gave biologically active human gastrin-I.

Other side reactions which may be important in certain cases are the formation of pGlu when Glu is the N-terminal amine component and also side-reactions due to the instability of protecting groups during the segment coupling reactions.

In the synthesis of the sequence of the toxin II of the scorpion *Androctonus australis*⁴¹⁴ we noticed a discrepancy between the 80% coupling yield observed for the coupling of the 18–31 segment onto 32–64-resin by amino acid analysis and the 97% yield obtained by sequencing. This difference may be due to the cyclisation of Glu(OBzl) at position 32 forming pGlu, which is not detectable by Edman degradation.

In our synthesis of the repetitive domain of the glutelin-2 protein from maize,^{201,202} a domain which consists of repeating Val-His-Leu-Pro-Pro-Pro units, the Boc/Bzl strategy was used and the His residue was left unprotected. Attempts to synthesise the trimer by coupling Boc-Val-His-Leu-Pro-Pro-Pro-OH to the resin-bound dimer H-Val-His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-O-resin led only to the formation of the N^α-acetylated dimer. This side reaction may be due to the acetylation of the resin which was carried out after the formation of the Boc-N^α protected dimer. This capping procedure also leads to the N-acetylation of the imidazole of the His residue, so that when the coupling between the resin-bound dimer containing His(Ac) residues and a third Boc-N^α-protected segment using DCC and HOBT is attempted, acetylation of the N^α terminal of the dimer takes place, preventing coupling. The problem was partially solved by using the Fmoc/*t*Bu strategy, but a definitive solution lies in finding a protecting group for His which is completely stable to the reaction conditions. In solid-phase segment couplings the reaction times are often quite long so that the stability of side-chain protecting groups to the coupling conditions should be carefully checked.

3.7. Monitoring of the coupling reaction

Monitoring of solid-phase segment coupling reactions is not necessarily straightforward. The qualitative ninhydrin test is useful for determining whether or not unreacted amino groups remain on the resin, although as the length of the peptide chain increases the test becomes less sensitive. Since during the course of a synthesis on a solid support the weight of the peptide-resin increases, this weight gain can, in principle, be used as a means for monitoring the reaction. The method has the advantage of simplicity and instances of its use for the monitoring of solid-phase segment coupling reactions are documented.^{406,430} However, it is often not a reliable or accurate guide to coupling yields. It should be used only as supporting evidence, when the coupling yield is determined using other techniques.

Amino acid analysis is widely used for determining the extent of segment coupling reactions but again, as the peptide attached to the solid support becomes longer, the information that amino acid analysis can provide is sometimes limited, since it can be difficult to judge the extent of incorporation of the new segment if it contains residues which are already present in the peptide sequence, especially if several of these residues are already present.

In the synthesis of ACTH by an *N* to *C* elongation strategy. Matsueda^{402,403} monitored the progress of the segment coupling steps by HPLC analysis of the solution of the peptide segment. Coupling was judged to be complete when no further uptake of the segment was observed.

Birr^{204,205} has described the synthesis of the mast cell degranulating peptide from bee venom, by a convergent solid-phase approach, as shown in Fig. 26. In this synthesis the Ddz group was used as the *N*^α protecting group for the protected peptide segments. The strong ultra-violet absorption of this group provides a means of monitoring the segment coupling reactions.

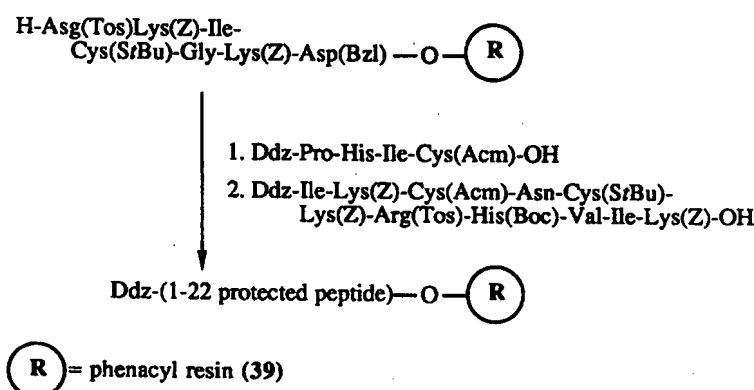


Fig. 26.

Coupling of Ddz-Ile-Lys(Z)-Cys(Acm)-Asn-Cys(*t*Bu)-Lys(Z)-Arg(Tos)-OH to the resin bound segment H-His(Boc)-Val-Ile-Lys(Z)-O-resin was achieved using DCC/HOBt and after three days the coupling was complete, as judged by the determination of the amount of Ddz group in the solution obtained after deprotection of this group with 5% TFA in DCM. This 1-11 protected peptide was detached from the resin by saponification and purified. The 12-15 protected segment Ddz-Pro-His-Ile-Cys(Acm)-OH was then coupled to the resin-bound 16-22 segment using DCC and HOBt. The coupling was carried out in DMF for three days, after which time the yield was estimated to be 73%. The purified Ddz-1-11 segment was coupled to the resin-bound 12-22 segment using a three-fold excess of peptide segment, DCC and HOBt in DMF solution for five days. The fully protected 1-22 sequence was released from the resin by treatment with NH_3 -saturated dioxan to give the desired *C*-terminal amide. Subsequent synthetic manipulations gave material with 35% of the activity of the natural peptide. Birr has also used this method for monitoring the coupling

reactions in the synthesis of insulin.^{448,449} The use of the Fmoc group as *N*^α protection also allows this type of monitoring of the coupling reaction to be used.

We have used solid-phase automatic sequencing to calculate the yields in solid-phase segment coupling reactions, by analysing aliquots of the different peptide-resins removed after each of the segment couplings. In the synthesis of the toxin-II from the scorpion *Androctonus australis*,⁴¹⁴ the results of the 'one segment previews' agreed very well with those obtained by amino acid analysis (all couplings were shown to have proceeded in 96–99% yield. Solid phase Edman sequencing is a very useful and accurate method for determining the coupling yields of segment couplings, particularly when amino acid analysis and ninhydrin tests do not give clear results.^{36,72,414}

3.8. Examples of convergent solid-phase peptide synthesis

3.8.1. *Rat atrial natriuretic factor*. The convergent solid-phase synthesis of rat atrial natriuretic factor has been described by the Merck, Sharpe and Dohme group.¹⁹⁷ This 26 residue peptide was synthesised by a segment coupling strategy on a polystyrene resin crosslinked with 1% *p*-divinylbenzene. The C-terminal peptide was synthesised on the resin by a stepwise procedure. The protected peptide segments Boc-Ala-Gln-Ser(Bzl)-Gly-OH, Boc-Arg(NO₂)-Ile-Gly-OH and Boc-Arg(NO₂)-Arg(NO₂)-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-Phe-Gly-Gly-OH were also synthesised on the same type of resin and were detached by transesterification using methanolic solutions of Et₃N,^{192,196} affording the methyl esters. These were purified by crystallisation, before saponification to give the corresponding peptide carboxylic acids. In addition to the protected peptide segments, single amino acids were also coupled to the peptide-resin in this synthesis, which is outlined in Fig. 27.

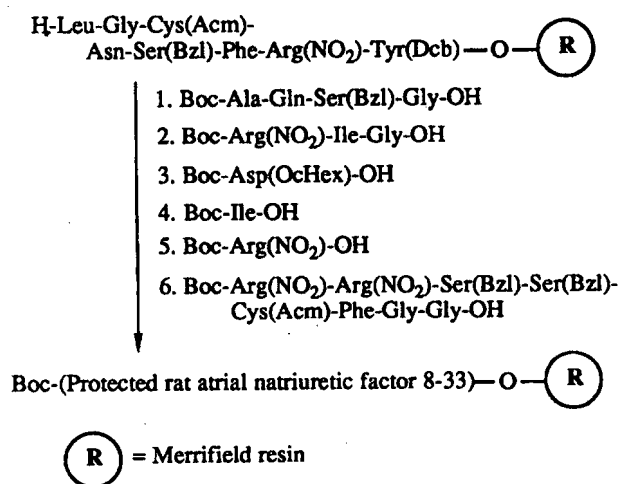


Fig. 27.

The coupling reactions were carried out using an excess of protected peptide segment, DCC and HOBt in DMF. Coupling times were between 18 and 24 h and coupling yields were judged to be very high by HPLC analysis of the peptide cleaved from the resin at several points during the synthesis. The coupling of the final octapeptide segment 6 was more problematical and required the use of *N*-hydroxysuccinimide and DCC in order to achieve acceptable coupling yields. The peptide resin was transformed by subsequent synthetic operations into the desired atrial natriuretic factor, which exhibited full biological activity. It is worthwhile emphasising the scale at which the synthesis was carried out; more than 10 g of the desired final peptide was obtained, illustrating that the convergent solid-phase synthesis approach is useful for the provision of multigram quantities of complex peptides.

3.8.2. *Prothymosin-α*. Prothymosin- α is the largest peptide which has been synthesised to date using a CSPPS strategy. Barlos¹¹¹ synthesised protected peptide segments spanning the 1–75 sequence of the protein by solid-phase techniques using the trityl resin (23). The C-terminal segment consisting of 34 amino acid residues and corresponding to the 76–109 sequence of the protein was synthesised on the resin (23) in a stepwise manner using the Fmoc/*t*Bu strategy. The trityl resin used had a functionalisation level of between 0.4 and 0.6 meq/g. The peptide segments were also synthesised on resin (23) and after cleavage and purification using chromatography on silica gel eluting with CHCl₃/MeOH, were coupled to the 76–109 peptide-resin, as shown in Fig. 28.

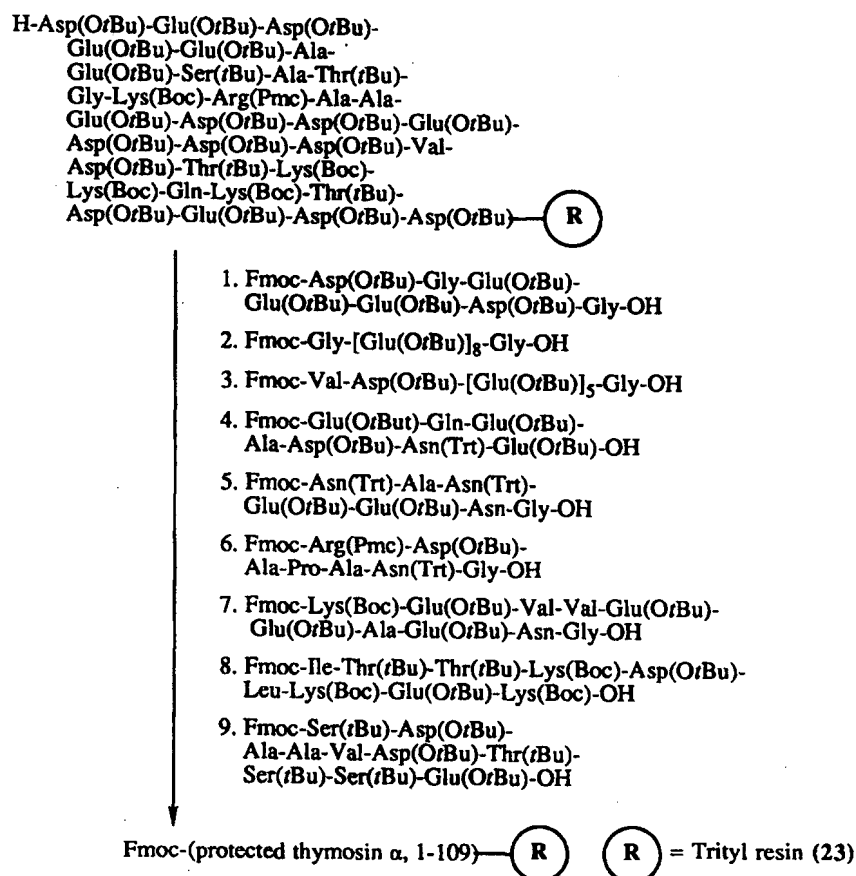


Fig. 28.

In the segment coupling steps, a five-fold excess of peptide segment/HOBt/DCC (1:1.5:1) in DMSO was used and the coupling reactions were complete after 6–18 h. Apart from segment 9, corresponding to the 1–10 sequence of the protein, which could only be coupled in 55% yield, all other segment couplings proceeded in very high yields as judged by HPLC analysis of material obtained from aliquots of the peptide resin taken throughout the synthesis, after removal of the peptide from the resin and subsequent removal of the protecting groups. Prothymosin α was obtained in 11% overall yield and the synthetic material had identical biological activity to the natural protein. This synthesis demonstrates that a CSPPS strategy can be used to synthesise peptides of over 100 amino acids in length.

3.8.3. *β -Amyloid protein*. Lansbury^{36,149–151} has described the synthesis of the β -amyloid protein

of Alzheimer's disease. The 1–17 sequence of the protein was first synthesised by segment coupling on the oxime resin (35) as shown in Fig. 29.

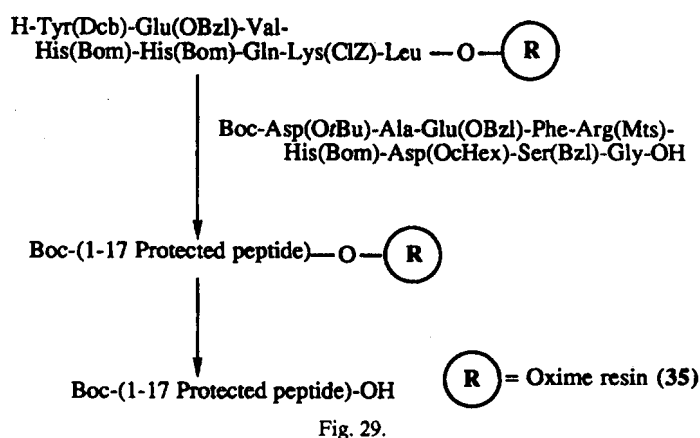


Fig. 29.

The C-terminal segment corresponding to the 10–17 sequence was synthesised on the oxime resin by a stepwise procedure. The 1–9 segment also synthesised on the oxime resin and was detached by treatment with HO-Pip followed by hydrolysis with Zn in AcOH, and purified by HPLC. Segment coupling was carried out using BOP reagent in DMF at 0–4°C for 14 h to afford the 1–17 sequence of the protein in a yield of 59%. The 1–17 segment was detached from the resin and used in the synthesis of the β A4-protein, outlined in Fig. 30.

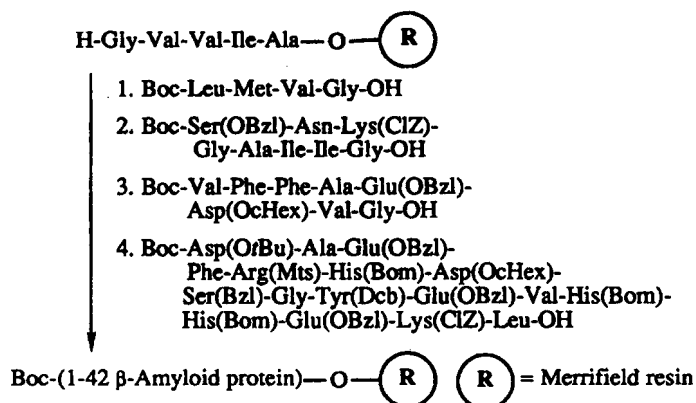


Fig. 30.

The synthesis was carried out on a standard Merrifield resin on which the C-terminal pentapeptide was synthesised in a stepwise manner. The requisite protected peptide segments 1, 2, and 3 all of which were synthesised on the resin (35) and detached using HO-Pip followed by treatment with Zn in AcOH, were then coupled sequentially to the peptide-resin. Each peptide segment was dissolved in DMF and coupled using BOP reagent and HOBT. The coupling yields for the segments were 95%, 70–90% and 80–90% respectively. In this way the 18–42 sequence of the peptide was built up on the solid support. The coupling of the 1–17 segment was somewhat more problematical. After deprotection of the Boc group and neutralisation of the peptide-resin, the 1–17 segment was

coupled in 50–85% yield by four successive room temperature coupling reactions, each using a progressively smaller amount of segment (a total of 2.3 equivalents). Model studies indicated that the amount of epimerisation in this last coupling step was ca. 8%. After treatment with HF and purification synthetic β -amyloid protein was obtained in an overall yield of 42%. This yield is lower than that (ca. 66%) that would be obtained in an efficient linear synthesis, assuming that a yield of 99.9% were possible in each coupling step, but since the material is devoid of single amino acid deletion impurities, the final purification is facilitated. This synthesis impressively demonstrates the power and potential of CSPPS.

OTHER CONVERGENT PEPTIDE SYNTHESIS STRATEGIES

Other convergent strategies for the synthesis of peptides are based upon the solid-phase synthesis of unprotected or of minimally protected peptide segments and their coupling in solution by chemical means^{101,102,450–453} or by using enzymes.^{454–457} We⁴⁵⁸ have reported on an example of the use of enzymes in the solid-phase coupling of protected peptide segments.

Blake⁴⁵⁹ has developed a useful segment coupling strategy, based on the coupling of minimally protected peptide segments using silver ion activation of the thiocarboxyl group. In this approach, a minimally protected peptide having a thiocarboxyl amino acid e.g. thiocarboxylglycine, as the C-terminal amino acid is synthesised on a solid-support. Boc-thiocarboxylglycine is first made to react with handle (61) in solution, forming the pre-formed handle⁵⁹ (62) which is then attached to a suitable resin, and peptide synthesis is carried out by standard procedures. The method is outlined in Fig. 31.

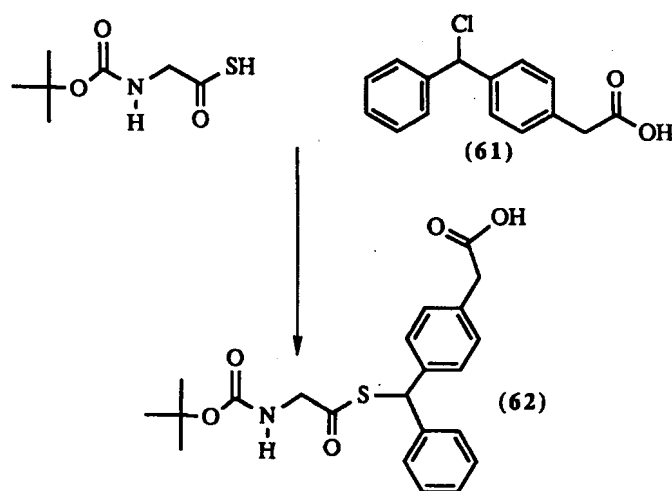


Fig. 31.

Once the desired sequence has been synthesised, the peptide is detached from the resin by HF treatment, which removes the majority of the protecting groups. The N^{α} -protecting group is chosen so as to withstand the conditions for the cleavage. After purification in aqueous medium, a process which is facilitated by the absence of protecting groups for the majority of the amino acid side-chains, the thiocarboxyl-terminal peptide is activated with silver ion and coupled with the free N^{α} group of another minimally protected peptide segment.

Blake synthesised the peptide segment (63) using the handle (61). This thiocarboxyl peptide was then coupled to the free peptide (64), synthesised on a standard Merrifield resin and detached with liquid HF. The coupling was carried out in aqueous solution, using silver nitrate to activate the

thiocarboxyl C-terminal, giving the peptide (65), as shown below. The coupling reaction was complete in 1 h at 40°C and (65) was obtained in 40% yield (Fig. 32).

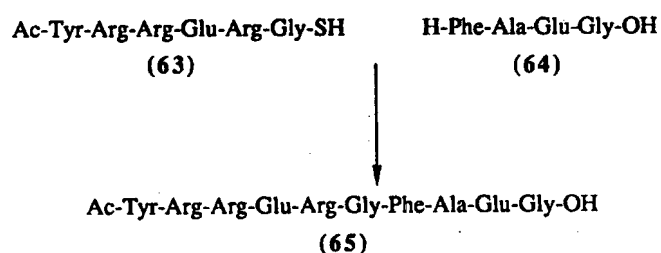


Fig. 32.

In order for this strategy to be generally applicable, all other amino groups apart from the one involved in the coupling reaction must be protected. Blake has used the Citr group for this purpose. Such a strategy has been applied to the synthesis of human β -endorphin.⁴⁶⁰ The 18–31 segment was synthesised on a Merrifield resin using the Boc/Bzl strategy. The last amino acid was incorporated as the Fmoc derivative so that in the cleavage step the N^α function remained protected. Cleavage of the peptide from the resin with HF removed all protecting groups except the Fmoc group. The peptide was then treated with Citr anhydride to protect the Lys N^ϵ amino group (Fig. 33).

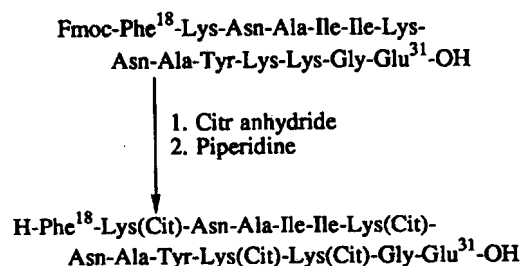


Fig. 33.

The 1–17 segment was synthesised on the solid support using the handle (61), as shown in Fig. 34.

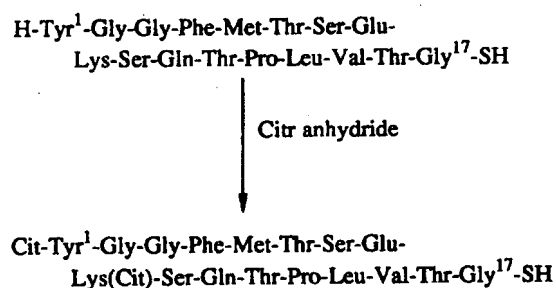


Fig. 34.

Detachment of this peptide from the resin gave the 1–17 segment with thiocarboxylglycine at the C-terminal. This peptide was treated with Citr anhydride to protect the N^α amino group and the N^ϵ amino group of the Lys at position 9. The two segments were then coupled by activation of the

thiocarboxyl group with silver nitrate, to give β -endorphin in 40% yield after purification. The same approach has been applied to the synthesis of human pancreatic growth hormone releasing factor,⁴⁶¹ to *S*-carbamoyl bovine apocytochrome C,⁴⁶² to α -inhibin-92,^{463,464} and to analogues of human β -endorphin.⁴⁶⁵

Aimoto⁴⁶⁶⁻⁴⁶⁸ has also reported on this type of approach in the synthesis of the DNA-binding domain of *c*-Myb protein, and of the DNA-binding protein of *Bacillus stearothermophilus*.

A variation on this procedure is to be found in Kent's⁴⁶⁹ synthesis of an HIV protease analogue. This analogue was synthesised in two halves. Both of these peptides were synthesised on a Merrifield resin, and detached with HF, removing all the protecting groups, which ensured excellent solubility of the peptide segments in aqueous medium. One of the peptides was synthesised with thiocarboxylglycine at the C-terminus and the other peptide had a bromoacetyl group at the N-terminus. After purification the two segments were joined by an S_N2 substitution reaction of the thiocarboxyl group at the bromoacetyl unit, giving the desired analogue.

Other examples of the synthesis of protein analogues by the chemical coupling of two unprotected peptide segments have been reported by Gaertner.⁴⁷⁰

ASSESSMENT AND PROSPECTS FOR THE FUTURE

From the quite extensive literature on CSPPS which has appeared over the last few years, it would perhaps be easy to conclude that the synthesis of large peptides and proteins by the solid-phase coupling of protected peptide segments is a well-established and relatively problem-free procedure. While it is true that certain problems, which only a decade or so ago were considered to be insurmountable, have been solved thanks to the considerable efforts of a number of research groups, CSPPS is still far from being a general method for the synthesis of peptide molecules of over 50 amino acid residues.

The aspect of CSPPS which has been most successfully addressed to date is the synthesis of protected peptide segments. Efficient methods for the solid-phase synthesis of protected peptide segments with different N^α - and side-chain-protecting groups now exist, although work will continue in this area in order to improve yields, to develop even milder methods for the cleavage of the peptide from the support, and above all to improve the level of compatibility between the different types of protecting groups that must be used.

However there are other fundamental issues in CSPPS which require much more research in order to arrive at workable solutions to the problems.

1. The risk of epimerisation of the protected peptide segments during the coupling reactions makes it desirable to have either Gly or Pro be at the C-terminal of the peptide. However, many naturally-occurring peptides are deficient in these residues or have an irregular distribution of them such that if the protected peptide segments are not to be excessively long, it will not be possible to have either Pro or Gly at the C-terminal. There is therefore a need to investigate new methods for the formation of amide bonds, compatible with the main protecting group strategies used in SPPS, which are epimerisation-free whatever the amino acid residue at the C-terminal.

Many of the solid-phase segment couplings described in the literature, in which the C-terminal residue is not Gly or Pro, do not indicate how or even if, the question of epimerisation was addressed. This gives a false sense of security, leading one to think that perhaps the problem is not significant or even does not exist. We believe that gas chromatography on chiral columns, coupled to mass spectrometry and using DCI instead of HCl for hydrolyses of peptide resins is the most general method⁴⁷¹ available for determining the chiral homogeneity of a peptide prepared in a convergent synthesis.

2. In this review we have drawn attention to several syntheses in which investigators had to

depart from their original synthetic plan because the coupling yield for one or other of the initially chosen segments was too low or sometimes was even zero. Very little is known of the causes of these low coupling yields which occur for a small but significant number of peptide sequences. This field is open for physicochemical research to attempt to establish the types of effects (conformation of the molecule in solution, or when attached to the polymer support, interchain associations, etc.) which are operating in each case. Understanding the origin of the phenomenon is probably the best way to attempt to solve the synthetic problem.

3. The poor solubility of many protected peptide segments is another severe limitation of CSPPS. Here again there is an opportunity for physicochemical study of the three-dimensional structure of protected peptide molecules in the polar aprotic solvents which are normally used for segment couplings. This might allow the poor solubility of certain peptide segments to be predicted without having to begin the synthetic work, allowing the synthesis to be designed appropriately. Another approach is to work with protection strategies which minimise the problem of poor solubility, by using protecting groups (for the *N*^α-terminal or for the side-chains) which modify the physicochemical properties of protected peptide segments in such a way that their purification and coupling may be carried out efficiently.

We believe that in its present state of development, CSPPS is a viable alternative for the synthesis of many medium-sized peptides. Furthermore, it is a magnificent field for basic research into peptide synthesis and we believe it should permit, in the foreseeable future, the synthesis of highly pure proteins consisting of 100, 200 or even 300 amino acid residues.

Acknowledgements—We thank Drs A. Grandas, E. Pedrosa and J. van Rietschoten for their contributions to the work carried out in our group on CSPPS and for their advice during the preparation of the manuscript. We are grateful to those colleagues who supplied us with information on their latest results in the field. Much of our work on CSPPS was carried out with funding from DGICYT, grant no PB 89-257.

REFERENCES AND NOTES

1. Kent, S. B. H. *Ann. Rev. Biochem.* **1988**, *57*, 957–989.
2. Bayer, E. *Angew. Chem., Int. Edn. Engl.* **1991**, *30*, 113–129.
3. Abbreviations used in this review for amino acids and for the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *138*, 9–37 and *J. Biol. Chem.* **1989**, *264*, 633–673. The following additional abbreviations are used: Ac, acetyl; Acn, acetamidomethyl; ACTH, adrenocorticotropin; Adoc, 1-(1-adamantyl)-1-methylethoxycarbonyl; Aib, α-aminoisobutyric acid; Asu, α-aminosuberic acid; BHA, benzhydramine (resin); Boc, *tert*-butoxycarbonyl; Bom, benzyloxymethyl; BOP, benzotriazol-1-yl *N*-oxytrisdimethylaminophosphonium hexafluorophosphate; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; Bpoc, 2-(*p*-biphenyl)-propyl(2)oxycarbonyl; BPTI, bovine pancreatic basic trypsin inhibitor; BrZ, 2-bromobenzyloxycarbonyl; *t*Bu, *tert*-butyl; Bum, *tert*-butoxymethyl; Bzl, benzyl; CDI, *N,N'*-carbonyldiimidazole; cHex, cyclohexyl; Cit, citrulline; Citr, citraconyl; CSPPS, convergent solid-phase peptide synthesis; ClZ, 2-chlorobenzyloxycarbonyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; Dcb, 2,6-dichlorobenzyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; Ddz, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl; Dha, dehydroalanine; DIEA, *N,N'*-diisopropylethylamine; DKP, diketopiperazine; DMAE, dimethylaminoethanol; DMF, *N,N*-dimethylformamide; Dmob, dimethoxybenzyl; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; Dnp, 2,4-dinitrophenyl; DPD, 2,2'-dipyridyl disulfide; Dpp, diphenylphosphinamide; Dts, dithiasuccinoyl; EACNOx, ethyl 2-(hydroxyimino)-2-cyanoacetate; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; FAB, fast atom bombardment; Fm, 9-fluorenyl; Fmoc, 9-fluorenylmethoxycarbonyl; For, formyl; HAL, hyper acid labile, [5-(4-hydroxymethyl-3,5-dimethoxyphenoxy)valeric acid]; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; HIV, human immunodeficiency virus; HMPA, hexamethylphosphorotriamide; HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid; HOBt, 1-hydroxybenzotriazole; HOObt, 1-oxo-2-hydroxydihydrobenzotriazine; HO-Pip, 1-hydroxypiperidine; HOSu, *N*-hydroxysuccinimide; HPLC, high performance liquid chromatography; *i*Pr, *iso*-propyl; LHRH, luteinizing hormone-releasing hormone (luliberin); Mbh, 4,4'-dimethoxybenzhydrazyl; MBHA, *p*-methylbenzhydramine (resin); MeBzl, *p*-methylbenzyl; MPLC, medium pressure liquid chromatography; Msc, 2-(methylsulfonyl)ethoxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Mts, mesitylene sulfonyl; Nbb, nitrobenzamido benzyl; NBS, *N*-bromosuccinimide; NMM, *N*-methylmorpholine; NMP, *N*-methyl-2-pyrrolidinone; NMR, nuclear magnetic resonance; NPE, 2-(2-nitrophenyl)ethyl; Nps, *o*-nitrophenylsulfenyl; Npys, 3-nitro-2-pyridinesulfenyl; OMPA, oxymethylphenyl acetic acid; PAL, peptide amide linker, [5-(4-(9-fluorenylmethoxycarbonyl)aminomethyl-3,5-dimethoxy)-valeric acid]; Pam, phenylacetamidomethyl; PEG, polyethyleneglycol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; SASRIN, super acid sensitive resin, (2-methoxy-4-alkoxybenzylalcohol-resin); SPPS, solid-phase peptide synthesis; Sulfmoc, 9-(2-sulfo)fluorenylmethoxycarbonyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetra-

methyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Tfa, trifluoroacetyl; TFE, 2,2,2-trifluoroethanol; THF, tetrahydrofuran; TMG, tetramethylguanidine; Tos, *p*-toluenesulfonyl; Trt, triphenylmethyl (trityl); Z, benzyloxy-carbonyl. The amino acid symbols used denote the L configuration unless otherwise indicated.

4. Smith, M. *Ann. Rev. Genet.* **1985**, *19*, 423–462.
5. Carter, P. *Biochem. J.* **1986**, *237*, 1–7.
6. Wells, J. A.; Vasser, M.; Powers, D. B. *Gene* **1985**, *34*, 315–323.
7. Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182–188.
8. Bain, J. D.; Diala, E. S.; Glabe, C. G.; Wacker, D. A.; Lyttle, M. H.; Dix, T. A.; Chamberlain, A. R. *Biochemistry* **1991**, *30*, 5411–5421.
9. Mendel, D.; Ellman, J. A.; Chang, Z.; Veenstra, D. L.; Kollman, P. A.; Schultz, P. G. *Science* **1992**, *256*, 1798–1802.
10. Dutta, A. S. *Adv. Drug Res.* **1991**, *21*, 145–286.
11. See Niu, C. I. In *Perspectives in Peptide Chemistry*; Eberle, A.; Geiger, R.; Wieland, T. Eds.; Karger, Basel, 1981, pp. 45–57.
12. Fischer, E.; Fournau, E. *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 2868–2877. See also *Nobel Lectures—Chemistry 1901–1921*, Elsevier, Amsterdam, 1966, p. 34.
13. Meienhofer, J.; Schnabel, E.; Bremer, H.; Brinkoff, O.; Zabel, R.; Sroka, W.; Klostermeyer, H.; Brandenburg, D.; Okuda, T.; Zahn, H. *Z. Naturforsch.* **1963**, *18b*, 1120–1121.
14. Katsoyannis, P. G.; Fukuda, K.; Tometsko, A.; Suzuki, K.; Tilak, M. *J. Am. Chem. Soc.* **1964**, *86*, 930–932.
15. Kung, Y.-T.; Du, Y.-C.; Huang, W.-T.; Chen, C.-C.; Ke, L.-T.; Hu, S.-C.; Jiang, R.-Q.; Chu, S.-Q.; Niu, C.-I.; Hsu, J.-Z.; Chang, W.-C.; Cheng, L.-L.; Li, H.-S.; Wang, Y.; Loh, T.-P.; Chi, A.-H.; Li, C.-H.; Shi, P.-T.; Yieh, Y.-H.; Tang, K.-L.; Hsing, C.-Y. *Scientia Sinica* **1965**, *14*, 1710–1716.
16. Sieber, P.; Kamber, B.; Hartmann, A.; Jöhl, A.; Riniker, B.; Rittel, W. *Helv. Chim. Acta* **1974**, *57*, 2617–2621.
17. Yajima, H.; Fujii, N. *Biopolymers* **1981**, *20*, 1859–1867.
18. Yajima, H.; Fujii, N. *J. Chem. Soc., Chem. Commun.* **1980**, 115–116.
19. For leading references see Galpin, I. J.; Hancock, F. E.; Handa, B. K.; Jackson, A. G.; Kenner, G. W.; McDowell, P.; Noble, P.; Ramage, R. *Tetrahedron* **1981**, *37*, 3043–3050.
20. See for example Pettit, G. R.; Singh, S. B.; Hogan, F.; Lloyd-Williams, P.; Herald, D. L.; Burkett, D. D.; Clewlow, P. J. *J. Am. Chem. Soc.* **1989**, *111*, 5463–5465.
21. See for example, Blout, E. R. *Biopolymers* **1981**, *20*, 1901–1912.
22. See for example Rinehart, K. L.; Gloer, J. B.; Hughes, R. G.; Renis, H. E.; McGovern, J. P.; Swynenberg, E. B.; Stringfellow, D. A.; Kuentzel, S. L.; Li, L. H. *Science* **1981**, *212*, 933–935.
23. See for example Ewing, W. R.; Harris, B. D.; Bhat, K. L.; Joulié, M. M. *Tetrahedron* **1986**, *42*, 2421–2428.
24. See for example Inouye, K.; Watanabe, K.; Tochino, Y.; Kobayashi, M.; Shigeta, Y. *Biopolymers* **1981**, *20*, 1845–1858.
25. Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
26. Merrifield, R. B. *Science* **1965**, *150*, 178–185.
27. Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E.; Meienhofer, J. Eds.; Academic Press, New York, 1979; Vol. 2, pp. 1–284.
28. Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. *Int. J. Peptide Protein Res.* **1987**, *30*, 705–739.
29. Letsinger, R. L.; Kornet, M. J. *J. Am. Chem. Soc.* **1963**, *85*, 3045–3046.
30. Bayer, E.; Eckstein, H.; Hägele, K.; König, W. A.; Brüning, W.; Hagenmaier, H.; Parr, W. *J. Am. Chem. Soc.* **1970**, *92*, 1735–1738.
31. See Ref. 1, pp. 969–971. Also see Kent, S. B. H. In *Peptides. Structure and Function. Proceedings of the 9th American Peptides Symposium*; Deber, C. M.; Hruby, V. J.; Kopple, K. D. Eds.; Pierce Chemical Co., Rockford, Illinois, 1985, pp. 407–414.
32. Bedford, J.; Hyde, C.; Johnson, T.; Jun, W.; Owen, D.; Quibell, M.; Sheppard, R. C. *Int. J. Peptide Protein Res.* **1992**, *40*, 300–307.
33. Barrow, C. J.; Zagorski, M. G. *Science* **1991**, *253*, 179–182.
34. Hilbich, C.; Kisters-Woike, B.; Reed, J.; Masters, C. L.; Beyreuther, K. *J. Mol. Biol.* **1991**, *218*, 149–163.
35. Burdick, D.; Soreghan, B.; Kwon, M.; Kosmoski, J.; Knauer, M.; Henschen, A.; Yates, J.; Cotman, C.; Glabe, C. *J. Biol. Chem.* **1992**, *267*, 546–554.
36. Hendrix, J. C.; Halverson, K. J.; Lansbury, P. T. *J. Am. Chem. Soc.* **1992**, *114*, 7930–7931.
37. Milton, R. C. de L.; Milton, S. C. F.; Kent, S. B. H. *Science* **1992**, *256*, 1445–1448.
38. Miller, M.; Schneider, J.; Sathyanarayana, B. K.; Toth, M. V.; Marshall, G. R.; Clawson, L.; Selk, L.; Kent, S. B. H.; Wlodawer, A. *Science* **1989**, *246*, 1149–1152.
39. Wlodawer, A.; Miller, M.; Jaskólski, M.; Sathyanarayana, B. K.; Baldwin, E.; Weber, I. T.; Selk, L. M.; Clawson, L.; Schneider, J.; Kent, S. B. H. *Science* **1989**, *245*, 616–621.
40. Clark-Lewis, I.; Aebersold, R.; Ziltener, H.; Schrader, J. W.; Hood, L. E.; Kent, S. B. H. *Science* **1986**, *231*, 134–139.
41. Jones, J. H.; Ridge, B. In *Amino acids, Peptides and Proteins*; Young, D. Ed.; Specialist Periodical Reports, The Chemical Society, London, 1972, Vol. 4, p. 379.
42. Sakakibara, S.; Shimonishi, Y.; Kishida, Y.; Okada, M.; Sugihara, H. *Bull. Chem. Soc. Japan* **1967**, *40*, 2164–2167.
43. Tam, J. P.; Heath, W. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1983**, *105*, 6442–6455, and refs. therein.
44. See for example *The Formation of Disulfide Bonds in Synthetic Peptides and Proteins*; Andreu, D.; Albericio, F.; Solé, N.; Munson, M.; Ferrer, M.; Barany, G. In *Peptide Synthesis and Purification Protocols*; Pennington, M. W.; Dunn, B. M. Eds.; Humana Press, Clifton, NJ, in press.

45. Carpino, L. A. *J. Am. Chem. Soc.* **1957**, *79*, 4427–4431.
46. McKay, F. C.; Albertson, N. F. *J. Am. Chem. Soc.* **1957**, *79*, 4686–4690.
47. Anderson, G. W.; McGregor, A. C. *J. Am. Chem. Soc.* **1957**, *79*, 6180–6183.
48. Carpino, L. A.; Han, G. A. *J. Org. Chem.* **1972**, *37*, 3404–3409.
49. Sieber, P.; Iselin, B. *Helv. Chim. Acta* **1968**, *51*, 614–622.
50. Sieber, P.; Iselin, B. *Helv. Chim. Acta* **1968**, *51*, 622–632.
51. Barany, G.; Merrifield, R. B. *J. Am. Chem. Soc.* **1977**, *99*, 7363–7365.
52. Bergmann, M.; Zervas, L. *Ber. Dtsch Chem. Ges.* **1932**, *65*, 1192–1201.
53. See *The Peptides*; Gross, E.; Meienhofer, J. Eds; Academic Press, New York, 1981, Volume 3.
54. For a review of photolabile protecting groups in organic synthesis see Pillai, V. N. R. *Synthesis* **1980**, 1–26.
55. Rich, D. H.; Gurwara, S. K. *J. Chem. Soc., Chem. Commun.* **1973**, 610–611.
56. Rich, D. H.; Gurwara, S. K. *J. Am. Chem. Soc.* **1975**, *97*, 1575–1579.
57. See Ref. 27, p. 57.
58. The situation is further complicated by the use of the term “handle” to describe a protecting group which aids the purification of peptides. See for example Refs. 373 and 374.
59. Barany, G.; Albericio, F. *J. Am. Chem. Soc.* **1985**, *107*, 4936–4942.
60. Giralt, E.; Albericio, F.; Andreu, D.; Eritja, R.; Martin, P.; Pedrosa, E. *An. Quim.* **1981**, *77C*, 120–125.
61. Gisin, B. F. *Helv. Chim. Acta* **1973**, *56*, 1476–1482.
62. Giralt, E.; Albericio, F.; Pedrosa, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1982**, *38*, 1193–1208.
63. Albericio, F.; Granier, C.; Labbe-Jullie, C.; Seagar, M.; Couraud, F.; van Rietschoten, J. *Tetrahedron* **1984**, *40*, 4313–4326.
64. Giralt, E.; Eritja, R.; Pedrosa, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1986**, *42*, 691–698.
65. Albericio, F.; Nicolás, E.; Josa, J.; Grandas, A.; Pedrosa, E.; Giralt, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1987**, *43*, 5961–5971.
66. Morize, I.; Surcouf, E.; Vaney, M. C.; Epelboin, Y.; Buehner, M.; Fridlansky, F.; Milgrom, E.; Mornon, J. P. *J. Mol. Biol.* **1987**, *194*, 725–739.
67. Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Int. J. Peptide Protein Res.* **1991**, *37*, 58–60.
68. Lloyd-Williams, P.; Gairi, M.; Albericio, F.; Giralt, E. *Tetrahedron* **1991**, *47*, 9867–9880.
69. Lloyd-Williams, P.; Gairi, M.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, in press.
70. Bayer, E.; Dengler, M.; Hemmasi, B. *Int. J. Peptide Protein Res.* **1985**, *25*, 178–186.
71. Pillai, V. N. R.; Renil, M.; Haridasan, V. K. *Indian J. Chem.* **1991**, *30B*, 205–212.
72. Kneib-Cordonier, N.; Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1990**, *35*, 527–538.
73. Voss, C.; DiMarchi, R.; Whitney, D. B.; Tjoeng, F. S.; Merrifield, R. B.; Tam, J. P. *Int. J. Peptide Protein Res.* **1983**, *22*, 204–213.
74. Giralt, E.; Eritja, R.; Pedrosa, E. *Tetrahedron Lett.* **1981**, *22*, 3779–3782.
75. Pedrosa, E.; Grandas, A.; de las Heras, X.; Eritja, R.; Giralt, E. *Tetrahedron Lett.* **1986**, *27*, 743–746.
76. Suzuki, K.; Nitta, K.; Endo, N. *Chem. Pharm. Bull.* **1975**, *23*, 222–224.
77. Gairi, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **1990**, *31*, 7363–7366.
78. Patchornik, A.; Amit, B.; Woodward, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 6333–6335.
79. Ajayaghosh, A.; Pillai, V. N. R. *Tetrahedron* **1988**, *44*, 6661–6666.
80. Ajayaghosh, A.; Pillai, V. N. R. *J. Org. Chem.* **1987**, *52*, 5714–5717.
81. Wang, S. S. *J. Org. Chem.* **1976**, *41*, 3258–3261.
82. Sheehan, J. C.; Umezawa, K. *J. Org. Chem.* **1973**, *38*, 3771–3774.
83. Tjoeng, F. S.; Tam, J. P.; Merrifield, R. B. *Int. J. Peptide Protein Res.* **1979**, *14*, 262–274.
84. Tjoeng, F. S.; Heavner, G. A. *J. Org. Chem.* **1983**, *48*, 355–359.
85. Bellof, D.; Mutter, M. *Chimia* **1985**, *39*, 317–320.
86. Abraham, N. A.; Fazal, G.; Ferland, J. M.; Rakhit, S.; Gauthier, J. *Tetrahedron Lett.* **1991**, *32*, 577–580.
87. Wang, S. S. *J. Am. Chem. Soc.* **1973**, *95*, 1328–1333.
88. Wang, S. S.; Kulesha, I. D. *J. Org. Chem.* **1975**, *40*, 1227–1234.
89. Chang, C. D.; Felix, A. M.; Jimenez, M. H.; Meienhofer, J. *Int. J. Peptide Protein Res.* **1980**, *15*, 485–494.
90. Pedrosa, E.; Grandas, A.; Saralegui, M. A.; Giralt, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1982**, *38*, 1183–1192.
91. Grandas, A.; Pedrosa, E.; Giralt, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1986**, *42*, 6703–6711.
92. Sabatier, J.-M.; Tessier-Rochat, M.; Granier, C.; van Rietschoten, J.; Pedrosa, E.; Grandas, A.; Albericio, F.; Giralt, E. *Tetrahedron* **1987**, *43*, 5973–5980.
93. Nokihara, K.; Hellstern, H.; Höfle, G. In *Peptides. Chemistry, Structure and Biology. Proceedings of the 11th American Peptides Symposium*; Rivier, J. E.; Marshall, G. R. Eds; ESCOM, Leiden, 1990, pp. 1046–1048.
94. Sheppard, R. C.; Williams, B. J. *Int. J. Peptide Protein Res.* **1982**, *20*, 451–454.
95. Sheppard, R. C.; Williams, B. J. *J. Chem. Soc., Chem. Commun.* **1982**, 587–589.
96. Mergler, M.; Tanner, R.; Gosteli, J.; Grogg, P. *Tetrahedron Lett.* **1988**, *29*, 4005–4008.
97. Mergler, M.; Nyfeler, R.; Tanner, R.; Gosteli, J.; Grogg, P. *Tetrahedron Lett.* **1988**, *29*, 4009–4012.
98. Surovoy, A.; Metzger, J.; Jung, G. In *Innovation and Perspectives in Solid-Phase Synthesis: Peptides, Polypeptides and Oligonucleotides*; Epton, R. Ed; Intercept, Andover, (UK), 1992, pp. 467–473.
99. Surovoy, A.; Metzger, J.; Jung, G. In *Chemistry of Peptides and Proteins*; Brandenburg, D.; Ivanov, V.; Voelter, W. Eds; Attempto Verlag, Tübingen, in press.
100. Lünig, B.; Norberg, T.; Tejbrant, J. *J. Chem. Soc., Chem. Commun.* **1989**, 1267–1268.

101. Flörsheimer, A.; Riniker, B. In *Peptides 1990. Proceedings of the 21st European Peptides Symposium*; Giralt, E.; Andreu, D. Eds; ESCOM, Leiden, 1991, 131–133.
102. Kamber, B.; Riniker, B. In *Peptides. Chemistry and Biology. Proceedings of the 12th American Peptides Symposium*; Smith, J. A.; Rivier, J. E. Eds; ESCOM, Leiden, 1992, pp. 525–526.
103. Riniker, B.; Flörsheimer, A.; Fretz, H.; Kamber, B. In *Peptides 1992. Proceedings of the 22nd European Peptides Symposium*; Schneider, C. H.; Eberle, A. N. Eds; ESCOM, Leiden, in press.
104. Albericio, F.; Barany, G. *Tetrahedron Lett.* **1991**, 32, 1015–1018.
105. Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1987**, 30, 206–216.
106. Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, 55, 3730–3743.
107. Rink, H. *Tetrahedron Lett.* **1987**, 28, 3787–3790.
108. Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Wenqing, Y.; Schäfer, W. *Tetrahedron Lett.* **1989**, 30, 3943–3946.
109. Barlos, K.; Gatos, D.; Kaposos, S.; Papaphotiu, G.; Schäfer, W.; Wenqing, Y. *Tetrahedron Lett.* **1989**, 30, 3947–3950.
110. Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. *Int. J. Peptide Protein Res.* **1991**, 37, 513–520.
111. Barlos, K.; Gatos, D.; Schäfer, W. *Angew. Chem., Int. Edn. Engl.* **1991**, 30, 590–593.
112. van Vliet, A.; Smulders, R. H. P. H.; Rietman, B. H.; Tesser, G. I. In *Innovations and Perspectives in Solid-Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides*; Epton, R. Ed; Intercept, Andover (UK), 1992, pp. 475–477.
113. van Vliet, A.; Smulders, R. H. P. H.; Rietman, B. H.; Eggen, I. F.; van der Werken, G.; Tesser, G. I. In *Peptides 1992. Proceedings of the 22nd European Peptides Symposium*; Schneider, C. H.; Eberle, A. N. Eds; ESCOM, Leiden, 1993, pp. 279–280.
114. Wang, S. S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1969**, 91, 6488–6491.
115. Wang, S. S.; Merrifield, R. B. *Int. J. Peptide Protein Res.* **1972**, 4, 309–318.
116. Akaji, K.; Kiso, Y.; Carpino, L. A. *J. Chem. Soc., Chem. Commun.* **1990**, 584–586.
117. Wolters, E. T. M.; Tesser, G. I.; Nivard, R. J. F. *J. Org. Chem.* **1974**, 39, 3388–3392.
118. Schielen, W. J. G.; Adams, H. P. H. M.; Nieuwenhuizen, W.; Tesser, G. I. *Int. J. Peptide Protein Res.* **1991**, 37, 341–346.
119. Wang, S. S. *J. Org. Chem.* **1975**, 40, 1235–1239.
120. Mutter, M.; Bellhof, D. *Helv. Chim. Acta* **1984**, 67, 2009–2016.
121. Tjoeng, F. S.; Zupec, M. E.; Eubanks, S. R.; Adams, S. P. In *Peptides. Structure and Function. Proceedings of the 9th American Peptides Symposium*; Deber, C. M.; Hruby, V. J.; Kopple, K. D. Eds; Pierce Chemical Co., Rockford, Illinois, 1985, pp. 265–268.
122. Liu, Y.-Z.; Ding, S.-H.; Chu, J.-Y.; Felix, A. M. *Int. J. Peptide Protein Res.* **1990**, 35, 95–98.
123. Bláha, I.; Nemeš, J.; Tözsér, J.; Oroszlán, S. *Int. J. Peptide Protein Res.* **1991**, 38, 453–458.
124. Rabanal, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1992**, 33, 1775–1778.
125. Eritja, R.; Robles, J.; Fernandez-Fornier, D.; Albericio, F.; Giralt, E.; Pedroso, E. *Tetrahedron Lett.* **1991**, 32, 1511–1514.
126. Albericio, F.; Eritja, R.; Giralt, E. *Tetrahedron Lett.* **1991**, 32, 1515–1518.
127. Gairi, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E., unpublished work.
128. Tesser, G. I.; Buis, J. T. W. A. R. M.; Wolters, E. T. M.; Bothé-Helmes, E. G. A. M. *Tetrahedron* **1976**, 32, 1069–1072.
129. Buis, J. T. W. A. R. M.; Tesser, G. I.; Nivard, R. J. F. *Tetrahedron* **1976**, 32, 2321–2325.
130. Schwyzler, R.; Felder, E.; Failli, P. *Helv. Chim. Acta* **1984**, 67, 1316–1327.
131. Katti, S. B.; Misra, P. K.; Haq, W.; Mathur, K. B. *J. Chem. Soc., Chem. Commun.* **1992**, 843–844.
132. Kaiser, E. T.; Mihara, H.; Laforet, G. A.; Kelly, J. W.; Walters, L.; Findeis, F. A.; Sasaki, T. *Science* **1989**, 243, 187–192.
133. Kaiser, E. T. *Acc. Chem. Res.* **1989**, 22, 47–54.
134. DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, 45, 1295–1300.
135. Losse, G.; Barth, A.; Schatz, K. *Justus Liebigs Ann. Chem.* **1964**, 677, 185–190.
136. Losse, G.; Hoffman, K.-H.; Hetzer, G. *Justus Liebigs Ann. Chem.* **1965**, 684, 236–242.
137. Scarr, R. B.; Findeis, M. A. *Peptide Res.* **1990**, 3, 238–241.
138. DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, 47, 3258–3261.
139. Nakagawa, S. H.; Kaiser, E. T. *J. Org. Chem.* **1983**, 48, 678–685.
140. Nakagawa, S. H.; Lau, H. S. H.; Kézdy, F. J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1985**, 107, 7087–7092.
141. Kroon, D. J.; Kaiser, E. T. *J. Org. Chem.* **1978**, 43, 2107–2113.
142. Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1989**, 111, 380–381.
143. Sasaki, T.; Kaiser, E. T. *Biopolymers* **1990**, 29, 79–88.
144. Mihara, H.; Kaiser, E. T. *Science* **1988**, 242, 925–927.
145. Sasaki, T.; Findeis, M. A.; Kaiser, E. T. *J. Org. Chem.* **1991**, 56, 3159–3168.
146. Findeis, M. A.; Kaiser, E. T. *J. Org. Chem.* **1989**, 54, 3478–3482.
147. Atherton, E.; Clive, D. L. J.; Sheppard, R. C. *J. Am. Chem. Soc.* **1975**, 97, 6584–6585.
148. See Atherton, E.; Gait, M. J.; Sheppard, R. C.; Williams, B. J. *Bioorganic Chem.* **1979**, 8, 351–370 and Atherton, E.; Sheppard, R. C. In *Solid Phase Peptide Synthesis. A Practical Approach*; Rickwood, D.; Hames, B. D. Eds.; IRL Press, Oxford, UK, 1989.

149. Lansbury, P. T.; Hendrix, J. C.; Coffman, A. I. *Tetrahedron Lett.* **1989**, 30, 4915–4918.
150. Hendrix, J. C.; Jarrett, J. T.; Anisfeld, S. T.; Lansbury, P. T. *J. Org. Chem.* **1992**, 57, 3414–3420.
151. Hendrix, J. C.; Lansbury, P. T. *J. Org. Chem.* **1992**, 57, 3421–3426.
152. Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, 1219–1222.
153. Jarrett, J. T.; Lansbury, P. T. *Tetrahedron Lett.* **1990**, 31, 4561–4564.
154. Ösapay, G.; Taylor, J. W. *J. Am. Chem. Soc.* **1990**, 112, 6046–6051.
155. Ösapay, G.; Taylor, J. W. *J. Am. Chem. Soc.* **1992**, 114, 6966–6973.
156. Ösapay, G.; Profit, A.; Taylor, J. W. *Tetrahedron Lett.* **1990**, 31, 6121–6124.
157. Kaiser, E. T.; Kézdy, F. J. *Science* **1984**, 223, 249–255.
158. Taylor, J. W.; Ösapay, G. *Acc. Chem. Res.* **1990**, 23, 338–344.
159. Bouvier, M.; Taylor, J. W. *J. Med. Chem.* **1992**, 35, 1145–1155.
160. Xu, M.; Nishino, N.; Mihara, H.; Fujimoto, T.; Izumiya, N. *Chem. Lett.* **1992**, 191–194.
161. Mullen, D. G.; Barany, G. *Tetrahedron Lett.* **1987**, 28, 491–494.
162. Mullen, D. G.; Barany, G. *J. Org. Chem.* **1988**, 53, 5240–5248.
163. Ramage, R.; Barron, C. A.; Bielecki, S.; Thomas, D. W. *Tetrahedron Lett.* **1987**, 28, 4105–4108.
164. Ramage, R.; Barron, C. A.; Bielecki, S.; Holden, R.; Thomas, D. W. *Tetrahedron* **1992**, 48, 499–514.
165. Ueki, M.; Amemiya, M. *Tetrahedron Lett.* **1987**, 28, 6617–6620.
166. Ramage, R.; Atrash, B.; Hopton, D.; Parrot, M. J. *J. Chem. Soc., Perkin Trans. I* **1985**, 1217–1226.
167. Ueki, M.; Kai, K.; Amemiya, M.; Horino, H.; Oyamada, H. *J. Chem. Soc., Chem. Commun.* **1988**, 414–415.
168. Mitchell, A. R.; Erickson, B. W.; Ryabtsev, M. N.; Hodges, R. S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1976**, 98, 7357–7362.
169. Mitchell, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, 43, 2845–2852.
170. Baleux, F.; Daunis, J.; Jaquier, R. *Tetrahedron Lett.* **1984**, 25, 5893–5896.
171. Calas, B.; Mery, J.; Parello, J.; Cave, A. *Tetrahedron* **1985**, 41, 5331–5339.
172. Baleux, F.; Calas, B.; Mery, J. *Int. J. Peptide Protein Res.* **1986**, 28, 22–28.
173. Bodansky, M.; Sheehan, J. T. *Chem. Ind. (London)* **1964**, 1423–1424.
174. Wright, D. E.; Agarwal, N. S.; Hruby, V. J. *Int. J. Peptide Protein Res.* **1980**, 15, 271–278.
175. Niu, C.-I.; Yang, S.-Z.; Ma, A.-Q.; Chen, Y.-N.; Jiang, Y.-Q.; Huang, W.-T. *Biopolymers* **1981**, 20, 1833–1843.
176. Blake, J.; Li, C. H. *Int. J. Peptide Protein Res.* **1971**, 3, 185–189.
177. Kessler, W.; Iselin, B. *Helv. Chim. Acta* **1966**, 49, 1330–1344.
178. Ohno, M.; Anfinsen, C. B. *J. Am. Chem. Soc.* **1967**, 89, 5994–5995.
179. Visser, S.; Roeloffs, J.; Kerling, K. E. T.; Havinga, E. *Recl. Trav. Chim. Pays-Bas* **1968**, 87, 559–571.
180. Murakami, Y.; Nakano, A.; Matsumoto, K.; Iwamoto, K. *Bull. Chem. Soc. Japan* **1978**, 51, 2690–2697.
181. Kaufmann, K.-D.; Bauschke, S. Z. *Chem.* **1980**, 20, 145–146.
182. Chang, J. K.; Shimizu, M.; Wang, S. S. *J. Org. Chem.* **1976**, 41, 3255–3258.
183. Mizoguchi, T.; Shigezane, K.; Takamura, N. *Chem. Pharm. Bull.* **1969**, 17, 411–412.
184. Mizoguchi, T.; Shigezane, K.; Takamura, N. *Chem. Pharm. Bull.* **1970**, 18, 1465–1474.
185. Epton, R.; Marr, G.; Willmore, G. A. *Int. J. Biol. Macromol.* **1983**, 5, 135–142.
186. Small, P. W.; Epton, R.; Marr, G.; Twaij, S. *Int. J. Biol. Macromol.* **1984**, 6, 189–194.
187. Marshall, D. L.; Liener, I. E. *J. Org. Chem.* **1970**, 35, 867–868.
188. Johnson, B. J.; Jacobs, P. M. *J. Chem. Soc., Chem. Commun.* **1968**, 73–75.
189. Flanagan, E.; Marshall, G. R. *Tetrahedron Lett.* **1970**, 2403–2406.
190. Wieland, T.; Lewalter, J.; Birr, C. *Justus Liebigs Ann. Chem.* **1970**, 740, 31–47.
191. Kenner, G. W.; McDermott, J. R.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1971**, 636–637.
192. Bodansky, M.; Sheehan, J. T. *Chem. Ind. (London)* **1966**, 1597–1598.
193. Beyerman, H. C.; Hindiks, H.; de Leer, E. W. B. *J. Chem. Soc., Chem. Commun.* **1968**, 1668.
194. Beyerman, H. C.; Kranenburg, P.; Syrier, J. L. M. *Recl. Trav. Chim. Pays Bas* **1971**, 90, 791–800.
195. Penke, B.; Birr, C. *Justus Liebigs Ann. Chem.* **1974**, 1999–2002.
196. Reddy, G. L.; Bikshapathy, E.; Nagaraj, R. *Tetrahedron Lett.* **1985**, 26, 4257–4260.
197. Lyle, T. A.; Brady, S. F.; Ciccarone, T. M.; Colton, C. D.; Paleveda, W. J.; Veber, D. F.; Nutt, R. F. *J. Org. Chem.* **1987**, 52, 3752–3759.
198. Barton, M. A.; Lemieux, R. U.; Savoie, J. Y. *J. Am. Chem. Soc.* **1973**, 95, 4501–4506.
199. Marshall, G. R.; Merrifield, R. B. *Biochemistry* **1965**, 4, 2394–2401.
200. Anwer, M. K.; Spatola, A. F. *Tetrahedron Lett.* **1992**, 33, 3121–3124.
201. Giralt, E.; Celma, C.; Ludevid, M. D.; Pedrosa, E. *Int. J. Peptide Protein Res.* **1987**, 29, 647–656.
202. Celma, C.; Albericio, F.; Pedrosa, E.; Giralt, E. *Peptide Res.* **1992**, 5, 62–71.
203. Tam, J. P.; Cunningham-Rundles, W. F.; Erickson, B. W.; Merrifield, R. B. *Tetrahedron Lett.* **1977**, 4001–4004.
204. Birr, C.; Wengert-Müller, M.; Buku, A. In *Peptides. Proceedings of the 5th American Peptides Symposium*; Goodman, M.; Meienhofer, J. Eds.; Halstead Press, New York, 1977, pp. 510–513.
205. Birr, C.; Wengert-Müller, M. *Angew. Chem., Int. Edn. Engl.* **1979**, 18, 147–148.
206. Voss, C.; Birr, C. *Hoppe-Seyler's Z. Physiol. Chem.* **1981**, 362, 717–725.
207. Birr, C. In *Aspects of the Merrifield Peptide Synthesis, Reactivity and Structure Concepts in Organic Chemistry*; Hafner, K.; Lehn, J.-M.; Rees, C. W.; von Ragué Schleyer, P.; Trost, B. M.; Zahradnik, R. Eds.; Springer-Verlag, Heidelberg, 1978; Vol. 8, p. 67.
208. Fridkin, M.; Patchornik, A.; Katchalski, E. *J. Am. Chem. Soc.* **1966**, 88, 3164–3165.
209. Fridkin, M.; Patchornik, A.; Katchalski, E. *J. Am. Chem. Soc.* **1968**, 90, 2953–2957.

210. Wieland, T.; Birr, C. *Angew. Chem., Int. Edn. Engl.* **1966**, *5*, 310.
211. Wieland, T.; Birr, C., *Chimia* **1967**, *21*, 581-582.
212. Inukai, I.; Nakano, K.; Murakami, M. *Bull. Chem. Soc. Japan* **1968**, *41*, 182-186.
213. Losse, G.; Neubert, K. *Tetrahedron Lett.* **1970**, 1267-1270.
214. Losse, G.; Madlung, C.; Lorenz, P. *Chem. Ber.* **1968**, *101*, 1257-1262.
215. See Kenner, G. W. *Angew. Chem.* **1959**, *71*, 741-742.
216. Kenner, G. W.; Seely, J. H. *J. Am. Chem. Soc.* **1972**, *94*, 3259-3260.
217. Arshady, R.; Kenner, G. W.; Ledwith, A. *J. Polymer Science, Polymer Chem. Ed.* **1974**, *12*, 2017-2025.
218. Hudson, D.; Kenner, G. W.; Sharpe, R.; Szelke, M. *Int. J. Peptide Protein Res.* **1979**, *14*, 177-185.
219. Hudson, D.; Kenner, G. W. *Int. J. Biol. Macromol.* **1980**, *2*, 63-67.
220. Rivaille, P.; Gautron, J. P.; Castro, B.; Milhaud, G. *Tetrahedron* **1980**, *36*, 3413-3419.
221. Schlatter, J. M.; Mazur, R. H. *Tetrahedron Lett.* **1977**, 2851-2852.
222. Jones, D. A. *Tetrahedron Lett.* **1977**, 2853-2856.
223. Khan, S. A.; Sivanandaiah, K. M. *Synthesis* **1978**, 750-751.
224. Ljungqvist, A.; Folkers, K. Z. *Naturforsch.* **1983**, *38b*, 1022-1024.
225. Tjoeng, F. S.; Hodges, R. S. *Tetrahedron Lett.* **1979**, 1273-1276.
226. Anwer, M. K.; Spatola, A. F. *Tetrahedron Lett.* **1981**, *22*, 4369-4372.
227. Anwer, M. K.; Spatola, A. F.; Bossinger, C. D.; Flanigan, E.; Liu, R. C.; Olsen, D. B.; Stevenson, D. *J. Org. Chem.* **1983**, *48*, 3503-3507.
228. Stevens, C. M.; Watanabe, R. *J. Am. Chem. Soc.* **1950**, *72*, 725-727.
229. Corey, E. J.; Suggs, J. W. *J. Org. Chem.* **1973**, *38*, 3223-3224.
230. Kunz, H.; Dombo, B. *Angew. Chem., Int. Edn. Engl.* **1988**, *27*, 711-713.
231. Kunz, H. *Angew. Chem., Int. Edn. Engl.* **1987**, *26*, 294-308.
232. Blankmeyer-Menge, B.; Frank, R. *Tetrahedron Lett.* **1988**, *29*, 5871-5874.
233. Guibé, F.; Dangles, O.; Balavoine, G.; Loffet, A. *Tetrahedron Lett.* **1989**, *30*, 2641-2644.
234. Dangles, O.; Guibé, F.; Balavoine, G.; Lavielle, S.; Marquet, A. *J. Org. Chem.* **1987**, *52*, 4984-4993.
235. Merzouk, A.; Guibé, F.; Loffet, A. *Tetrahedron Lett.* **1992**, *33*, 477-480.
236. Lloyd-Williams, P.; Jou, G.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **1991**, *32*, 4207-4210.
237. Tam, J. P.; Tjoeng, F. S.; Merrifield, R. B. *Tetrahedron Lett.* **1979**, 4935-4938.
238. Tam, J. P.; Tjoeng, F. S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1980**, *102*, 6117-6127.
239. Whitney, D. B.; Tam, J. P.; Merrifield, R. B. *Tetrahedron* **1984**, *40*, 4237-4244.
240. Kubiak, T.; Whitney, D. B.; Merrifield, R. B. *Biochemistry* **1987**, *26*, 7849-7855.
241. Tam, J. P.; DiMarchi, R. D.; Merrifield, R. B. *Int. J. Peptide Protein Res.* **1980**, *16*, 412-425.
242. Gross, E.; Noda, K.; Nisula, B. *Angew. Chem.* **1973**, *85*, 672-673.
243. Noda, K.; Gazis, D.; Gross, E. *Int. J. Peptide Protein Res.* **1982**, *19*, 413-419.
244. Rosamund, J. D.; Ferger, M. F. *J. Med. Chem.* **1976**, *19*, 873-876.
245. Glass, J. D.; Talansky, A.; Grzonka, Z.; Schwarz, I. L.; Walter, R. *J. Am. Chem. Soc.* **1974**, *96*, 6476-6480.
246. Stahl, G. L.; Walter, R.; Smith, C. W. *J. Am. Chem. Soc.* **1979**, *101*, 5383-5394.
247. Kemp, D. S.; Galakatos, N. G. *J. Org. Chem.* **1986**, *51*, 1821-1829.
248. Kemp, D. S.; Galakatos, N. G.; Bowen, B.; Tan, K. *J. Org. Chem.* **1986**, *51*, 1829-1838.
249. Kemp, D. S. *Biopolymers* **1981**, *20*, 1793-1804.
250. Mensi, N.; Isied, S. S. *J. Am. Chem. Soc.* **1987**, *109*, 7882-7884.
251. Arbo, B.; Isied, S. S. *Int. J. Peptide Protein Res.* in press.
252. Elmore, D. T.; Guthrie, D. J. S.; Wallace, A. D.; Bates, S. R. E. *J. Chem. Soc., Chem. Commun.* **1992**, 1033-1034.
253. See for example: *Yajima, H.; Fujii, N. *J. Chem. Soc., Perkin Trans. I* **1981**, 789-796; Yajima, H.; Fujii, N. *J. Chem. Soc., Perkin Trans. I* **1981**, 797-803; Yajima, H.; Fujii, N. *J. Chem. Soc., Perkin Trans. I* **1981**, 804-810; Yajima, H.; Fujii, N. *J. Chem. Soc., Perkin Trans. I* **1981**, 811-818; Yajima, H.; Fujii, N. *J. Chem. Soc., Perkin Trans. I* **1981**, 819-830; Yajima, H.; Fujii, N. *J. Chem. Soc., Perkin Trans. I* **1981**, 831-841; *Kenner, G. W.; Ramage, R.; Sheppard, R. C. *Tetrahedron* **1979**, *35*, 2767-2769; Galpin, I. J.; Hancock, F. E.; Handa, B. K.; Jackson, A. G.; Kenner, G. W.; Ramage, R.; Singh, B. *Tetrahedron* **1979**, *35*, 2771-2778; Galpin, I. J.; Hancock, F. E.; Handa, B. K.; Jackson, A. G.; Kenner, G. W.; Ramage, R.; Singh, B.; Tyson, R. G. *Tetrahedron* **1979**, *35*, 2779-2783; Galpin, I. J.; Kenner, G. W.; Ohlsen, S. R.; Ramage, R.; Sheppard, R. C.; Tyson, R. G. *Tetrahedron* **1979**, *35*, 2785-2790; Galpin, I. J.; Hancock, F. E.; Handa, B. K.; Jackson, A. G.; Kenner, G. W.; Ramage, R.; Singh, B. *Tetrahedron* **1979**, *35*, 2791-2794; Galpin, I. J.; Kenner, G. W.; Ramage, R. *Tetrahedron* **1980**, *36*, 2241-2245; Galpin, I. J.; Handa, B. K.; Kenner, G. W.; Ohlsen, S. R.; Ramage, R. *Tetrahedron* **1980**, *36*, 2247-2253; Galpin, I. J.; Hudson, D.; Jackson, A. G.; Kenner, G. W.; Ramage, R. *Tetrahedron* **1980**, *36*, 2255-2257; Galpin, I. J.; Handa, B. K.; Jackson, A. G.; Kenner, G. W.; McDowell, P.; Ohlsen, S. R.; Ramage, R. *Tetrahedron* **1980**, *36*, 2259-2262; Galpin, I. J.; Hallett, A.; Kenner, G. W.; Noble, P.; Ramage, R.; Seely, J. H. *Tetrahedron* **1981**, *37*, 3017-3023; Galpin, I. J.; Hudson, D.; Kenner, G. W.; Noble, P.; Ramage, R. *Tetrahedron* **1981**, *37*, 3025-3029; Galpin, I. J.; Kenner, G. W.; Morgan, B. A.; Noble, P.; Ramage, R. *Tetrahedron* **1981**, *37*, 3031-3036; Galpin, I. J.; Kenner, G. W.; Ramage, R.; Thorpe, W. D. *Tetrahedron* **1981**, *37*, 3037-3041; *Kawasaki, K.; Camble, R.; Dupuis, G.; Romovacek, H.; Storey, H. T.; Yanaihara, C.; Hofmann, K. *J. Am. Chem. Soc.* **1973**, *95*, 6815-6824; Romovacek, H.; Dowd, S. R.; Kawasaki, K.; Nishi, N.; Hofmann, K. *J. Am. Chem. Soc.* **1979**, *101*, 6081-6091.
254. Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. *Nature* **1975**, *258*, 598-599.
255. We refer the reader to the extensive classical literature on peptide purification. See for example: Jaeger, E.; Deffner, M. In *Methoden der Organischen Chemie (Houben-Weyl)*; Wünsch, E. Ed.; Georg Thieme Verlag, Stuttgart, 1974,

- Vol. 15, Part II; and the relevant sections in *Methods in Enzymology, Enzyme Structure*; Hirs, C. H. W. ed.; Academic Press, New York, 1967, Vol. XI.
256. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–2925.
 257. For application of such techniques in CSPPS, see Ref. 197.
 258. See for example: Kaufmann, K. D.; Kunzek, H.; Dölling, R.; Halatsch, W. R.; Nieke, E. M.; Rose, K. B.; Bauschke, S.; Schönherr, C. *Z. Chem.* **1980**, *20*, 99–100.
 259. Lindqvist, B.; Storgards, T. *Nature* **1955**, *175*, 511–512.
 260. Porath, J.; Flodin, P. *Nature* **1959**, *183*, 1657–1659.
 261. Porath, J. *Biochem. Biophys. Acta* **1960**, *39*, 193–207.
 262. Flodin, P. *J. Chromatogr.* **1961**, *5*, 103–115.
 263. Andrews, P. *Biochem. J.* **1964**, *91*, 222–233.
 264. Nyström, E.; Sjövall, J. *Biochem. J.* **1964**, *92*, 10 P.
 265. Mutt, V.; Nyström, E.; Sjövall, J. *J. Chromatogr.* **1966**, *24*, 205–207.
 266. Nyström, E.; Sjövall, J. *J. Chromatogr.* **1966**, *24*, 208–212.
 267. Gut, V.; Cimrova, M. *Coll. Czech. Chem. Commun.* **1969**, *34*, 1620–1623.
 268. Zeiger, A. R.; Anfinsen, C. B. *J. Am. Chem. Soc.* **1973**, *95*, 880–886.
 269. Galpin, I. J.; Kenner, G. W.; Ohlsen, S. R.; Ramage, R. *J. Chromatogr.* **1975**, *106*, 125–129.
 270. Galpin, I. J.; Handa, B. K.; Kenner, G. W.; Moore, S.; Ramage, R. *J. Chromatogr.* **1976**, *123*, 237–242.
 271. Galpin, I. J.; Jackson, A. G.; Kenner, G. W.; Noble, P.; Ramage, R. *J. Chromatogr.* **1978**, *147*, 424–428.
 272. Galpin, I. J.; Handa, B. K.; Kenner, G. W.; Moore, S.; Ramage, R. In *Chromatography of Synthetic and Biological Polymers*; Ellis Horwood Ltd., Chichester, UK, 1978; Vol. I, pp. 331–338.
 273. Tesser, G. I.; Adams, P. J. H. M.; Kortenaar, P. B. W. T.; Boots, H. A. *Int. J. Peptide Protein Res.* **1984**, *24*, 192–194.
 274. For examples of purification using gel-filtration in CSPPS see Refs 62, 63, 64, 65, 68, 73, 90, 91, 92, 139, 140, 141, 204, 205, 206, 220, 240.
 275. Colin, H.; Guiochon, G. *J. Chromatogr.* **1977**, *141*, 289–312.
 276. Bakalyar, S. B.; Henry, R. A. *J. Chromatogr.* **1976**, *126*, 327–345.
 277. Lundanes, E.; Greibrokk, T. *J. Chromatogr.* **1978**, *149*, 241–254.
 278. Snyder, L. R.; Dolan, J. W.; Gant, J. R. *J. Chromatogr.* **1979**, *165*, 3–30.
 279. Dolan, J. W.; Gant, J. R.; Snyder, L. R. *J. Chromatogr.* **1979**, *165*, 31–58.
 280. Naleway, J. J.; Hoffman, N. E. *J. Liquid Chromatogr.* **1981**, *4*, 1323–1338.
 281. Gabriel, T. F.; Michalewsky, J.; Meienhofer, J. *J. Chromatogr.* **1976**, *129*, 287–293.
 282. Gabriel, T. F.; Jimenez, M. H.; Felix, A. M.; Michalewsky, J.; Meienhofer, J. *Int. J. Peptide Protein Res.* **1977**, *9*, 129–136.
 283. Naider, F.; Sipzner, R.; Steinfeld, A. S.; Becker, J. M. *J. Chromatogr.* **1979**, *176*, 264–269.
 284. Hearn, M. T. W. In *Methods in Enzymology, Enzyme Purification and Related Techniques*; Jakoby, W. B. Ed.; Academic Press, New York, 1984; Vol. 104, pp. 190–212.
 285. Hancock, W. S.; Bishop, C. A.; Hearn, M. T. W. *FEBS Lett.* **1976**, *72*, 139–142.
 286. Mönch, W.; Dehnen, W. *J. Chromatogr.* **1977**, *140*, 260–262.
 287. Molnár, I.; Horváth, C. *J. Chromatogr.* **1977**, *142*, 623–640.
 288. Hearn, M. T. W.; Hancock, W. S.; Hurrell, J. G. R.; Fleming, R. J.; Kemp, B. *J. Liq. Chromatogr.* **1979**, *2*, 919–933.
 289. Biemond, M. E. F.; Sipman, W. A.; Olivé, J. *J. Liq. Chromatogr.* **1979**, *2*, 1407–1435.
 290. Nachtmann, F. *J. Chromatogr.* **1979**, *176*, 391–397.
 291. Hearn, M. T. W.; Bishop, C. A.; Hancock, W. S.; Harding, D. R. K.; Reynolds, G. D. *J. Liq. Chromatogr.* **1979**, *2*, 1–21.
 292. Bishop, C. A.; Harding, D. R. K.; Meyer, L. J.; Hancock, W. S.; Hearn, M. T. W. *J. Chromatogr.* **1980**, *192*, 222–227.
 293. Pedroso, E.; Grandas, A.; Amor, J.-C.; Giralt, E. *J. Chromatogr.* **1987**, *409*, 281–290.
 294. See for example: Xue, C. B.; Becker, J. M.; Naider, F. *Int. J. Peptide Protein Res.* **1991**, *37*, 476–486.
 295. Tarr, G. E.; Crabb, J. W. *Anal. Biochem.* **1983**, *131*, 99–107.
 296. For examples of purification by HPLC in CSPPS see Refs 36, 63, 64, 65, 123, 142, 143, 150, 151.
 297. Meek, J. L.; Rossetti, Z. L. *J. Chromatogr.* **1981**, *211*, 15–28.
 298. Sasagawa, T.; Okuyama, T.; Teller, D. C. *J. Chromatogr.* **1982**, *240*, 329–340.
 299. Houghten, R. A.; DeGraw, S. T. *J. Chromatogr.* **1987**, *386*, 223–228.
 300. Houghten, R. A.; Ostresh, J. M. *BioChromatography* **1987**, *2*, 80–84.
 301. Fong, G. W. K.; Grushka, E. *Anal. Chem.* **1978**, *50*, 1154–1161.
 302. Hancock, W. S.; Bishop, C. A.; Prestidge, R. L.; Harding, D. R. K.; Hearn, M. T. W. *Science* **1978**, *200*, 1168–1170.
 303. Hearn, M. T. W.; Grego, B.; Hancock, W. S. *J. Chromatogr.* **1979**, *185*, 429–444.
 304. Hearn, M. T. W.; Grego, B. *J. Chromatogr.* **1984**, *296*, 309–319.
 305. Ulyashin, V. V.; Deigin, V. I.; Ivanov, V. T.; Ovchinnikov, Y. A. *J. Chromatogr.* **1981**, *215*, 263–277.
 306. Ryba, M. *Chromatographia* **1982**, *15*, 227–230.
 307. See for example: Tomatis, R.; Guarneri, M.; Guggi, A.; Salvadori, S.; Rocchi, R. *Int. J. Peptide Protein Res.* **1979**, *14*, 347–355.
 308. Jimenez, M. H.; Winter, D. P.; Felix, A. M. *J. Chromatogr.* **1971**, *63*, 167–171.
 309. Knight, M.; Kask, A. M.; Tamminga, C. A. *J. Liq. Chromatogr.* **1984**, *7*, 351–362.
 310. Anfinsen, C. B.; Ontjes, D.; Ohno, M.; Corley, L.; Eastlake, A. *Proc. Natl. Acad. Sci. USA* **1967**, *58*, 1806–1811.

311. Naithani, V. K.; Föhles, J. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 1173–1181.
312. Berndt, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1979**, *360*, 765–772.
313. Knight, M.; Ito, Y.; Kask, A. M.; Tamminga, C. A.; Chase, T. N. *J. Liq. Chromatogr.* **1984**, *7*, 2525–2533.
314. O'Neill, I. A. *Synlett* **1991**, 661–662.
315. Regnier, F. E. *Nature* **1991**, *350*, 634–635.
316. Ball, H. L.; Mascagni, P. In *Proceedings of the Second Japan Symposium on Peptide Chemistry*; Yanaihara, N. Ed.; ESCOM, Leiden, 1993, in press.
317. Grandas, A.; Pedrosa, E.; Figueras, A.; Rivera, J.; Giralt, E. *Biomed. Environ. Mass Spec.* **1988**, *15*, 681–684.
318. Celma, C.; Giralt, E. *Biomed. Environ. Mass Spec.* **1990**, *19*, 235–239.
319. Toniolo, C.; Bonora, G. M.; Schaaper, W. M. M. *Int. J. Peptide Protein Res.* **1984**, *23*, 389–393.
320. Toniolo, C.; Bonora, G. M.; Moretto, V.; Bodansky, M. *Int. J. Peptide Protein Res.* **1985**, *25*, 425–430.
321. Toniolo, C.; Bonora, G. M.; Heimer, E. P.; Felix, A. M. *Int. J. Peptide Protein Res.* **1987**, *30*, 232–239.
322. Atherton, E.; Woolley, V.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1980**, 970–971.
323. Giralt, E.; Rizo, J.; Pedrosa, E. *Tetrahedron* **1984**, *40*, 4141–4152.
324. Live, D. H.; Kent, S. B. H. In *Elastomers and Rubber Elasticity*; ACS Symposium Series 193; Mark, J.; Lol, J. Eds.; American Chemical Society, Washington, D.C., 1982, pp. 501–514.
325. Live, D. H.; Kent, S. B. H. In *Peptides. Structure and Function. Proceedings of the Eighth American Peptides Symposium*; Hruby, V. J.; Rich, D. H. Eds.; Pierce Chemical Co., Rockford, Illinois, 1983, pp. 65–68.
326. Ludwick, A. G.; Jelinski, L. W.; Live, D.; Kintanar, A.; Dumais, J. J. *J. Am. Chem. Soc.* **1986**, *108*, 6493–6496.
327. Leibfritz, D.; Mayr, W.; Oekonomopulos, R.; Jung, G. *Tetrahedron* **1978**, *34*, 2045–2050.
328. Milton, R. C. de L.; Milton, S. C. F.; Adams, P. A. *J. Am. Chem. Soc.* **1990**, *112*, 6039–6046.
329. Westall, F. C.; Robinson, A. B. *J. Org. Chem.* **1970**, *35*, 2842–2844.
330. Yamashiro, D.; Blake, J.; Li, C. H. *Tetrahedron Lett.* **1976**, 1469–1472.
331. Milton, S. C. F.; Milton, R. C. de L. *Int. J. Peptide Protein Res.* **1990**, *36*, 193–196.
332. Stewart, J. M.; Klis, W. A. In *Innovations and Perspectives in Solid-Phase Peptide Synthesis. Peptides, Polypeptides and Oligonucleotides*; SPCC (UK) Ltd., 1990, pp. 1–9.
333. Hendrix, J. C.; Halverson, K.; Jarrett, J. T.; Lansbury, P. T. *J. Org. Chem.* **1990**, *55*, 4517–4518.
334. Toniolo, C.; Bonora, G. M.; Mutter, M.; Maser, F. *J. Chem. Soc., Chem. Commun.* **1983**, 1298–1299.
335. Weygrand, F.; Steglich, W.; Bjarnason, J.; Akhtar, R.; Khan, N. M. *Tetrahedron Lett.* **1966**, 3483–3487.
336. Weygrand, F.; Steglich, W.; Bjarnason, J.; Akhtar, R.; Chytil, N. *Chem. Ber.* **1968**, *101*, 3623–3641.
337. Blaakmeer, J.; Tijssen-Klasen, T.; Tesser, G. I. *Int. J. Peptide Protein Res.* **1991**, *37*, 556–564.
338. Toniolo, C.; Bonora, G.; Mutter, M.; Pillai, V. N. R. *Makromol. Chem.* **1981**, *182*, 1997–2005.
339. Toniolo, C.; Bonora, G.; Mutter, M.; Pillai, V. N. R. *Makromol. Chem.* **1981**, *182*, 2007–2014.
340. El Rahman, S. A.; Anzinger, H.; Mutter, M. *Biopolymers* **1980**, *19*, 173–187.
341. Narita, M.; Fukunaga, T.; Wakabayashi, A.; Ishikawa, K.; Nakano, H. *Int. J. Peptide Protein Res.* **1984**, *23*, 306–314.
342. Narita, M.; Nagasawa, S.; Chen, J.-Y.; Sato, H.; Tanaka, Y. *Makromol. Chem.* **1984**, *185*, 1069–1078.
343. Narita, M.; Ohkawa, N.; Nagasawa, S.; Isokawa, S. *Int. J. Peptide Protein Res.* **1984**, *24*, 129–134.
344. Isokawa, S.; Asakura, T.; Narita, M. *Makromolecules* **1985**, *18*, 871–877.
345. Isokawa, S.; Tominaga, I.; Asakura, T.; Narita, M. *Makromolecules* **1985**, *18*, 878–881.
346. Bartl, R.; Kloppe, K.-D.; Frank, R. In *Peptides. Chemistry and Biology. Proceedings of the 12th American Peptides Symposium*; Smith, J. A.; Rivier, J. E. Eds.; ESCOM, Leiden, 1992, pp. 505–506.
347. Bartl, R.; Kloppe, K.-D.; Frank, R. In *Peptides 1992. Proceedings of the 22nd European Peptides Symposium*; Schneider, C. H.; Eberle, A. N. Eds.; ESCOM, Leiden, 1993, pp. 277–278.
348. Haack, T.; Mutter, M. *Tetrahedron Lett.* **1992**, *33*, 1589–1592.
349. Haack, T.; Zier, A.; Nefzi, A.; Mutter, M. In *Peptides 1992. Proceedings of the 22nd European Peptides Symposium*; Schneider, C. H.; Eberle, A. N. Eds.; ESCOM, Leiden, 1993, pp. 595–596.
350. Narita, M.; Doi, M.; Sugawara, H.; Ishikawa, K. *Bull. Chem. Soc. Japan* **1985**, *58*, 1473–1479.
351. Narita, M.; Ishikawa, K.; Sugawara, H.; Doi, M. *Bull. Chem. Soc. Japan* **1985**, *58*, 1731–1737.
352. Narita, M.; Chen, J.-Y.; Sato, H.; Kim, Y. *Bull. Chem. Soc. Japan* **1985**, *58*, 2494–2501.
353. Paterson, Y.; Rumsey, S. M.; Benedetti, E.; Némethy, G.; Scheraga, H. A. *J. Am. Chem. Soc.* **1981**, *103*, 2947–2955.
354. Narita, M.; Ishikawa, K.; Chen, J.-Y.; Kim, Y. *Int. J. Peptide Protein Res.* **1984**, *24*, 580–587.
355. Narita, M.; Ogura, T.; Sato, K.; Honda, S. *Bull. Chem. Soc. Japan* **1986**, *59*, 2433–2438.
356. Narita, M.; Honda, S.; Umeyama, H. *Bull. Chem. Soc. Japan* **1987**, *60*, 4127–4131, and refs therein.
357. Narita, M.; Umeyama, H.; Sasaki, C. *Int. J. Peptide Protein Res.* **1989**, *33*, 340–344.
358. For a useful classification of solvents see: Snyder, L. R. *J. Chromatogr.* **1974**, *92*, 223–230.
359. Narita, M.; Honda, S.; Umeyama, H.; Obana, S. *Bull. Chem. Soc. Japan* **1988**, *61*, 281–284.
360. Nishino, N.; Xu, M.; Mihara, H.; Fujimoto, T. *Chem. Lett.* **1992**, 327–330.
361. Narita, M.; Honda, S.; Obana, S. *Bull. Chem. Soc. Japan* **1989**, *62*, 342–344.
362. Narita, M.; Umeyama, H.; Isokawa, S.; Honda, S.; Sasaki, C.; Kakei, H. *Bull. Chem. Soc. Japan* **1989**, *62*, 780–785.
363. Narita, M.; Umeyama, H.; Yoshida, T. *Bull. Chem. Soc. Japan* **1989**, *62*, 3582–3586.
364. Nishino, N.; Mihara, H.; Makinose, Y.; Fujimoto, T. *Tetrahedron Lett.* **1992**, in press.
365. Kuroda, H.; Chen, Y.-N.; Kimura, T.; Sakakibara, S. *Int. J. Peptide Protein Res.* **1992**, *40*, 294–299.
366. Seebach, D.; Thaler, A.; Beck, A. K. *Helv. Chim. Acta* **1989**, *72*, 857–867.
367. Halverson, K.; Fraser, P. E.; Kirschner, D. A.; Lansbury, P. T. *Biochemistry* **1990**, *29*, 2639–2644.
368. Kimura, T.; Takai, M.; Masui, Y.; Morikawa, T.; Sakakibara, S. *Biopolymers* **1981**, *20*, 1823–1832.

369. Bayer, E.; Mutter, M. *Nature* **1972**, *237*, 512–513.
370. Mutter, M.; Hagenmaier, H.; Bayer, E. *Angew. Chem., Int. Edn. Engl.* **1971**, *10*, 811–812.
371. See for example: Toniolo, C.; Bonora, G. M.; Mutter, M. *J. Am. Chem. Soc.* **1979**, *101*, 450–454.
372. Rizo, J.; Albericio, F.; Romero, G.; Garcia-Echeverria, C.; Claret, J.; Muller, C.; Giralt, E.; Pedroso, E. *J. Org. Chem.* **1988**, *53*, 5386–5389.
373. See for example: Macrae, R.; Young, G. T. *J. Chem. Soc., Perkin Trans. I* **1975**, 1185–1191.
374. Ramage, R.; Raphy, G. *Tetrahedron Lett.* **1992**, *33*, 385–388.
375. Rizo, J.; Albericio, F.; Giralt, E.; Pedroso, E. *Tetrahedron Lett.* **1992**, *33*, 397–400.
376. Suzuki, K.; Sasaki, Y.; Endo, N. *Chem. Pharm. Bull.* **1976**, *24*, 1–9.
377. Kemp, D. S.; Roberts, D. C. *Tetrahedron Lett.* **1975**, 4629–4632.
378. Kunz, H. *Chem. Ber.* **1976**, *109*, 2670–2683.
379. Veber, D. F.; Paleveda, W. J.; Lee, Y. C.; Hirschmann, R. *J. Org. Chem.* **1977**, *42*, 3286–3288.
380. Kunz, H.; Barthels, R. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 783–784.
381. Kessler, H.; Becker, G.; Kogler, H.; Wolff, M. *Tetrahedron Lett.* **1984**, *25*, 3971–3974.
382. Eckert, H.; Seidel, C. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 159–160.
383. Merrifield, R. B.; Bach, A. E. *J. Org. Chem.* **1978**, *43*, 4808–4816.
384. Mutter, M.; Oppliger, H.; Zier, A. *Makromol. Chem., Rapid Commun.* **1992**, *13*, 151–157.
385. Anzinger, H.; Mutter, M.; Bayer, E. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 686–687.
386. Rabanal, F.; Albericio, F.; Giralt, E., to be published.
387. Ball, H. L.; Grecian, C.; Kent, S. B. H.; Mascagni, P. In *Peptides. Chemistry, Structure and Biology. Proceedings of the 11th American Peptides Symposium*; Rivier, J. E.; Marshall, G. R. Eds; ESCOM, Leiden, 1990, pp. 435–436.
388. Ball, H. L.; Kent, S. B. H.; Mascagni, P. In *Peptides 1990. Proceedings of the 21st European Peptides Symposium*; Giralt, E.; Andreu, D. Eds; ESCOM, Leiden, 1991, pp. 323–325.
389. Ball, H. L.; Kent, S. B. H.; Mascagni, P. *Int. J. Peptide Protein Res.* **1992**, *40*, 370–379.
390. Weygrand, F.; Ragnarsson, U. Z. *Naturforsch.* **1966**, *21b*, 1141–1144.
391. Weygrand, F.; Obermeier, R. Z. *Naturforsch.* **1968**, *23b*, 1390.
392. Omenn, G. S.; Anfinsen, C. B. *J. Am. Chem. Soc.* **1968**, *90*, 6571–6572.
393. See Niu, C. I.; Yang, S. Z.; Chen, Y. N.; Ma, A. Q.; Shi, P. T.; Jiang, Y. Q.; Xu, L. G.; Huang, W. T. In *Peptides. Synthesis, Structure, Function. Proceedings of the Seventh American Peptides Symposium*; Rich, D. H.; Gross, E. Eds; Pierce Chemical Co., Rockford, Illinois, 1981, pp. 143–152, for one approach to segment coupling on a solid support.
394. Losse, A. *Tetrahedron Lett.* **1971**, 4989–4992.
395. Losse, A. *Tetrahedron* **1973**, *29*, 1203–1208.
396. Albericio, F.; Pons, M.; Pedroso, E.; Giralt, E. *J. Org. Chem.* **1989**, *54*, 360–366.
397. Atherton, E.; Brown, E.; Priestley, G.; Sheppard, R. C.; Williams, B. J. In *Peptides. Synthesis Structure, Function. Proceedings of the Seventh American Peptide Symposium*; Rich, D. H.; Gross, E. Eds; Pierce Chemical Company, Illinois, 1981, 163–175.
398. Atherton, E.; Cameron, L. R.; Cammish, L. E.; Dryland, A.; Goddard, P.; Priestly, G. P.; Richards, J. D.; Sheppard, R. C.; Wade, J. D.; Williams, B. J. In *Innovations and Perspectives in Solid-Phase Synthesis. Peptides, Polypeptides and Oligonucleotides*; SPCC (UK) Ltd., 1990, pp. 11–25.
399. Felix, A. M.; Merrifield, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 1385–1391.
400. Matsueda, R.; Maruyama, H.; Kitazawa, E.; Takahagi, H.; Mukaiyama, T. *Bull. Chem. Soc. Japan* **1973**, *46*, 3240–3247.
401. Mukaiyama, T.; Matsueda, R.; Suzuki, M. *Tetrahedron Lett.* **1970**, 1901–1904.
402. Matsueda, R.; Maruyama, H.; Kitazawa, E.; Takahagi, H.; Mukaiyama, T. *J. Am. Chem. Soc.* **1975**, *97*, 2573–2575.
403. Maruyama, H.; Matsueda, R.; Kitazawa, E.; Takahagi, H.; Mukaiyama, T. *Bull. Chem. Soc. Japan* **1976**, *49*, 2259–2267.
404. Yajima, H.; Kiso, Y. *Chem. Pharm. Bull.* **1974**, *22*, 1087–1094.
405. Ljungqvist, A.; Folkers, K. *Acta Chem. Scand.* **1984**, *38B*, 375–379.
406. Lelievre, D.; Trudelle, Y.; Heitz, F.; Spach, G. *Int. J. Peptide Protein Res.* **1989**, *33*, 379–385.
407. Clark, J. H.; Miller, J. M. *J. Chem. Soc., Chem. Commun.* **1976**, 229–230.
408. Clark, J. H.; Holland, H. L.; Miller, J. M. *Tetrahedron Lett.* **1976**, 3361–3364.
409. Whitney, D. B.; Tam, J. P.; Merrifield, R. B. In *Peptides. Structure and Function. Proceedings of the Eighth American Peptides Symposium*; Hruby, V. J.; Rich, D. H. Eds; Pierce Chemical Co., Rockford, Illinois, 1983, pp. 167–170.
410. Itoh, M. *Bull. Chem. Soc. Japan* **1973**, *46*, 2219–2221.
411. Rink, H.; Born, W.; Fischer, J. A. In *Peptides. Chemistry, Structure and Biology. Proceedings of the Eleventh American Peptides Symposium*; Rivier, J. E.; Marshall, G. R. Eds; ESCOM, Leiden, 1990, pp. 1041–1042.
412. Sato, K.; Abe, H.; Kato, T.; Izumiya, N. *Bull. Chem. Soc. Japan* **1977**, *50*, 1999–2004.
413. Camble, R.; Petter, N. N. In *Peptides 1976. Proceedings of the Fourteenth European Peptides Symposium*; Loffet, A. Ed.; Editions de l'Université de Bruxelles, 1976, pp. 299–307.
414. Grandas, A.; Albericio, F.; Josa, J.; Giralt, E.; Pedroso, E.; Sabatier, J. M.; van Rietschoten, J. *Tetrahedron* **1989**, *45*, 4637–4648.
415. Di Bello, C.; Lucchiari, A.; Buso, O.; De Castiglione, R.; Gozzini, L. *Gazz. Chim. Ital.* **1986**, *116*, 221–228.
416. Di Bello, C.; Lucchiari, A.; Buso, O.; Tonellato, M. *Int. J. Peptide Protein Res.* **1984**, *23*, 61–71.
417. Curtius, T. *Ber. Dtsch. Chem. Ges.* **1902**, *35*, 3226–3228.
418. Visser, S.; Kerling, K. E. T. *Recl. Trav. Chim. Pays Bas* **1970**, *89*, 880–884.

419. Weber, U.; André, M. In *Peptides 1974. Proceedings of the 13th European Peptides Symposium*; Wolman, Y. Ed.; John Wiley and Sons, New York-Israel Universities Press, Jerusalem, 1975, 153-157.
420. Weber, U.; André, M. *Hoppe Seyler's Z. Physiol. Chem.* **1975**, *356*, 701-714.
421. Noda, T.; Morita, K.; Uzawa, T.; Kinoshita, H.; Hori, M. *Experientia* **1987**, *43*, 890-892.
422. Sakakibara, S.; Kishida, Y.; Kikuchi, Y.; Sakai, R.; Kakiuchi, K. *Bull. Chem. Soc. Japan* **1968**, *41*, 1273.
423. Gisin, B. F.; Merrifield, R. B.; Tosteson, D. C. *J. Am. Chem. Soc.* **1969**, *91*, 2691-2695.
424. Losse, G.; Klengel, H. *Tetrahedron* **1971**, *27*, 1423-1434.
425. Gisin, B. F.; Dhundale, A. R. *Int. J. Peptide Protein Res.* **1979**, *14*, 356-363.
426. Neubert, K.; Jakubke, H-D. *J. Prakt. Chem.* **1975**, *317*, 448-454.
427. Yajima, H.; Kawatani, H.; Watanabe, H. *Chem. Pharm. Bull.* **1970**, *18*, 1333-1339.
428. Yajima, H.; Kawatani, H. *Chem. Pharm. Bull.* **1971**, *19*, 1905-1913.
429. Yajima, H.; Kiso, Y.; Okada, Y.; Watanabe, H. *J. Chem. Soc., Chem. Commun.* **1974**, 106-107.
430. Noda, K.; Terada, S.; Mitsuyasu, N.; Waki, M.; Kato, T.; Izumiya, N. *Mem. Fac. Science, Kyushu Univ.* **1970**, *7C*, 189-201.
431. Kullmann, W.; Gutte, B. *Int. J. Peptide Protein Res.* **1978**, *12*, 17-26.
432. Kullmann, W. *Tetrahedron Lett.* **1980**, *21*, 589-592.
433. Karlsson, S. M.; Ragnarsson, U. *Acta Chem. Scand.* **1974**, *28B*, 376-378.
434. Ragnarsson, U.; Ragnarsson, S. M.; Hamberg, U. *Int. J. Peptide Protein Res.* **1975**, *7*, 307-312.
435. Larsson, L. E.; Melin, P.; Ragnarsson, U. *Int. J. Peptide Protein Res.* **1976**, *8*, 39-44.
436. Protein Synthesis Group, *Scientia Sinica* **1975**, *18*, 745-768.
437. Wong, C-H.; Chen, S-T.; Ho, C-L.; Wang, K-T. *Biochem. Biophys. Acta* **1978**, *536*, 376-389.
438. Du, Y. C.; Shen, J. H.; Wu, W. Y.; Wu, C.R.; Wang, K. Z.; Gu, B. X.; Fu, P. *Biopolymers* **1986**, *25*, S85-S96.
439. Anantharamaiah, G. M.; Sivanandaiah, K. M. *Ind. J. Chem.* **1978**, *16B*, 797-802.
440. Birr, C.; Voss, C. In *Peptides. Synthesis, Structure, Function. Proceedings of the 7th American Peptides Symposium*; Rich, D. H.; Gross, E. Eds; Pierce Chemical Co., Rockford, Illinois, 1981, pp. 177-180.
441. Ho, P.; Slavazza, D.; Chang, K.; Bassi, K.; Chang, D. In *Peptides. Chemistry, Structure and Biology. Proceedings of the 11th American Peptides Symposium*; Rivier, J. E.; Marshall, G. R. Eds; ESCOM, Leiden, 1990, 993-995.
442. Benoiton, N. L.; Kuroda, K. *Int. J. Peptide Protein Res.* **1981**, *17*, 197-204.
443. Bayer, E.; Gil-Av, E.; König, W. A.; Nakaparksin, S.; Oró, J.; Parr, W. *J. Am. Chem. Soc.* **1970**, *92*, 1738-1740.
444. Goodman, M.; Keogh, P.; Anderson, H. *Bioorganic Chem.* **1977**, *6*, 239-247.
445. Steinauer, R.; Chen, F. M. F.; Benoiton, N. L. *J. Chromatogr.* **1985**, *325*, 111-126.
446. Di Bello, C.; Marigo, A.; Buso, O.; Lucchiari, A. *Tetrahedron Lett.* **1977**, 1135-1136.
447. Kaufmann, K-D.; Dölling, R. *J. Prakt. Chem.* **1978**, *320*, 814-824.
448. Birr, C.; Voss, C. In *Peptides. Synthesis, Structure, Function. Proceedings of the 7th American Peptides Symposium*; Rich, D. H.; Gross, E. Eds; Pierce Chemical Co., Rockford, Illinois, 1981, pp. 139-142.
449. Birr, C.; Pipkorn, R. *Angew. Chem., Int. Edn. Engl.* **1979**, *18*, 536-538.
450. Mergler, M.; Nyfeler, R.; Gosteli, J. *Tetrahedron Lett.* **1989**, *30*, 6741-6744.
451. Mergler, M.; Nyfeler, R.; Gosteli, J.; Tanner, R. *Tetrahedron Lett.* **1989**, *30*, 6745-6748.
452. Aimoto, S. *Bull. Chem. Soc. Japan* **1988**, *61*, 2220-2222.
453. Aimoto, S.; Mizoguchi, N.; Hojo, H.; Yoshimura, S. *Bull. Chem. Soc. Japan* **1989**, *62*, 524-531.
454. Homandberg, G. A.; Komoriya, A.; Chaiken, I. M. *Biochemistry* **1982**, *21*, 3385-3389.
455. Nakatsuka, T.; Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 3808-3810.
456. Nishino, N.; Xu, M.; Mihara, H.; Fujimoto, T. *J. Chem. Soc., Chem. Commun.* **1992**, 648-650.
457. Xaus, N.; Albericio, F.; Jorba, X.; Calvet, S.; Clapés, P.; Torres, J. L.; Valencia, G. *Int. J. Peptide Protein Res.* **1992**, *39*, 528-532.
458. Slomczynska, U.; Albericio, F.; Cárdenas, F.; Giralt, E. *Biomed. Biochim. Acta* **1991**, *50*, 567-573.
459. Blake, J. *Int. J. Peptide Protein Res.* **1981**, *17*, 273-274.
460. Blake, J.; Li, C. H. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 4055-4058.
461. Blake, J.; Westphal, M.; Li, C. H. *Int. J. Peptide Protein Res.* **1984**, *24*, 498-504.
462. Blake, J. *Int. J. Peptide Protein Res.* **1986**, *27*, 191-200.
463. Blake, J.; Yamashiro, D.; Ramasharma, K.; Li, C. H. *Int. J. Peptide Protein Res.* **1986**, *28*, 468-476.
464. Yamashiro, D.; Li, C. H. *Int. J. Peptide Protein Res.* **1988**, *31*, 322-334.
465. Cheng, H.-C.; Yamashiro, D. *Int. J. Peptide Protein Res.* **1991**, *38*, 70-78.
466. Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Japan* **1991**, *64*, 111-117.
467. Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Japan* **1992**, *65*, 3055-3063.
468. Hojo, H.; Kwon, Y. D.; Aimoto, S. In *Peptide Chemistry 1991*; Suzuki, A. Ed.; Protein Research Foundation, Osaka, Japan, 1992, pp. 115-120.
469. Schnölzer, M.; Kent, S. B. H. *Science* **1992**, *256*, 221-225.
470. See for example Gaertner, H. F.; Rose, K.; Cotton, R.; Timms, D.; Camble, R.; Offord, R. *Bioconjugate Chem.* **1992**, *3*, 262-268.
471. Celma, C.; Giralt, E. *J. Chromatogr.* **1991**, *562*, 447-458.