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CHEMICAL SYNTHESIS OF PEPTIDES AND PROTEINS

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PERSPECTIVES AND SUMMARY

Advances in the methodology of peptide synthesis have always been stimulated by the existence of interesting target molecules. Peptide synthesis originated in the work of Emil Fischer, whose goal was to prepare by total chemical synthesis molecules with the properties of the "albumoses," that is, proteins. This early work led to the first methods for the formation of "peptide" bonds, amide linkages between alpha-amino acids, and established the importance of optical purity in synthetic products. In the 1930s, attempts to synthesize a variety of small peptide substrates for the recently discovered proteolytic enzymes led to the development of the first reversible protecting group for use in peptide synthesis (1). The discovery of naturally occurring complex biologically active peptides stimulated an explosion of synthetic activity and methods development that lasted through the 1950s and 1960s and laid the basis for the modern era of peptide and protein synthesis.

The past few years have seen extraordinary progress in the chemical synthesis of peptides and proteins because of an explosive growth in biological research related to these molecules. Biologically active peptides are the objects of intense interest in the research community, and are the subjects of research programs in hundreds of industrial and academic laboratories around the world. In the past 15 years we have seen the discovery of a vast array of naturally occurring peptides with potent and specific biological activities, particularly neuropeptide hormones and neurotransmitters. As Lynch & Snyder (2) pointed out in a recent review, there are now more than 50 neuropeptides whose molecular structures are known, many of which exist within closely related families of molecules. Together with other recently discovered peptide families, such as the atrial natriuretic factors (3) and antibiotic peptides (4, 5), these provide important targets for chemical peptide synthesis. Chemical synthesis has a variety of applications in research on biologically active peptides, including proof of structure and the demonstration that the appropriate activities actually reside in a particular molecule. Most importantly, chemical synthesis allows the systematic variation of structure with the aim of developing peptides for therapeutic use.

Immune responses to synthetic peptides constitute another important area of current biological research. Sera raised against synthetic peptides are highly specific reagents for the corresponding native proteins from which the peptide amino acid sequence was derived (6, 7). These antipeptide sera are powerful reagents for the detection and characterization of proteins known only through the cDNA sequence of their genes. This and earlier work (8) have promised the development of synthetic peptide-based vaccines, and several candidate vaccines have been developed for human (9, 10) and animal (11) diseases. Of greater long-term significance is the impetus the vaccine objective has given to the rapidly growing body of research devoted to defining the structural features responsible for the immune response to synthetic peptides (12, 13).

Even more recently, there has been an upsurge in interest in protein chemistry. To a large extent, this has been stimulated by the establishment of a pharmaceutical industry based on the production of proteins by genetic engineering means. Modern molecular biology research has led to the availability of amino acid sequence data determined by the cloning and cDNA sequencing of genes. In many cases, the proteins themselves have never been isolated and their properties are largely or completely unknown. Chemical synthesis of peptide fragments, or of the whole molecule, can be used to study the properties of such gene products, and also to help redesign known proteins as second-generation products for human pharmaceutical use. Recent improvements in synthetic and analytical methodology have made the total

chemical synthesis of small proteins in the research laboratory a practical and accessible goal (14, 15).

Chemical synthesis has played an essential role in the areas of research described above. The improved synthetic capabilities that are the cornerstone of current peptide and protein research originated in a revolutionary development, the invention of the solid phase method by Bruce Merrifield (16). The magnitude of this contribution was recognized by the award of the 1984 Nobel Prize in Chemistry to Merrifield (17). Recent improvements in the solid phase method itself (18) and in associated chemistry (19) have extended the usefulness of solid phase peptide synthesis to the routine preparation of peptides up to 50 amino acid residues in length. This has made synthetic peptides readily available to a very large number of biological researchers, and has changed the way in which many research problems are approached.

This review article provides a critical perspective on the current status of the methods of chemical peptide synthesis actually used in the research laboratory. The emphasis is on the principles underlying the various techniques, with discussion of the strengths and weaknesses of different approaches in light of these principles. The aim is to provide an interpretive overview of synthetic methods that a nonspecialist can use as a guide, but that an expert will not find seriously deficient.

CHEMICAL METHODOLOGY

Strategies

The aim of peptide and protein synthesis is the production of pure compounds of known structure. Common elements in the chemical synthesis of both peptides and proteins are the assembly of protected peptide chains, their deprotection, purification, and characterization. In this section a highly critical perspective will be provided on possible strategies and on the chemistries actually in current use for the practical chemical synthesis of polypeptides. No attempt is made to be comprehensive, and the reader in search of compilations of all methodology is referred to recent reviews (20, 21), which serve that purpose.

SOLID PHASE SYNTHESIS VERSUS SOLUTION SYNTHESIS The three basic approaches to the assembly of protected peptide chains are shown in Figure 1. Fundamentally, these are combinations selected from the stepwise elongation of the peptide chain or its assembly from a number of synthetic fragments, with the chemistry being carried out in solution or on a solid support. It is a measure of the power of the solid phase principle that stepwise solid phase

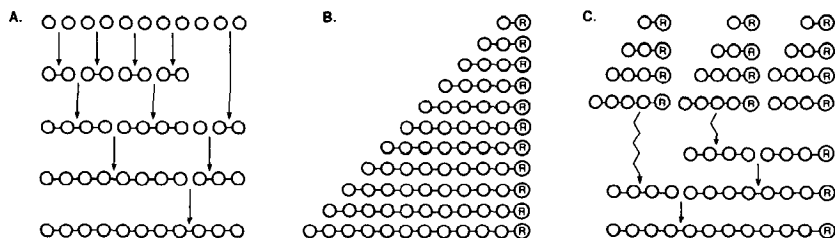


Figure 1 Three fundamental strategies for the assembly of peptide chains. **A.** Convergent fragment condensation, carried out in solution; **B.** Stepwise solid phase synthesis, in which the peptide chain is built up from the C-terminal residue while bound to a polymeric resin support; **C.** Sequential solid phase fragment condensation. The protected fragments are generated by stepwise solid phase synthesis, released in protected form, and condensed on a resin support.

peptide synthesis is used to the virtual exclusion of other synthetic methods for the preparation of synthetic peptides in the research laboratory. We briefly discuss the reasons for this.

Fragment condensation in solution Convergent fragment condensation synthesis, particularly of long peptides, has always provided the intellectual challenge sought by chemists facing the apparently mundane task of synthesizing polypeptide chains. This classical solution synthetic strategy is shown in Figure 1A. In principle, the advantage of this approach lies in the easy purification and characterization of intermediates after each peptide bond-forming step, based on ready discrimination between reactants and the target product. However, there are inherent flaws in this approach that severely limit its practical use.

The most serious of these problems is the poor solubility of some protected peptides in organic solvents. It is the common experience of researchers attempting a fragment condensation synthesis that one or more of their carefully designed fragments has such limited solubility as to render peptide bond formation impossible (22). This is because of a fundamental tendency of protected peptide chains to form intermolecular aggregates rather than interact with organic solvents (23). No general means of overcoming this problem has been developed. Other serious problems include racemization of the activated C-terminal amino acid in peptides (24), and the lack of highly resolving methods for the purification of protected peptide fragments.

Finally, the sophisticated methods worked out by the direct scientific descendants of Emil Fischer require high levels of training and experience. The fragment condensation approach is, of its essence, not a general one. While this makes it intellectually stimulating and experimentally challenging, at the same time it prohibits the use of the approach in most laboratories. In

practice, solution synthesis is so demanding that even the best laboratories can make only limited use of total synthesis due to time and expense constraints.

Stepwise solid phase synthesis The fundamental strategy of solid phase peptide synthesis (SPPS) is shown in Figure 1B. This approach to the assembly of protected peptide chains involves the attachment of the C-terminal amino acid to a "solid" support and the addition of the subsequent amino acids in a stepwise fashion (16). The techniques used are simple, particularly the purification of resin-bound intermediates by filtration and washing. However, this places much more stringent requirements on the chemistry, because all resin-bound intermediates are carried over into the final product. In the early development of solid phase peptide synthesis this was the principal objection raised to the method, but over the past decade the chemistry has been refined to the point where this is no longer a valid criticism.

Stepwise solid phase peptide synthesis has fundamental physical and chemical advantages over the solution approach (25). In particular, the attachment of a protected peptide to a swollen resin support overcomes the poor solubility of protected peptide intermediates (26). Because of this, stepwise solid phase synthesis is more generally applicable than solution methods. A comparison of the solid phase and solution methods for the synthesis of peptides is given in Table 1. Shortcomings of the stepwise solid phase approach, including sequence-dependent difficulties in chain assembly, have to a large extent been overcome as discussed in detail below, and are not obstacles to practical peptide synthesis.

Solid phase fragment condensation The third basic strategy for the assembly of protected peptide chains is shown in Figure 1C. The target sequence is

Table 1 Comparison of the solution and solid phase methods for the chemical synthesis of peptides

	Solution	Solid phase
Strategy	Segment/convergent	Stepwise
Side-chain protection	Maximal	Maximal
Solubility problems	Frequent/serious	Occasional/minor
Chemistry	Highly specialized	Simple/general
Automation	No	Yes
Purity	Excellent	Acceptable
Manpower		
Skill level:	High + experience	Less
Amount:	50×	1×

assembled by consecutive condensation of fragments on a solid support using protected fragments prepared by stepwise SPPS (27, 27a). As in stepwise SPPS, purification of resin-bound intermediates is performed by filtration and washing. One of the attractions of this approach lies in the straightforward preparation of the protected fragments. A particularly imaginative and powerful application of the solid phase method was the completely automated production of gram quantities of fully protected peptide hydrazides, ready for conversion to the azides for coupling (28).

This approach overcomes the problem of the insolubility for the resin-bound peptide intermediates. It also reduces the racemization problem by allowing the condensations to occur in the absence of base. However, the protected fragments not bound to the resin will still be difficult to purify and characterize. This is of particular importance because the fragments have been synthesized by stepwise SPPS and will contain closely related impurities. In addition, the non-resin-bound protected fragments will still be subject to the fundamental solubility problems described above.

The solid phase fragment condensation approach reduces but does not eliminate the problems inherent to the solution fragment condensation approach.

Stepwise Solid Phase Peptide Synthesis

ASSEMBLY OF THE PROTECTED PEPTIDE CHAIN The basic strategy of stepwise solid phase peptide synthesis is shown in Figure 1B, and fundamental aspects of the solid phase method are summarized in Table 2. Protected peptide chains are assembled on an insoluble support, which in macroscopic terms behaves as a solid. The most commonly used support is a cross-linked polymeric resin, produced as a suspension copolymer of styrene-divinylbenzene (S-DVB) in the form of beads approximately 50 microns in diameter. This beaded polystyrene resin was introduced by Merrifield in his original publication of the solid phase method (16), and is illustrated in Figure 2 (16a).

Table 2 Fundamental aspects of the solid phase method

-
-
1. Peptide covalently attached to a solid support
 2. "Solid" support: swollen, interpenetrating polymer network
 3. Synthesis occurs within the swollen support
 4. Peptide and resin mutually enhance one another's solvation
 5. Covalently attached peptide chains effectively in solution
 6. No effect of chain length on synthetic efficiency
 7. Reactions are rapid and obey normal (solution) kinetics
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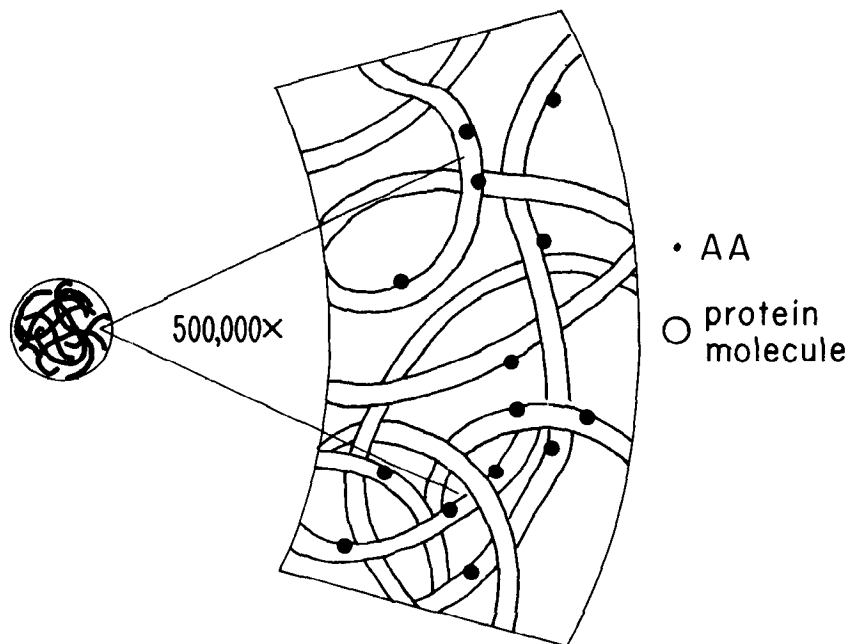


Figure 2 The peptide-resin support used in solid phase synthesis. Beads of loosely cross-linked polymer are swollen in an organic solvent and peptide synthesis takes place inside the swollen polymer network. The swollen peptide-resin is approximately 80–90% solvent by volume, and there are on the order of 10^{12} growing peptide chains per polymer bead. [After Merrifield, (16a)]

Role of the resin support The peptide-resin is a unique copolymer species that has favorable physical chemical properties that were unanticipated in the original development of the solid phase method but that are crucial to its success. In particular, the swollen peptide-resin exerts a strong solvating effect on the covalently attached peptide chains. The origin of this solubilizing effect has been shown to lie in the thermodynamics of linear peptides covalently attached by one end only to an interpenetrating polymer network (29). The net result is that peptide-resin beads swell to an ever greater extent as the protected peptide chain is elongated.

There are several consequences of this. First, there is no effective limit to the chain length or amount of peptide that can be grown within the swollen resin beads. Thus, very high initial substitutions (1 to 8+ mmol/g S-DVB; i.e. 10–90% of the polystyrene aromatic rings bearing a peptide chain) can be used in the solid phase synthesis of peptides and proteins (S. B. H. Kent, unpublished), reducing the amount of solvent needed for synthesis (30) and allowing the use of high concentrations of reagents for deprotection and

coupling. However, the use of high substitutions of the resin in SPPS can lead to increased aggregation of peptide chains within the swollen support for some “difficult” sequences, with increased problems in chain assembly (see *Difficult sequences* section, below).

Implicit in the picture of the peptide-resin presented above (Figure 2) is the fact that the resin-bound peptide is effectively in solution within the swollen support. This has been directly confirmed by nmr measurements that show rotational and diffusional mobilities typical of peptides in free solution (31), and explains at a molecular level the normal (solution) kinetics observed for reaction between soluble activated amino acids and the resin-bound peptide chain. The highly solvated state of the peptide-resin should be maintained throughout the synthetic cycle in order to maximize reaction yields. Maximal swelling also allows unhindered diffusion of molecules between the inside and outside of the swollen beads and is essential for efficient washing of the peptide-resin. Protocols that use solvents to shrink the peptide-resin will hinder diffusion and reduce the effectiveness of SPPS, contrary to popular belief (32).

The properties of peptide-resins are not substantially modified by the chemical nature of the initial chemical moiety pendant on the resin polymer backbone. Polystyrene (16) and polyacrylamide (33, 34) resins (often erroneously termed “polyamide” supports) are both polyethylene derivatives and are equally effective supports for the synthesis of protected peptides in either polar or relatively apolar organic solvents. This is contrary to oft-repeated claims with respect to the poor solvation properties of polystyrene-based peptide-resins in polar organic solvents (35, 36). This incorrect picture stemmed from a fundamental misunderstanding of the physical chemistry involved (34–36), and led to erroneous predictions, such as the inability of polar solvents [e.g. dimethylformamide (DMF)] to swell polystyrene resins, which have not been borne out by common experimental practice (16, 18, 29).

Protecting group tactics In the assembly of peptide chains by chemical means it is necessary to protect reactive functionalities other than those involved in peptide bond formation. Such protection must be reversible without damage to the assembled peptide chain. For stepwise SPPS at least two types of protection are necessary: temporary protection of the alpha-amino group, removable after formation of each peptide bond; and, side chain protection removable after assembly of the complete peptide chain.

Over the past four decades an enormous amount of chemical ingenuity has gone into the development of protecting groups for use in synthetic peptide chemistry (37). In practice, the strategy of maximal protection of all side chain functionalities (except Asn, Gln side chain amides) has been routinely

employed. There are two sets of side chain-protecting groups, which are dictated by the N-alpha protecting group strategy used: the Boc-amino acid, or the Fmoc-amino acid approaches. These N-alpha-protecting groups were developed by Louis Carpino (38, 40a-c) and by others (39a,b) and together are used for essentially all current practical peptide synthesis.

The fundamental operations of one cycle of chain extension in stepwise SPPS using Boc- or Fmoc-chemistries are shown in Figure 3, and the relative characteristics of the two sets of protecting groups are summarized in Table 3. Both of these protecting group strategies are practical ways of achieving the total chemical synthesis of peptides by stepwise SPPS. Their use has unfortunately been clouded by rhetoric, rather than based on factual chemical data, and has broken down along national lines with the Boc-chemistry used predominantly in Japan and North America, and the Fmoc-chemistry used in Europe.

The N-alpha Boc, benzyl-based side chain protection strategy relies on the principle of graduated acid lability of the protecting groups. It is a more mature chemistry, and a great deal of effort has been put into identifying chemical side reactions and minimizing their effects (20, 21). Despite frequent assertions to the contrary, there are no substantiated reports of damage to the protected peptide chain due to repeated acidolytic removal of the N-alpha Boc group by trifluoroacetic acid during chain assembly. Boc-protected amino acids are inexpensive and available in high purity. Although work could usefully be done on His and Trp side chain protection, satisfactory protecting groups exist for all amino acids (21, 41, 42).

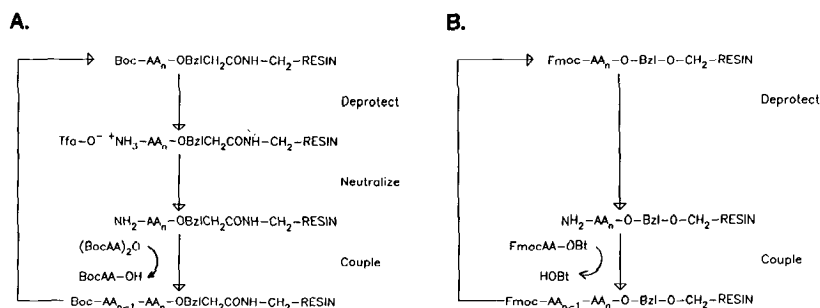


Figure 3 Chemistries used for the assembly of protected peptide chains by stepwise solid phase synthesis. **A.** N-alpha Boc-chemistry. The N-alpha Boc group is removed by treatment with trifluoroacetic acid. After washing, the resulting amine salt is neutralized with a tertiary amine. The peptide bond is formed by reaction of the resin-bound peptide with a Boc-amino acid symmetric anhydride. **B.** N-alpha Fmoc-chemistry. The N-alpha Fmoc group is removed by treatment with a secondary amine, such as piperidine, to give the neutral peptide-resin. After washing, the peptide bond is formed by reaction with the Fmoc-amino acid hydroxy-benzotriazole active ester.

The Fmoc-based strategy uses different mechanisms for removal of the N-alpha- and side chain-protecting groups: a secondary amine for the N-alpha-protecting group, with side chain-protecting groups labile to trifluoroacetic acid (Table 3). Fmoc-based SPPS was first introduced by Meienhofer (43) only 10 years ago. Less is known about side reactions, and satisfactory side chain-protecting groups (44) have not been available for all amino acid residues, particularly His (45) and Arg (45, 46), and to some extent Cys. However, thanks to the work of a number of laboratories (47-49) this situation is virtually corrected, making a full range of useful Fmoc-amino acid derivatives available (Table 3). A major problem with the Fmoc approach has been the limited solubilities of the activated Fmoc-amino acids in the solvents commonly used in peptide synthesis, leading to reports of extraordinarily slow coupling kinetics (50).

Peptide bond formation In practice, despite many alternatives, essentially all peptide bonds are currently formed using the coupling agent dicyclohexylcarbodiimide (DCC),¹ either through "in situ" activation of the carboxyl component in the presence of the amino component, or through the

Table 3 Protected amino acid derivatives used in stepwise solid phase peptide synthesis

A. General characteristics

	N ^{alpha} Boc	N ^{alpha} Fmoc
N ^{alpha} lability:	TFA (S _N 1)	Piperidine (beta-elimination)
Side-chain groups:	Benzyl-based	tert. Butyl-based
Side-chain lability:	HF, RSO ₃ H	TFA
Cost/purity:	Cheap/excellent	Expensive/good
Solubilities:	High	Low-moderate
Period in use:	30 years	10 years

B. Side chain-protecting groups

Amino acid	N ^{alpha} Boc	N ^{alpha} Fmoc
Asp, Glu	OBzl, OcHxl	Otert. Butyl
Ser, Thr	Bzl	tert. Butyl
Lys	2ClZ	Boc
Tyr	BrZ	tert. Butyl
Cys	4MeBzl, MeOBzl	Acm, tert. Butyl
His	DNP, Tos ^a , Z	Trityl, Boc
Arg	Tos, Mts	Mtr ^a , Pmc
Trp	Formyl ^a	unprotected
Met, Asn, Gln	unprotected	unprotected

^aNot completely satisfactory

prior formation of a symmetric anhydride or an active ester. Side reactions, which can occur with Arg, Asn, and Gln, are minimized by coupling as the HOBt esters (51). Most significantly, racemization does not occur in stepwise SPPS (52), even though alkoxyoxazolones can be formed from activated N-alpha urethane-protected amino acids (53), because these are less susceptible to deprotonation and solid phase peptide bond formation is carried out in the absence of added base.

A breakthrough in SPPS methodology occurred with the development of fully automated synthesis involving the separate formation of symmetric anhydrides or active esters immediately prior to use (54, 55). This provided a new dimension in flexibility of activation chemistry for SPPS, allowing the use of appropriate activated amino acid derivatives in optimal solvents. Amino acid symmetric anhydrides, the favored derivative for peptide bond formation (56), are formed rapidly by reaction with DCC in dichloromethane, but are not formed to any appreciable extent in polar solvents such as DMF. Using automated chemistry adjusted for each amino acid, the symmetric anhydride is formed in dichloromethane and then converted to a solution in DMF for optimal chain assembly (54, 55, 57).

Coupling reactions carried out in polar solvents can give dramatic improvement in yields compared with earlier DCM-based protocols. Similar results can be obtained in manual SPPS by "in situ" activation with DCC, followed by addition of DMF to the coupling reaction (S. B. H. Kent, unpublished). The automated separate activation and solvent exchange avoids the problem of storage of expensive, labile activated amino acids in favor of the use of the readily available, stable Fmoc- or Boc-amino acids. Some work has been carried out with alternative activated derivatives, particularly of the Fmoc-amino acids. Thus, active esters of pentafluorophenol have been proposed for use in SPPS (58), as have active esters of 3,4-dihydro-3-hydroxy-4-oxobenzotriazine (HOOBt) (59). This latter derivative is known to generate a chain-terminating by-product (60), and due care must be taken to avoid this known side reaction (59).

Documentation of chain assembly In solid phase synthesis, purification consists only of filtration and washing of the resin-bound intermediates. Because of this, it is necessary to drive all reactions involved in the chain assembly to completion. It is important to have accurate information on the yields at all steps of the chain assembly so that the chemistry can be improved in general and, if necessary, for a particular synthesis. Documentation of the efficiency of chain assembly is also the first step in ensuring the production of a high-purity peptide of known structure (see *Characterization* section below).

Recently, a major advance in SPPS has been accomplished by the develop-

ment of a practical and effective method of taking, under computer control, samples of the peptide-resin at any stage of an automated synthesis (see below). After completion of the synthesis, a set of peptide-resin samples representing all intermediate stages of the chain assembly is available for analysis. For example, the quantitative ninhydrin reaction (61) can be used to measure residual uncoupled amine at the end of each cycle of amino acid addition, as an indicator of coupling yields. The method is sensitive and accurate, giving reliable data down to less than 0.1% residual amine (>99.9% coupling) with a reproducibility of $\pm 0.05\%$. The conditions of the ninhydrin reaction are sufficiently vigorous (5–10 min at 100°C in pyridine) to reveal amine that was unavailable for coupling, e.g. from aggregates (see *Difficult sequences* section below), or from Schiff's base formation (62).

Quantitative Edman degradation of the protected peptide-resin product can also be used to document the efficiency of chain assembly in Boc-chemistry stepwise SPPS (63). This provides essential confirmation of the correct sequence of amino acids in the product, and can also give accurate data on the cumulative efficiency of the N-alpha Boc removal steps and the coupling yields (63a). The absence of side reactions in the Edman degradation of protected peptides results in very low backgrounds and higher yields, allowing longer runs and thus making the data more reliable and accurate (63). Data obtained in this way are in excellent agreement with quantitative ninhydrin data, confirming the validity of the latter as an accurate indicator of the efficiency of chain assembly for Boc-chemistry stepwise SPPS (57). This agreement also shows complete removal of the N-alpha Boc group at each step of the chain assembly, a result also obtained by an elegant and sensitive assay of residual Boc groups by pyrolysis-gas chromatography of peptide-resin samples taken after the deprotection step (64). Sequencing of the protected resin-bound peptides is possible only with side chain-protecting groups and peptide-resin linkages, both of which are stable to the acid conditions of the Edman degradation. Thus this technique is not applicable to the resin-bound products of standard Fmoc-chemistry SPPS.

Feedback control of automated SPPS (65) would require a rapid, noninterfering method for sensitive, accurate analysis at key steps of each synthetic cycle as it was occurring. In addition, it must be possible to carry out more effective chemistry to correct any incomplete reactions detected, because simply increasing the time of the coupling step or repetition of the coupling (double coupling) under the same conditions has little or no effect (26, 57). Up to the present, neither the effective means of monitoring nor appropriate corrective measures have existed.

Side reactions Over the past 10 years, intensive efforts have resulted in the identification and correction of side reactions occurring at every cycle of

Boc-chemistry stepwise SPPS, regardless of amino acid sequence (62, 66–68). In many instances, the side reactions were caused by the presence on the resin support of extraneous functional groups that participated in the chemistry (62, 66). The problems were cured by the use of chemically defined, clean, peptide-resins that were stable to the conditions of chain assembly (67). As shown in Table 4, this has resulted in a reduction of the total level of side reactions at every cycle of SPPS from approximately 5% per cycle to less than 0.1% per cycle. In the past, these unavoidable side reactions were the single greatest source of problems in Boc-chemistry stepwise SPPS. Close examination of Fmoc-chemistry SPPS will reveal similar chronic side reactions peculiar to that chemistry (e.g. 69). Regardless of the chemical tactics used in stepwise SPPS, any side reactions must be identified, their mechanisms elucidated, and their occurrence eliminated.

Residue- or sequence-specific side reactions have also been the subject of extensive investigation in Boc-chemistry SPPS. Thus, the mechanism of diketopiperazine formation for Pro-containing dipeptide-resins has been elucidated and conditions adjusted to reduce this side reaction to negligible levels (70). Similarly, the mechanism of pyrrolidone carboxylic acid formation from N-terminal glutamine has been elucidated and conditions worked out to minimize its occurrence (71). In both cases, the recommended conditions for avoiding these side reactions coincide with the preferred conditions for optimized chain assembly, namely deprotection with high concentrations of trifluoroacetic acid (TFA), rapid washing, and coupling with separately activated amino acids in polar solvents (see below).

Difficult sequences The most serious potential problem in stepwise SPPS is incomplete peptide bond formation, giving rise to peptides with one or more internal amino acids missing but with properties similar to the target sequence. Even after the elimination of chronically occurring side reactions (see

Table 4 Chronic side reactions in SPPS chain assembly

	Standard resin	Clean, stable resin
Chain loss ^a	1.3%	0.01%
Trifluoroacetylation ^b	2.0%	0.02%
Deletion ^c	1.0%	0.05%
secBoc termination ^d	0.7%	0.02%
	5.0% per residue	0.1% per residue

^aPrevented by use of TFA-stable peptide-resin linkage (67).

^bPrevented by use of hydroxymethyl-free resin and TFA-stable peptide-resin linkage (66).

^cPrevented by the use of aldehyde-free resin (62).

^dPrevented by the use of pure tert.Boc-amino acids (68).

above), incomplete coupling can still occur and is more prevalent in some sequences than in others.

Based on a study of 750 Boc-chemistry coupling reactions (72), it was concluded that difficult sequences have the characteristics shown in Table 5. A similar phenomenon has been observed in Fmoc-chemistry SPPS (36, 73). The primary source of the problem was the sequence-dependent tendency of the resin-bound peptide to form intermolecular aggregates, despite the favorable effects of covalent attachment to the swollen polymer support (see above) (26). In a definitive experiment (74), it was shown that difficult coupling was due to the internal sequence of the peptide, rather than the identity of the two amino acids involved in peptide bond formation. The use of high resin loadings exaggerates the phenomenon (75). A general way of overcoming this problem was to carry out couplings in a dipolar aprotic solvent such as dimethylformamide, a good solvent for protected peptide chains. Combined with the favorable physical chemical properties of the peptide-resin discussed above, this has resulted in a universally applicable chain assembly chemistry for the chemical synthesis of peptides using stepwise SPPS (26).

Nevertheless, sequence-dependent coupling difficulties still arise at a low level, typically in the 0.5–2% range. The effect of repetition of the coupling step in the SPPS cycle is minimal, averaging only a 0.3% per residue increase for the second coupling with no further increase with subsequent couplings (26, 57). Approaches to the solution of this problem include the use of heat in the coupling step (76), and the quantitative conversion of residual unreacted resin-bound peptide chains to nonreactive (terminated) species (77). This “capping” procedure, in combination with suitable purification strategies to select out only full-length peptide chains, could eliminate the formation of deletion peptides. Due note should be taken of the fact that it has never been proved that this strategy works in practice. It must be demonstrated that it is possible to quantitatively block peptide chains that did not react with an activated amino acid derivative, and that additional side reactions are not caused in the process.

Sequence-dependent variability in the rate and extent of removal of the

Table 5 Characteristics of difficult sequences in stepwise SPPS (72)

-
1. 2–15% incomplete coupling reaction
 2. Several amino acid residues in a row
 3. Maximum occurrence at 5–15 residues from the resin
 4. Sequence dependent
 5. Loading dependent
 6. Activated beta-branched amino acids (Ile, Thr, Val) exaggerate the poor couplings
 7. Weak or no correlation with N-terminal amino acid of peptide-resin
-

Mechanism: intermolecular aggregation of the protected peptide chains (26).

N-alpha Fmoc group has been observed (50). This represents a substantial disadvantage of Fmoc-chemistry stepwise SPPS compared with the Boc-chemistry approach. Because of the use of TFA, an excellent solvent for protected peptide chains, removal of the N-alpha Boc group is generally complete and shows no sequence dependence.

Optimized solid phase peptide synthesis Consideration of the principles and chemistries described above has allowed the development of substantially improved methods of peptide synthesis over the past few years. These are summarized in Table 6. Particularly in the case of the Boc-chemistry SPPS, these methods represent a highly optimized synthetic chemistry that takes advantage of the thermodynamics of the peptide-resin to maximize solvation and minimize the occurrence and severity of sequence-dependent difficulties (26). Effective swelling solvents such as DMF are used throughout the synthetic cycle, with rapid and efficient flow washes of the peptide-resin suspension (18). The chemistry has been adjusted to minimize or eliminate side reactions in the chain assembly. Suitable resin loadings have been used to reduce solvent and reactant volumes, thus keeping concentrations as high as possible and increasing the rates and yields of reactions. These optimized protocols have been adapted for automated assembly of the protected peptide chain.

Automated synthesis Merrifield's original vision of the stepwise solid phase method included the goal of a fully automated chain assembly chemistry (16).

Table 6 Optimized Boc-chemistry for the solid phase synthesis of peptides (18, 57, 88)

Strategy:	Stepwise SPPS
Resin:	Copoly(styrene-divinylbenzene)
Peptide-resin linkage:	4-(carboxamidomethyl)benzyl ester 4-methylbenzhydrylamide
Protected amino acids:	N ^{alpha} Boc, benzyl-based (side chain)
Coupling (in DMF):	Symmetric anhydrides; HOBt esters
Washing protocols:	Flow washes (DMF)
Documentation:	Quantitative ninhydrin; Edman degradation
Automation:	Microprocessor controlled; Parallel activation/solvent exchange; Resin sampling
Deprotection:	HF; TFMSA
Purification:	Reverse-phase HPLC
Characterization:	Analytical HPLC; amino acid analysis; Edman degradation; peptide mapping; SDS-PAGE; isoelectric focusing; mass spectrometry

Several versions of instruments for this purpose soon appeared, but in retrospect they can be seen to have been premature in that the chemistry was not sufficiently developed to allow effective automation of the method. The first instrument incorporating an optimized solid phase peptide synthesis chemistry was described in 1984 (54, 55) and is now found in several hundred laboratories around the world.

This automated peptide synthesizer carries out optimized chain assembly chemistries as summarized in Table 6 (14, 18, 26, 57). Solid amino acids are automatically dissolved from the dry powder in a sequential cartridge delivery system, and converted either to the symmetric anhydride or to an active (HOBt) ester immediately before use. For the symmetric anhydrides, a solvent exchange/concentration operation yields the activated derivative in DMF solution ready for addition to the deprotected, neutralized peptide-resin. Simultaneously with the dissolution and activation of the amino acid, the peptide-resin is deprotected, washed, and neutralized (for Boc-chemistry) in DMF, and is ready to receive the activated derivative. After coupling for a preset time, the peptide-resin is washed and a small (few mg) sample of the peptide-resin deposited in a fraction collector tube for documentation of the chain assembly (see above).

Unique features of this instrument included the separate activation/solvent exchange chemistries, and the automatic use of specific chemistries for different amino acid residues, or different chemistries for the same residue at different positions in the sequence. Furthermore, the chemistries, which are performed simultaneously, are automatically coordinated for maximum overlap, giving the minimum overall cycle times for the synthesis. Together with extremely reproducible operation and the automatic provision of samples to evaluate synthetic efficiencies, these capabilities have made the instrument an excellent tool for the development of new methods for SPPS. Applications of new chemistries on this instrument have been described, particularly for the chemical synthesis of proteins (14) (see below), and for the rapid synthesis of peptides (18).

Future developments The future may see the use of more reactive amino acid derivatives in SPPS, such as the acid chlorides, as recently suggested for solution synthesis by Carpino (79). If suitable means of formation are found for the acid chloride or comparably reactive derivatives, this will allow much more rapid coupling reactions. The problem of sequence-dependent coupling difficulties may be completely eliminated by the use of rigid supports, such as glass fiber discs (80) or porous glass beads (81), which may, with sufficiently low loadings, prevent the formation of the intermolecular aggregates that interfere with coupling. In addition, if the problem of limited solubility of the activated amino acid derivatives can be overcome, the Fmoc-chemistry SPPS

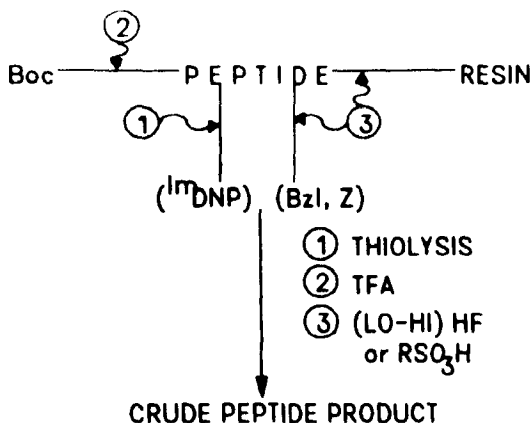
may predominate in the future, because of the convenience of TFA-based deprotection (46). Finally, alternative protecting group chemistries promise increased flexibility for solid phase synthesis and may eventually offer a practical alternative to currently used chemistries (81a).

DEPROTECTION AND CLEAVAGE FROM THE RESIN SUPPORT After the protected peptide chain has been assembled on the resin support, all the protecting groups must be removed and the covalent link between the resin support and the peptide cleaved in order to release the free peptide into solution.

Standard N-alpha Fmoc, tButyl-based side chain-protection SPPS chemistry is compatible with final deprotection in TFA, with added scavengers such as water or phenol to trap reactive coproducts (46, 82). Thus far, there are relatively few studies of shortcomings of this final deprotection scheme, with the exception of incomplete removal (46) or rearrangement (83) of the Mtr-group from Arg residues. Other side reactions do occur and remain to be studied in detail.

The operations involved in the final deprotection of peptides synthesized with N-alpha Boc, benzyl-based side chain-protection chemistry are summarized in Figure 4. In a very important contribution to synthetic peptide chemistry, a two-stage deprotection protocol using hydrogen fluoride (19) was introduced for Boc-chemistry SPPS with the protecting groups given in Table 3B. The same two-stage approach was subsequently extended to the use of trifluoromethanesulfonic acid (TFMSA) in TFA (84). In the first stage, removal of most protecting groups takes place by S_N2 acidolysis [a mechanism of side chain deprotection first introduced by Kiso (85)] at low temperature in a low-acidity function medium consisting primarily of dimethyl sulfide and either HF or TFA/TFMSA. The peptide-resin bond is stable to this step. After removal of the coproducts and reagents used for this first step, the remaining protecting groups are removed and the peptide cleaved from the resin by means of S_N1 acidolysis at low temperature in a high-acidity function medium. Either of these strong acid cleavage protocols, or more standard protocols using HF (86–88) or TFMSA (89, 90), give very satisfactory products.

The principal drawback to the use of HF in synthetic peptide chemistry is its extraordinary toxicity, which necessitates the use of expensive apparatus for safe use. TFMSA, on the other hand, is much less toxic than HF and can be used in ordinary laboratory glassware. (*CAUTION: both TFMSA and TFA can cause severe burns. Appropriate safety precautions must be taken when handling these chemicals.) Unlike HF, which is a weak acid in aqueous solution (91), TFMSA is a very strong acid in the presence of water and must be removed from the peptide before lyophilization to ensure a nonhygroscopic-



- ① THIOLYSIS
- ② TFA
- ③ (LO-HI) HF or RSO_3H

NOTES: ③ peptide- $\text{OCH}_2\text{BzICH}_2\text{CONH-CH}_2\text{-Resin}$ gives peptide alpha- COOH .
 peptide- NH(4MeBHA)-Resin gives peptide alpha- CONH_2 .

Figure 4 Deprotection of the assembled peptide and cleavage from the resin. ① the histidine DNP side chain-protecting group is removed by thiolysis; ② the N-alpha Boc group is removed; ③ all remaining protecting groups and the linkage to the resin are cleaved by treatment with strong acid (HF or TFMSA). The crude, deprotected peptide is released into solution, as the free acid from substituted benzyl ester resin, or as the alpha-carboxamide from benzhydrylamine resin.

ic, stable product. This is best achieved by passing the precipitated crude peptide, dissolved in dilute acetic acid, through a quaternary ammonium acetate column to give the peptide as the acetate salt. Similar treatment of HF-cleaved peptides is recommended to control counterion composition (S. Sakakibara, personal communication).

Several residue-specific problems can occur in the strong acid deprotection of peptides. Reaction of the Glu side chain with aromatic scavengers is controlled by reduced temperature reaction (92). Cyclization of the Asp side chain to form a succinimide moiety can occur both in chain assembly and in the final deprotection; this has been minimized by new side chain-protecting groups and temperature control in the deprotection (41). Reaction of the methionine side chain thioether with tert.butylating by-products formed from the N-alpha Boc group in HF (92a) or TFMSA (92b) can be avoided by removal of the Boc group prior to the final deprotection (Figure 4). Other residue-specific side reactions have been described (88). Contrary to recent reports (46), Asp-Pro bonds are completely stable to strong acid deprotection,

but are labile to the dilute acetic acid often used in the work up of the cleaved peptide. Well-known artefacts such as this can be avoided by the proper design of work up conditions.

Purification of the Crude Peptide

The crude product resulting from the deprotection/cleavage of a peptide synthesized by stepwise SPPS contains a variety of by-products in addition to the target compound. Even with optimized SPPS, there will typically be small amounts of a family of peptides with a single internal amino acid missing from the target sequence (deletion peptides, formed during chain assembly), and a family of peptides with chemical modifications due to side reactions in the final deprotection. The key to successful purification is the initial formation of a high-purity crude product. With the synthetic procedures summarized in Table 6, crude peptides in the 30–45-residue range are typically 85–60 mole percent the target peptide.

In principle, purification should be carried out by the consecutive application of several procedures based on different separation mechanisms. In practice in the research laboratory, most peptides can be purified in a single step by preparative reverse-phase HPLC, as summarized in Figure 5 (88).

CRUDE PEPTIDE PRODUCT

1. 6M GN.HCL, 20% HS-CH₂-CH₂-OH
pH 8.5, 2 hr, 50°C
2. OXIDIZE (DISULFIDES)
3. PREP. RP-HPLC (C₄)

PURIFIED PEPTIDE

HOMOGENEITY

Anal. RP-HPLC
(vary pH; C₄, C₈)

Isoelectric focusing
(immobilized pH. grad.)

COVALENT STRUCTURE

Sequence analysis
Mass spectrometry

Peptide mapping
(disulfides)

Figure 5 Purification and characterization of the synthetic peptide. The crude peptide is incubated at basic pH with thiol to remove any residual protecting groups, reduce any oxidized methionine, and reverse any strong acid-induced rearrangements. After formation of disulfide bonds (if appropriate), the peptide is purified by reverse-phase HPLC. The homogeneity of the product is established by HPLC under different conditions, or by isoelectric focusing. The covalent structure of the synthetic peptide is established by amino acid analysis, by sequence determination in conjunction with peptide mapping, and confirmed by mass spectrometry.

Open column reverse-phase chromatography can also be used to purify synthetic peptides with similar efficiencies. Another useful approach for the purification of synthetic peptides is displacement chromatography on reverse-phase HPLC supports (93). Reverse-phase HPLC may not be satisfactory for the purification of products containing a high proportion of late-eluting impurities, because the more hydrophobic components can displace the target peptide.

At about 50 amino acid residues in length, this simple HPLC approach to peptide purification generally begins to break down. This can be attributed to the onset of relatively stable, slowly exchanging folded structures of the peptide chain (94). Thus, a single molecular species will give a variety of chromatographically separable components, making the separation an impossibly complex task. Either the peptide chain must be folded, prior to purification, into a single structure stable to the conditions of reverse-phase HPLC, or conformationally independent separation techniques such as isoelectric focusing in immobilized pH gradients, ion exchange chromatography, and high-resolution gel filtration (all in the presence of appropriate denaturants) must be used to purify the target polypeptide chain prior to folding to form the target protein. Together with the folding itself, the purification of the long peptide chains that fold to form proteins presents the most important current challenge in chemical peptide synthesis.

Characterization of the Synthetic Product

The stepwise solid phase method is not an unambiguous synthetic technique. Because the intermediate resin-bound products are not characterized, and because of the large number of operations involved in a synthesis, there is abundant opportunity for error. Accurate documentation of a carefully controlled synthetic process, as described above, substantially reduces this ambiguity and is the first step in obtaining a reliable product of known structure. The synthetic product must be fully characterized, both with respect to homogeneity and covalent structure, as summarized in Figure 5.

Homogeneity of the purified product should be determined, under conditions different from those used in the purification, by highly resolving analytical techniques such as reverse-phase HPLC for peptides, and isoelectric focusing for proteins (88). In the past, the identity of the purified peptide was established by careful comparison with the corresponding naturally occurring compound (95). More recently, where the goal of synthesis has become to relate the systematic variation of structure to effects on activity, it has become necessary to independently prove the molecular identity of synthetic peptides and proteins by complete structural characterization of the purified synthetic product.

The first step in the determination of the covalent structure of a synthetic peptide is measurement of its amino acid composition. This is most accurately

and reliably performed by ion exchange chromatography after hydrolysis, with post column ninhydrin detection according to the classic methods of Spackman, Stein, & Moore (96). Sequence analysis of the peptide is performed by the automated Edman degradation (97), in combination with peptide mapping and amino acid analysis/sequence determination of the separated fragments for longer peptides.

The most important recent development in the structural characterization of synthetic peptides is the use of mass spectrometry. Technical developments in that field have made the ionization and accurate mass analysis of large peptides and small proteins feasible at the precise juncture where improved synthetic methods have led to increased interest in these compounds. Because of unpredictable variation in ionization efficiencies, the product peptide should first be purified to homogeneity, then analyzed by mass spectrometry. Techniques such as fast atom bombardment ionization in a standard mass spectrometer or plasma desorption/time-of-flight mass spectrometry (97a) routinely give an accurate mass measurement for the molecular ion. Identity of this measurement with the predicted mass for the target sequence proves the absence of covalent modifications of the product and, together with the Edman degradation and peptide mapping data, unambiguously defines the covalent structure of the synthetic peptide or protein.

At present only a limited amount of sequence data can be directly obtained from mass spectral measurements, but in the near future it will be possible to routinely determine the complete covalent structure of purified synthetic peptides by mass spectrometry. Particularly exciting developments are two-stage techniques involving ion storage followed by fragmentation/mass analysis (98a,b), and the use of wet chemistry, such as enzymatic digestions carried out in the source of the mass spectrometer, in combination with mass spectral measurements (99).

The next stage in the characterization of synthetic peptides, and especially synthetic proteins, will be the routine determination of the three-dimensional structure of the purified products. This will be particularly important for systematic structure-function studies of proteins, and for the rational design of novel proteins of predetermined structure. Nuclear magnetic resonance techniques (100) will be the most effective for this purpose, particularly in combination with synthetic incorporation of probe nuclei (e.g. ^{13}C , ^{15}N) and nuclei to simplify nmr spectra (e.g. ^2H) at specific sites in the molecules. This will allow the precise characterization of similarities and differences in the three-dimensional structure of synthetic protein molecules in solution.

SYNTHETIC PEPTIDES

The aim of peptide synthesis is the production of pure compounds of defined structure. The optimized stepwise solid phase synthetic methodology de-

scribed above is quite effective in this respect and essentially any peptide up to about 45 residues in length can be prepared in a straightforward fashion (18). An example of this is shown in Figure 6. The crude synthetic 31-residue peptide hormone human beta-endorphin contained 84 mol% the target peptide and was readily purified to a very high degree of homogeneity.

Goals of peptide synthesis in the research laboratory include the verification of the structure and/or the activity of a naturally occurring peptide, the exploration of structure-function relationships in biologically active peptides, and the design of modified molecules with more desirable properties. One of the finest achievements of synthetic peptide chemistry in the determination of the structure of a natural product was the total chemical synthesis of several candidate structures of the membrane-active peptide alamethicin, using a novel and elegant fragment solid phase approach (101a,b). The resulting data

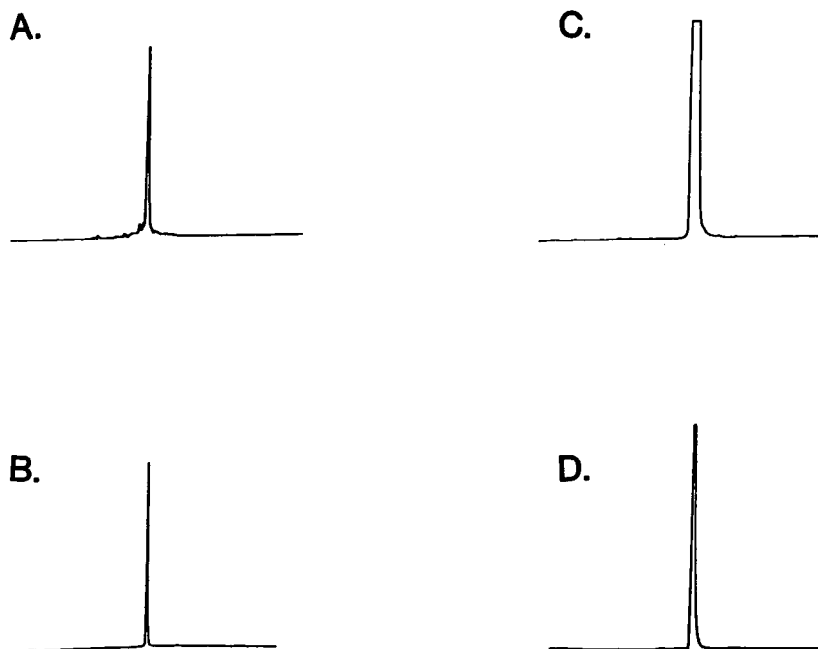


Figure 6 The neuropeptide hormone human beta-endorphin (31 amino acid residues) was synthesized according to the improved methods of SPPS described in the text (18). **A.** Reverse-phase HPLC analysis of crude product after TFMSA deprotection and cleavage. Vydac C4 column, 0–60% CH_3CN gradient vs 0.1% TFA in water, detection at 214 nm. The target peptide was 84 mol% of the total products. **B.** Synthetic peptide after purification by reverse-phase HPLC. Analytical conditions as in **A.** **C.** Highly overloaded analytical HPLC of the purified peptide. Conditions as in **A** and **B.** **D.** Reanalysis of the purified synthetic beta-endorphin by reverse-phase HPLC at pH 8.

established the correct structure of this unusual molecule, corrected the structure assigned to this peptide on the basis of nmr data, and served as the essential basis to correct misinterpretation of a mass spectral study (102) of the peptide.

There are relatively few biologically active peptides for which a definitive understanding of structure-function relationships has been achieved. A typical body of such work, on growth hormone releasing factors, has recently been summarized (103). The first steps toward the design of secondary structural elements important for biological activity have included the hypothesis that amphipathic helices may be common motifs in peptide hormones (104).

An important development in the research use of peptide synthesis has been the parallel synthesis of large numbers of peptides. This concept was introduced by Geysen and his coworkers (105) for the simultaneous preparation of hundreds of peptides bound to solid supports for use in immunoassay screening to locate antibody-binding sites (B-cell epitopes) in viral surface proteins. The approach is quite general, and has been extended to the parallel synthesis of free peptides (106). These techniques have great utility for specialized research problems, particularly for the generation of large numbers of analogues for rapid screening in pharmaceutical research. The caveats (above) concerning the ambiguity of SPPS and the need for direct confirmation of the structure of the product peptides should be borne in mind.

Another important development in biological research has been the systematic generation of antisera to synthetic peptides corresponding to defined regions of the amino acid sequence of a protein. This technique was first introduced by Walter & Doolittle (6), and has proved to be of great utility in the research laboratory. Antisera raised against peptides corresponding to the N- or C-terminal regions of proteins and, more rarely, against internal sequences (7), selectively recognize and have high affinities for the native protein. These antisera are enormously useful for cytochemical studies, for use in Western blots to identify proteins after separation by gel electrophoresis, and for immunoaffinity purification of proteins that, in some cases, were known only through the predicted amino acid sequence from cDNA cloning and sequencing (6). In a novel and important application, antisera raised against synthetic peptides have also been used to systematically locate the structures on viral surface proteins responsible for virus binding to cell-surface receptors (107). It was then possible to design and synthesize a peptide that would mimic this structure and inhibit virus binding. A similar approach was recently used to locate the AIDS virus-binding site on the CD4 cell-surface protein found on T helper cells (107a). These findings also illustrated the power of an alternative approach to epitope location, the use of long (20–30 residue) peptides (9), an approach supported by the recently

reported structure of the major histocompatibility antigen (type I) molecule that showed an antigen-binding cleft accommodating 15–20 amino acids (108).

The use of synthetic peptides as vaccines to immunize against human pathogens was first proposed 20 years ago (8). Recent progress in this field has included the realization that synthetic peptides can contain both B-cell epitopes and T-cell epitopes, structures that induce the proliferation of T_{helper} cells (12, 13). One of the first such peptides identified was derived from the M-protein of the hepatitis B virus envelope (109), and contained immunodominant B- and T-cell viral epitopes. Such peptides can be powerful immunogens without attachment to an immunogenic carrier, and are thus more attractive candidates for use in a vaccine.

SYNTHETIC PROTEINS

One of the most exciting developments in synthetic peptide chemistry in the past few years has been the reproducible total chemical synthesis of proteins (14, 15). As with peptides, the strategic aim is to define structure-function relationships in order to develop a predictive understanding of protein structure and properties. The ultimate goal is to design proteins (such as enzymes, antibodies, hormones, mitogens, and structural proteins) with predetermined specific properties. The properties of proteins are determined by the precise three-dimensional structure of the folded peptide chain. In this sense, the most fundamental property of the linear sequence of amino acids in a protein is its ability to code for the formation of this stable folded structure (110). Only when the mechanisms that determine protein folding are understood can the rational design of proteins be undertaken.

The goal of protein synthesis must be the production of homogeneous molecular species of defined covalent and three-dimensional structure. A notable early achievement was the total chemical synthesis of human insulin by classical fragment condensation solution methods, with chemically directed pairing of Cys residues to form disulfide bonds (111). Both the naturally occurring structure and a number of alternatively paired disulfide analogues were synthesized. Solution methods have also been used for total syntheses of proteins such as epidermal growth factor (112). This molecule and several transforming growth factors have been synthesized by manual solid phase methods (113, 114), but the systematic exploitation of chemical protein synthesis for structure-function studies has proved to be beyond the capabilities of solution or manual solid phase methods.

The improvements in the peptide synthesis methodology described above have allowed the total chemical synthesis of proteins in the research laboratory. In this respect, key methodology improvements were the development of highly optimized stepwise SPPS, effective automation of this chemistry for

the rapid, reproducible assembly of very long protected peptide chains, and improved procedures for deprotection of the product peptide. Empirical methods of protein folding, the use of high-resolution chromatographic and electrophoretic techniques for purification and analysis, and structural characterization of the synthetic proteins by mass spectrometry and nuclear magnetic resonance, have also played an important role.

A summary of the features of this approach to the chemical synthesis of proteins is given in Table 7. A recent total chemical synthesis of human transforming growth factor- α (TGF- α) (115) serves as an example of what can be achieved (Figure 7). Assembly of the 50-residue target sequence was documented by quantitative ninhydrin determinations and by quantitative Edman degradation and showed an average yield of 99.65% per residue. After deprotection using the S_{N2} - S_{N1} HF procedure (19), the crude peptide product was folded and oxidized to form disulfide bonds, then purified before characterization by mass spectrometry. The synthetic protein had potent receptor-binding, mitogenic, and colony-forming activities.

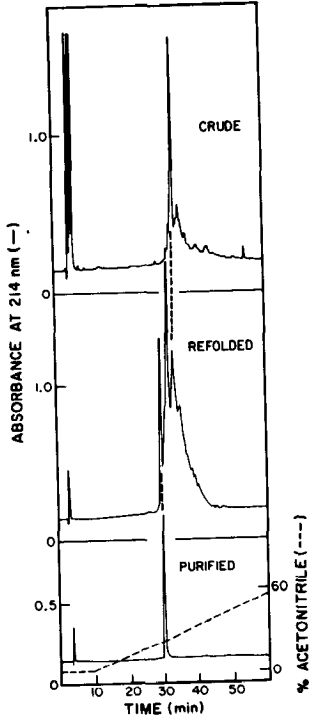
The most fundamental problem in the chemical synthesis of proteins is the folding of the peptide chain into the correct three-dimensional structure and the characterization of the folded structure. In the case of the TGF- α synthesis described above, a unique structure of the three disulfide bonds was determined by peptide mapping (115). This served as good, but indirect, proof that a single defined folded state of the peptide chain existed in the synthetic protein. Analogy of the disulfides with those of epidermal growth factor isolated from natural sources (116), together with the potent and specific biological activities of the synthetic TGF- α , suggested that this was a functionally correct folding.

For designed proteins, even for analogues as simple as the replacement of a single amino acid residue, it cannot be assumed that the three-dimensional structure remains unaltered compared to the parent structure (117). There will always be ambiguity as to whether functional changes are due directly to alteration of a particular side chain or arise indirectly from a different folding of the peptide chain. This ambiguity is true for altered proteins produced by

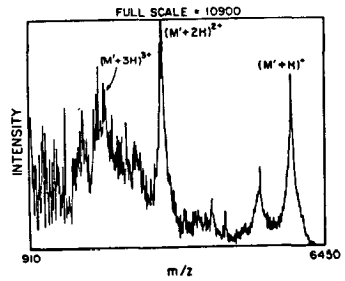
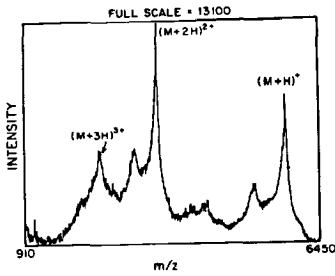
Table 7 The stepwise SPPS approach to the chemical synthesis of proteins (14, 57)

-
1. Fully automated chain assembly
 2. 15–30 amino acid residues per day
 3. Resin loading: 1 mmol/gram S-DVB
 4. Double coupling chemistry
 5. Quantitative Edman degradation of the protected peptide-resin
 6. Boc-chemistry, S_{N2} - S_{N1} acid deprotection
 7. Up to 1 gram crude product from 0.3 mmole scale synthesis
-

HUMAN TGF α



α TGF -



genetic engineering (118) or by chemical means. To interpret the altered activities of engineered proteins, direct and precise measurement of the three-dimensional structure of the protein is essential. Modern nmr techniques (100) have a unique role to play in this respect. Once the parent structure of a protein has been defined by a fully assigned and interpreted nmr spectrum, the similarities and differences in the three-dimensional structure of analogues can be precisely defined by rapid measurements in solution.

Proteins are made up of structural/functional domains that typically contain peptide chains 100–200 amino acids in length. The ultimate challenge facing synthetic peptide chemistry is the total chemical synthesis of these domains in pure form. A number of recent synthetic achievements have demonstrated that this is not beyond the capabilities of current methods. Automated total chemical synthesis of the 140-amino-acid lymphokine murine IL-3 (14) and a series of analogues (15) illustrates the power and limitations of current stepwise SPPS. The synthetic approach to protein engineering definitively established the essential role of a single disulfide bond in the activity of this molecule (15). It was not possible to prepare a homogenous synthetic protein of this size using current methods (14). However, applications of methodology improvements, such as capping and affinity purification, currently under development (S. B. H. Kent, unpublished), should make the synthesis of pure protein domains feasible by stepwise SPPS.

In special instances, it is already possible to chemically engineer structural domains through semisynthetic methods (119). Until recently, almost all protein semisynthesis used fragments that were generated by cleavage of the parent molecule and subsequently modified before re-ligation. The SPPS methods described above are easily capable of producing large numbers of analogues of such peptides, rapidly and in high purity. For example, six analogues of the 39-residue peptide cytochrome *c*(66–104) were produced in a matter of weeks and reacted with the naturally derived Cyt *c*(1–65) homoserine lactone fragment to produce a family of mutant cytochrome *c*

←

Figure 7 Chemical synthesis of the protein mitogen, human transforming growth factor alpha (115). *Top*, the covalent structure of the 50-residue molecule. Note the three disulfide bonds. *Left middle*, HPLC analyses of the synthetic product: (*top*) total crude products; (*middle*) folded, oxidized crude product; (*bottom*) HPLC-purified synthetic protein. For HPLC conditions see Figure 6A. *Right middle*, Immobilized pH gradient isoelectric focusing analysis of the purified synthetic product, at two loadings (5, 10 microgram). *Bottom left*, Plasma desorption time-of-flight mass spectrum of purified synthetic TGF-alpha; measured mass of the molecular ion, 5546.3 amu, in agreement with the calculated mass for the protein with three disulfide bonds. *Bottom right*, Mass spectrum of the synthetic TGF-alpha after reduction with thiol. The measured mass increased by 5.9 amu, corresponding to the reduction of three disulfide bonds.

molecules (P. Mascagni, C. J. Wallace, and S. B. H. Kent, unpublished results).

Ligation of large protected peptide fragments to form proteins has theoretical advantages (purification and characterization of intermediates; ready purification of the product protein) and practical problems (insolubility and difficult purification of the protected fragments) similar to those of fragment condensation peptide synthesis (see above). In a recent notable advance, a 92-amino-acid residue peptide was synthesized from intermediate fragments generated by stepwise SPPS, purified in unprotected form, and chemically ligated with minimal protection (120). This technique has the potential to be a powerful general approach to the synthesis of protein domains.

The key to routine chemical synthesis of proteins in the future may be the enzymatic ligation of unprotected peptide fragments 30–50 residues in length. Enzyme-catalyzed peptide bond formation is one of the oldest approaches in peptide synthesis (121), and the potential advantages of using fragments prepared by stepwise SPPS has not escaped recent attention (122). However, a more definitive approach would be to use highly specific proteases as regenerable stoichiometric reagents, rather than as catalysts, for the ligation of unprotected peptides. As in the enzyme-catalyzed approach, the thermodynamic driving force for the formation of the peptide bond would derive from the use of peptide esters. These peptide esters would be reacted, under appropriate conditions, with a serine protease to form the acyl-enzyme intermediate. This would then be used, perhaps in a packed column reactor, to react with the amino-component of the peptide bond (a large unprotected peptide). The highly specific binding pocket of the protease would provide the steric juxtaposition necessary for the formation of the peptide bond, and would eliminate the need for any side chain protection, even of Lys residues.

In combination with computer-aided structure prediction techniques (123), the production of designed proteins and their complete structural characterization will provide a feedback loop that will enable us to improve our understanding of protein structure and function. Chemical synthesis of proteins has a unique and powerful contribution to make in this area. By means of chemical synthesis, nmr probe nuclei can be incorporated at any predetermined single atom sites in the unperturbed protein molecule to help solve the nmr structure and to act as sensitive reporters of structure in analogues.

In addition, chemical synthesis can be used to incorporate non-genetically encoded structures into the protein molecule in a completely general fashion. For example, D-amino acids (124) can be incorporated, if desired. Artificial amino acids with isosteric side chains can be used to investigate the functional importance of particular residues. Fixed secondary structural elements, such as mimics of beta-turns, can be introduced and the effects of folding, function, and stability investigated. Similarly, the role of cis-trans isomerization

about X-Pro bonds in protein folding (125) can be investigated in a novel way by the incorporation of dipeptide analogues fixed in either *cis*- or *trans*-geometry. It is even possible to imagine geometrically constrained analogues of enzyme molecules that could be used to define the quantitative contribution to catalysis attributed to the passage of the enzyme molecule itself across the reaction coordinate in the E.S complex. In this way it might be possible to construct transition-state analogues of the enzyme molecule rather than of the substrate.

CONCLUSIONS

There are numerous biologically active peptides that provide interesting synthetic targets. Powerful general methods exist for the chemical synthesis of peptides up to about 50 amino acids in length. Future improvements will be in the speed of synthesis, reduced cost, and increased convenience. The next few years will see the development of truly automated procedures that will assemble the amino acid sequence, deprotect, and carry out a purification step to deliver the purified product, all without operator intervention (126). The excessive sophistication (complexity) that has been a feature of past synthetic methods will be replaced by truly elegant, simple approaches.

The total chemical synthesis of proteins is feasible with straightforward extensions of existing techniques and will have a revolutionary impact. In particular, chemical synthesis will play a central role in deciphering the way in which the amino acid sequence of the peptide chain determines the precisely folded three-dimensional structure of a protein. This question is the central problem in modern biology and must be answered before we can fully understand the fundamental questions in developmental and neurobiology. From a synthetic chemist's viewpoint, the precisely folded three-dimensional structure of a protein is perhaps the ultimate synthetic goal.

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Literature Cited

1. Bergmann, M., Zervas, L. 1932. *Ber. Dtsch. Chem. Ges.* 65:1192-201
2. Lynch, D. R., Snyder, S. H. 1986. *Ann. Rev. Biochem.* 55:773-99
3. Balasubramanian, T. M., Needleman, P., Currie, M. G., Geller, D. M., Cole, B. R., Marshall, G. R. 1984. *Peptides 1984*, ed. U. Ragnarsson, pp. 509-12. Stockholm: Almquist & Wiksell
4. Selsted, M. E., Brown, D. M., DeLange, R. J., Lehrer, R. I. 1983. *J. Biol. Chem.* 258:14485-89
5. Zasloff, M. 1987. *Proc. Natl. Acad. Sci. USA* 84:5449-53
6. Walter, G., Doolittle, R. F. 1983. *Genet. Eng.* 5:61-91
7. Lerner, R. A. 1982. *Nature* 299:592-96
8. Arnon, R. 1980. *Ann. Rev. Microbiol.* 34:593-618
9. Neurath, A. R., Kent, S. B. H., Prince, A. M., Strick, N., Brotman, B., Sproul, P. 1985. *Vaccine* 4:35-37
10. Herrington, D. A., Clyde, D. F., Losonsky, G., Cortesia, M., Murphy, J. R., et al. 1987. *Nature* 328:257-59
11. Pfaff, E., Mussgay, M., Bohm, H. O., Schultz, G. E., Schaller, H. 1982. *EMBO J.* 1:869-74
12. Good, M. F., Maloy, W. L., Lunde, M. N., Margalit, H., Cornette, J. L., et al. 1987. *Science* 235:1059-62
13. Guillet, J. G., Lai, M.-Z., Briner, T. J., Smith, J. A., Gefter, M. 1986. *Nature* 324:260-62
14. Clark-Lewis, I., Aebersold, R., Ziltener, H., Schrader, J. W., Hood, L. E., Kent, S. B. H. 1986. *Science* 231:134-39
15. Clark-Lewis, I., Lopez, A. F., Vadas, M., Schrader, J. W., Hood, L. E., Kent, S. B. H. 1988. *Proc. 5th Int. Lymphokine Workshop*, ed. C. W. Pierce. Clifton, NY: Humana. In press
16. Merrifield, R. B. 1963. *J. Am. Chem. Soc.* 85:2149-54
- 16a. Merrifield, R. B. 1974. *Biopolymer Topics #3*. Palo Alto: Beckman Spinco Division
17. Merrifield, R. B. 1986. *Science* 232:341-47
18. Kent, S. B. H., Parker, K. F. 1988. *Biochemistry*. In press
19. Tam, J. P., Heath, W. F., Merrifield, R. B. 1983. *J. Am. Chem. Soc.* 105:6442-55
20. Erickson, B. W., Merrifield, R. B. 1976. *The Proteins*, ed. H. Neurath, R. L. Hill, 2:257-527. New York: Academic
21. Barany, G., Merrifield, R. B. 1980. See Ref. 37, 2:1-284
22. Kiyama, S., Fujii, N., Yajima, H., Moriga, M., Takagi, A. 1984. *Int. J. Pept. Protein Res.* 23:174-86
23. Pillai, V. N., Mutter, M. 1981. *Acc. Chem. Res.* 14:122-30
24. Fridkin, M., Patchornik, A. 1974. *Ann. Rev. Biochem.* 43:419-43
25. Kent, S. B. H. 1980. *Biomedical Polymers*, ed. E. P. Goldberg, A. Nakajima, pp. 213-42. New York: Academic
26. Kent, S. B. H. 1985. *Peptides: Structure and Function. Proc. 9th Am. Pept. Symp., Toronto*, ed. C. M. Deber, V. J. Hruby, K. D. Kopple, pp. 407-14. Rockford, Ill: Pierce Chemical Co.
27. Whitney, D. B., Tam, J. P., Merrifield, R. B. 1983. *Peptides: Structure and Function. Proc. 8th Am. Pept. Symp.*, ed. V. J. Hruby, D. H. Rich, pp. 167-70. Rockford, Ill: Pierce Chemical Co.
- 27a. Kubiak, T., Whitney, D. B., Merrifield, R. B. 1987. *Biochemistry* 26:7849-55
28. Chang, J. K., Shimizu, M., Wang, S.-S. 1976. *J. Org. Chem.* 41:3255-58
29. Sarin, V. K., Kent, S. B. H., Merrifield, R. B. 1980. *J. Am. Chem. Soc.* 102:5463-70
30. Edelstein, M. S., Hughes, J. L., Foster, J. E. 1987. *10th Am. Pept. Symp. (Abstrs.)*, ed. G. R. Marshall, Abstr. #P-32. St. Louis: Washington Univ.
31. Live, D. H., Kent, S. B. H. 1982. *Elastomers and Rubber Elasticity. Am. Chem. Soc. Symp. Ser.*, ed. J. E. Mark, pp. 501-15. Washington, DC: Am. Chem. Soc.
32. Hancock, W. S., Prescott, D. J., Vageolos, P. R., Marshall, G. 1973. *J. Org. Chem.* 38:774-81
33. Stahl, G. L., Walter, R., Smith, C. W. 1979. *J. Am. Chem. Soc.* 101:5383-94
34. Sheppard, R. C. 1973. *Peptides 1971*, ed. H. Nesvadba, pp. 111-25. Amsterdam: North-Holland
35. Atherton, E., Sheppard, R. C. 1981. *Perspectives in Peptide Chemistry*, ed. A. Eberle, R. Geiger, T. Wieland, pp. 101-17. Basel: Karger

36. Atherton, E., Sheppard, R. C. 1985. See Ref. 26, pp. 415-18
37. Gross, E., Meienhofer, J., eds. 1980. *The Peptides: Analysis, Synthesis, Biology*, Vol. 3: *Protection of Functional Groups in Peptide Synthesis*. New York: Academic. 379 pp.
38. Carpino, L. A. 1957. *J. Am. Chem. Soc.* 79:4427-31
- 39a. McKay, F. C., Albertson, N. F. 1957. *J. Am. Chem. Soc.* 79:4686-90
- 39b. Anderson, G. W., McGregor, A. C. 1957. *J. Am. Chem. Soc.* 79:6180-83
- 40a. Carpino, L. A., Han, G. Y. 1970. *J. Am. Chem. Soc.* 92:5748-49
- 40b. Carpino, L. A., Han, G. Y. 1972. *J. Org. Chem.* 37:3404-9
- 40c. Carpino, L. A. 1987. *Acc. Chem. Res.* 20:401-7
41. Tam, J. P., Wong, T. W., Riemen, M. W., Tjoeng, F. S., Merrifield, R. B. 1979. *Tetrahedron Lett.* 4033-36
42. Yajima, H., Takeyama, M., Kanaki, J., Nishimura, O., Fujino, M. 1978. *Chem. Pharm. Bull.* 26:3752-57
43. Chang, C.-D., Meienhofer, J. 1978. *Int. J. Pept. Protein Res.* 11:246-49
44. Chang, C.-D., Waki, M., Ahmad, M., Meienhofer, J., Lundell, E. O., Haug, J. D. 1980. *Int. J. Pept. Protein Res.* 15:59-66
45. Atherton, E., Cammish, L. E., Goddard, P., Richards, J. D., Sheppard, R. C. 1984. See Ref. 54, pp. 153-56
46. Wade, J. D., Fitzgerald, S. P., McDonald, M. R., McDougall, J. G., Tregear, G. W. 1986. *Biopolymers* 25:S21-S37
47. Ramage, R., Green, J. 1987. *Tetrahedron Lett.* 28:2287-90
48. Sieber, P., Riniker, B. 1987. *Tetrahedron Lett.* 28:6031-34
49. Nakagawa, Y., Nishiuchi, Y., Emura, J., Sakakibara, S. 1980. *Peptide Chemistry 1980. Proc. Jpn. Pept. Symp.*, ed. K. Okawa, pp. 41-46. Osaka: Protein Res. Found.
50. Dryland, A., Sheppard, R. C. 1986. *J. Chem. Soc. Perkin Trans. 1*:125-37
51. Mojsov, S., Mitchell, A. R., Merrifield, R. B. 1980. *J. Org. Chem.* 45:555-60
52. Kent, S. B. H., Mitchell, A. R., Barany, G., Merrifield, R. B. 1978. *Anal. Chem.* 50:155-59
53. Benoiton, N. L. 1983. See Ref. 37, 5:218-84
54. Kent, S. B. H., Hood, L. E., Beilan, H., Meister, S., Geiser, T. 1984. *Peptides 1984: Proc. 18th Eur. Pept. Symp. Sweden*, ed. U. Ragnarsson, pp. 185-88. Stockholm: Almqvist and Wiksell
55. Kent, S. B. H., Hood, L. E., Beilan, H., Bridgham, H., Marriot, M., et al. 1985. *Peptide Chemistry 1984. Proc. Jpn. Pept. Symp. 1984*, ed. N. Isumiya, pp. 217-22. Osaka: Protein Res. Found.
56. Hagenmeier, H., Frank, H. 1972. *Hoppe Seyler's Z. Physiol. Chem.* 353:1973-76
57. Kent, S. B. H., Clark-Lewis, I. 1985. *Synthetic Peptides in Biology and Medicine*, ed. K. Alitalo, P. Partanen, A. Vaheri, pp. 29-57. Amsterdam: Elsevier
58. Atherton, E., Sheppard, R. C. 1985. *J. Chem. Soc. Chem. Commun.*, pp. 165-66
59. Atherton, E., Cameron, L., Meldal, M., Sheppard, R. C. 1986. *J. Chem. Soc. Chem. Commun.*, pp. 1763-65
60. Konig, W., Geiger, R. 1970. *Chem. Ber.* 103:2034-40
61. Sarin, V. K., Kent, S. B. H., Tam, J. P., Merrifield, R. B. 1981. *Anal. Biochem.* 117:147-57
62. Kent, S. B. H. 1984. *Peptides: Structure and Function*, ed. V. J. Hruby, D. H. Rich, pp. 99-102. Rockford, Ill: Pierce Chemical Co.
63. Kent, S. B. H., Riemen, M., LeDoux, M., Merrifield, R. B. 1982. *Methods in Protein Sequence Analysis*, ed. M. Elzinga, pp. 205-13. New Jersey: Humana
- 63a. Tregear, G. W. 1977. *Biochemistry* 16:2817-23
64. Kalbag, S. M., Snyder, E. S., Voelker, P. J., Hughes, J. L. 1985. See Ref. 26, pp. 269-72
65. Cameron, L., Meldal, M., Sheppard, R. C. 1987. *J. Chem. Soc. Chem. Commun.* pp. 270-72
66. Kent, S. B. H., Mitchell, A. R., Engelhard, M., Merrifield, R. B. 1979. *Proc. Natl. Acad. Sci. USA* 76:2180-84
67. Mitchell, A. R., Kent, S. B. H., Engelhard, M., Merrifield, R. B. 1978. *J. Org. Chem.* 43:2845-52
68. Kent, S. B. H., Merrifield, R. B. 1983. *Int. J. Pept. Protein Res.* 22:57-65
69. Fuller, W. D., Sigler, G. F., Chaturvedi, N. C., Verlander, M. S. 1983. See Ref. 27, pp. 79-82
70. Gisin, B. F., Merrifield, R. B. 1972. *J. Am. Chem. Soc.* 94:3102-6
71. Dimarchi, R. D., Tam, J. P., Kent, S. B. H., Merrifield, R. B. 1982. *Int. J. Pept. Protein Res.* 19:88-93
72. Meister, S. M., Kent, S. B. H. 1984. See Ref. 62, pp. 103-6
73. Atherton, E., Wolley, V., Sheppard, R. C. 1980. *J. Chem. Soc. Chem. Commun.*, pp. 970-71
74. Live, D. L., Kent, S. B. H. 1984. See Ref. 62, pp. 65-68
75. Kent, S. B. H., Merrifield, R. B. 1981. *Peptides 1980: Proc. 16th Eur. Pept.*

- Symp.*, ed. K. Brunfeldt, pp. 328-33. Copenhagen: Scriptor
76. Tam, J. P. 1985. See Ref. 26, pp. 423-25
77. Barany, G., Merrifield, R. B. 1980. See Ref. 37, 2:159-66
78. Deleted in proof
79. Carpino, L. A., Cohen, B. J., Stephens, K. E. Jr., Sadat-Aalee, S. Y., Tien, J.-H., Langridge, D. C. 1986. *J. Org. Chem.* 51:3734-36
80. Kent, S. B. H., Neurath, A. R., Aebersold, R., Parker, K. F., Strick, N. 1986. *Vaccines 86: Molecular and Chemical Basis of Resistance to Viral Diseases*, ed. R. A. Lerner, R. M. Chanock, F. Brown, pp. 365-69. New York: Cold Spring Harbor Press
81. Buttner, K., Fischer, W. H., Zahn, H. 1987. See Ref. 30, Abstr. #P-33
- 81a. Barany, G., Albericio, F. 1985. *J. Am. Chem. Soc.* 107:4936-42
82. Scanlon, D. B., Eefting, M. A., Lloyd, C. J., Burgess, A. W., Simpson, R. J. 1987. *J. Chem. Soc. Chem. Commun.*, pp. 516-18
83. Sieber, P. 1987. *Tetrahedron Lett.* 28:1637-40
84. Tam, J. P., Heath, W. F., Merrifield, R. B. 1986. *J. Am. Chem. Soc.* 108:5242-51
85. Kiso, Y., Ukawa, K., Akita, T. 1980. *J. Chem. Soc. Chem. Commun.*, pp. 101-2
86. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., Sugihara, H. 1967. *Bull. Chem. Soc. Jpn.* 40:2164-67
87. Matsueda, G. R. 1982. *Int. J. Pept. Protein Res.* 20:26-34
88. Clark-Lewis, I., Kent, S. B. H. 1988. *Receptor Biochemistry and Methodology: The Use of HPLC in Protein Purification and Characterization*, ed. A. R. Kerlavage. New York: Liss. In press
89. Yajima, H., Fujii, N. 1983. See Ref. 37, 5:65-109
90. Bergot, B. J., Noble, R. F., Geiser, T. 1987. *Peptides 1986: Proc. 19th Eur. Pept. Symp.*, ed. D. Theodoropoulos, pp. 97-101. Berlin: de Gruyter
91. Pauling, L. 1956. *J. Chem. Educ.* 33:16-17
92. Feinberg, R. S., Merrifield, R. B. 1975. *J. Am. Chem. Soc.* 97:3485-96
- 92a. Noble, R. L., Yamashiro, D., Li, C. H. 1976. *J. Am. Chem. Soc.* 98:2324-28
- 92b. Swerdloff, M. D., Brambilla, R. 1987. See Ref. 30, Abstr. #P-83
93. Burke, L., Hodges, R. S. 1987. See Ref. 30, Abstr. #P-42
94. Regnier, F. E. 1987. *Science* 238:319-23
95. Yamashiro, D., Li, C. H. 1985. *Int. J. Pept. Protein Res.* 26:299-304
96. Spackman, D. H., Stein, W. H., Moore, S. 1958. *Anal. Chem.* 30:1190-206
97. Hewick, R. M., Hunkapiller, M. W., Hood, L. E., Dreyer, W. J. 1981. *J. Biol. Chem.* 256:7990-97
- 97a. Chait, B. T., Gisin, B. F., Field, F. H. 1982. *J. Am. Chem. Soc.* 104:5157-62
- 98a. Hunt, D. F., Yates, J. R. III, Shabanowitz, J., Winston, S., Hauer, C. R. 1986. *Proc. Natl. Acad. Sci. USA* 83:6233-37
- 98b. Hunt, D. F., Shabanowitz, J., Yates, J. R. III. 1987. *J. Chem. Soc. Chem. Commun.*, pp. 548-50
99. Chait, B. T., Field, F. H. 1985. *J. Am. Chem. Soc.* 107:6743-44
100. Wuthrich, K. 1986. *NMR of Proteins and Nucleic Acids*. New York: Wiley-Interscience, 292 pp.
- 101a. Gisin, B. F., Kobayashi, S., Davis, D. G., Hall, J. E. 1977. *Peptides. Proc. 5th Am. Pept. Symp.*, ed. M. Goodman, J. Meienhofer, pp. 215-17. New York: Wiley
- 101b. Gisin, B. F., Davis, D. G., Borowska, Z. K., Hall, J. E., Kobayashi, S. 1981. *J. Am. Chem. Soc.* 103:6373-77
102. Paudey, R. C., Cook, J. C. Jr., Rinehart, K. L. Jr. 1977. *J. Am. Chem. Soc.* 99:8469-83
103. Ling, N., Zeytin, F., Bohlen, P., Esch, F., Brazeau, P., et al. 1985. *Ann. Rev. Biochem.* 54:403-23
104. Kaiser, E. T., Kezdy, F. J. 1984. *Science* 223:249-55
105. Geysen, H. M., Meloen, R. H., Barteling, S. J. 1984. *Proc. Natl. Acad. Sci. USA* 81:3998-4002
106. Houghten, R. A. 1985. *Proc. Natl. Acad. Sci. USA* 82:5131-35
107. Neurath, A. R., Kent, S. B. H., Strick, N., Parker, K. 1986. *Cell* 46:429-36
- 107a. Jameson, B. A., Rao, P., Hood, L. E., Kent, S. B. H. 1988. *Science*. In press
108. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., Wiley, D. C. 1987. *Nature* 329:506-12
109. Neurath, A. R., Kent, S. B. H., Strick, N. 1984. *Science* 224:392-95
110. White, F. H. 1961. *J. Biol. Chem.* 236:1353-60
111. Sieber, P., Eisler, K., Kamber, B., Riniker, B., Rittel, W., et al. 1978. *Hoppe Seyler's Z. Physiol. Chem.* 359:113-23
112. Akaji, K., Fujii, N., Yajima, H., Hayashi, K., Mizuta, K., et al. 1985. *Chem. Pharm. Bull.* 33:184-201
113. Tam, J. P., Sheikh, M. A., Solomon,

- D. S., Ossowski, L. 1986. *Proc. Natl. Acad. Sci. USA* 83:8082-86
114. Heath, W. F., Merrifield, R. B. 1986. *Proc. Natl. Acad. Sci. USA* 83:6367-71
115. Woo, D. D.-L., Clark-Lewis, I., Chait, B. T., Kent, S. B. H. 1988. *Protein Eng.* In press
116. Savage, C. R. Jr., Hash, J. H., Cohen, S. 1973. *J. Biol. Chem.* 248:7669-72
117. Matthews, B. W. 1987. *Biochemistry* 26:6885-88
118. Alber, T., Dao-pin, S., Wilson, K., Wozniak, J. A., Cook, S. P., Matthews, B. W. 1987. *Nature* 330:41-46
119. Offord, R. E. 1987. *Protein Eng.* 1:151-57
120. Blake, J., Yamashiro, D., Ramashara, K., Li, C. H. 1986. *Int. J. Pept. Protein Res.* 28:468-76
121. Jakubke, H.-D., Kuhl, P., Konnecke, A. 1985. *Angew. Chem. Int. Ed. Engl.* 24:85-93
122. Nakatsuka, T., Sasaki, T., Kaiser, E. T. 1987. *J. Am. Chem. Soc.* 109:3808-10
123. Eisenberg, D., McLachlan, A. D. 1986. *Nature* 319:199-203
124. Richter, K., Egger, R., Kreil, G. 1987. *Science* 238:200-2
125. Kelley, R. F., Richards, F. M. 1987. *Biochemistry* 26:6765-74
126. Lahm, H.-W., Udenfriend, S., Meienhofer, J. 1983. See Ref. 27, pp. 69-72



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