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Large-Scale Synthesis of Peptides

Abstract: *Recent advances in the areas of formulation and delivery have rekindled the interest of the pharmaceutical community in peptides as drug candidates, which, in turn, has provided a challenge to the peptide industry to develop efficient methods for the manufacture of relatively complex peptides on scales of up to metric tons per year. This article focuses on chemical synthesis approaches for peptides, and presents an overview of the methods available and in use currently, together with a discussion of scale-up strategies. Examples of the different methods are discussed, together with solutions to some specific problems encountered during scale-up development. Finally, an overview is presented of issues common to all manufacturing methods, i.e., methods used for the large-scale purification and isolation of final bulk products and regulatory considerations to be addressed during scale-up of processes to commercial levels.* © 2000 John Wiley & Sons, Inc.
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INTRODUCTION

For almost half a century, since du Vigneaud first presented his pioneering synthesis of oxytocin to the world in 1953,[1] the pharmaceutical community has been excited about the potential of peptides as "Nature's Pharmaceuticals." Further discoveries, including Merrifield's solid-phase synthesis (SPPS) method,[2] introduced a decade later, recombinant techniques for expressing peptides and proteins in microorganisms,[3] and most recently methods for producing peptides and proteins in transgenic animals[4] and plants,[5] have all combined to increase the availability and lower the cost of producing peptides. For many years, however, the major obstacle to the success of peptides as pharmaceuticals was their lack of oral bioavailability and, therefore, relatively few peptides reached the marketplace as approved drugs. As a result, several major pharmaceutical companies abandoned their research efforts in the area, in favor of small molecule mimics of peptide or protein lead compounds. In recent years, though, advances in the areas of formulation and novel delivery systems have revitalized the field, leading to several highly success-

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ful "blockbuster" peptide drugs, such as the luteinizing hormone releasing hormone (LH-RH) analogues, leuprolide (Lupron), and goserelin (Zoladex). The resulting resurgence of interest in peptides as pharmaceutical products has provided once more a challenge to the peptide industry to develop economically viable methods for manufacturing relatively complex peptides in large quantities. The challenge is being met, and a number of new peptide drugs are now in late-stage development with target requirements, at commercial launch, of up to hundreds of kilograms or even metric tons.

While, as noted above, a variety of methods are now available for the commercial-scale manufacture of peptides, chemical synthesis is still the most universal approach, since it permits access to all possible sequences, including those that contain unnatural D-amino acids or amino acids that are completely synthetic in origin, which is not routinely possible with methods based on biotechnology. Furthermore, it is noteworthy that two of the pioneers of the field—du Vigneaud and Merrifield—were both awarded Nobel Prizes for their work on chemical synthesis of peptides by solution-phase and solid-phase methods, respectively. The general technology of chemical synthesis approaches is the subject of a recent, comprehensive review.[6] This article, therefore, will focus on the practical aspects of the application of chemical synthesis methods to the large-scale production of peptides. First, a brief overview of the approaches, which are available and in use currently for large-scale manufacturing, will be presented, together with a discussion of strategies for scale-up. Each of the commonly-used strategies will next be discussed in more depth, together with selected examples. Finally, an overview of the methods used for large-scale purification and isolation of final, bulk products will be presented, together with a discussion of regulatory issues commonly encountered during scale-up of processes to commercial levels.

STRATEGIES

Solution-Phase Methods

It is not surprising that, with almost 50 years of experience since du Vigneaud first published the synthesis of oxytocin,[1] most of the approved peptide pharmaceuticals in use today are manufactured using solution-phase methodology. While the method has been shown to be useful for the synthesis of very long peptides and even small proteins, as can be seen from Table I, the longest peptides manufactured for commercial use by this method are the calcitonins (32 amino acids). More commonly, the method is used to produce small- to medium-length peptides in quantities of up to hundreds of kilograms or even metric tons per year. Such products include angiotensin converting enzyme (ACE) inhibitors, the dipeptides sweetener, Aspartame, HIV protease inhibitors, oxytocin, desmopressin, and LH-RH analogues, such as leuprolide and goserelin.

Solid-Phase Methods

When Merrifield first introduced the solid-phase method in 1963,[2] his discovery was met with considerable skepticism in the scientific community. This was based on comparison with the well-established solution-phase methodology, in which intermediates and fragments were routinely isolated, purified, and characterized. In contrast, in Merrifield's solid-phase method, no purification was possible until after cleavage of the fully assembled peptide from the solid support, when most of the by-products accumulated during the synthesis were simultaneously cleaved. Furthermore, when the solid-phase method was introduced, the analytical methods and purification techniques available to the peptide chemist were nowhere near as powerful or discriminating as those in routine use today, and therefore the purity of the peptides produced by the Merrifield method was in question. Nevertheless, from the beginning, the technique proved to be an extremely valuable addition to the research chemist's repertoire, especially since it could be automated, permitting rapid synthesis of relatively complex sequences. The subsequent introduction of alternate protecting group strategies, particularly the 9-fluorenylmethyloxycarbonyl Fmoc/*t*-butyl combination,[7] in place of the *t*-Boc/benzyl combination originally proposed by Merrifield, increased the versatility of the method. Finally, as more powerful analytical methods and purification techniques became available, especially those based on reverse-phase high performance liquid chromatography (RP-HPLC), the technique has been increasingly exploited as a manufacturing method for a number of commercial products, such as LH-RH and analogues, somatostatin and salmon calcitonin (Table I).

Hybrid Approaches

While both the solution-phase and solid-phase approaches have clearly proven to be effective for the manufacture of a relatively wide variety of products, each strategy has its limitations. For example, the relatively lengthy development times required for so-

Table I Some Approved Peptide Pharmaceuticals and Their Methods of Manufacture

Peptide	Length	Method[a]
Oxytocin	9	C
Vasopressin analogues		
Pitressin	9	C
Lypressin	9	C
Desmopressin	9	C, SP
Terlipressin	12	C, SP
Atosiban	9	C
Adrenocorticotropic hormone (ACTH) (1–24)	24	C
Insulin	51	E, S, R
Glucagon	29	E, SP, R
Secretin	27	E
Calcitonins		
Human	32	C
Salmon	32	C, SP
Eel	32	C, SP
Dicarba-Eel (Elcatonin)	31	C, SP
Luteinizing hormone–releasing hormone (LH-RH) and analogues	10	C, SP
Leuprolide	9	C
Deslorelin	9	SP
Triptorelin	10	SP
Goserelin	10	SP
Buserelin	9	SP
Parathyroid hormone (PTH) (1–34)	34	SP
Corticotropin releasing factors		
Human	41	SP
Ovine	41	SP
Growth hormone releasing factor (1–29)	29	SP
Somatostatin and analogues	14	C, SP
Lanreotide	8	SP
Octreotide	8	C
Thyrotropin releasing hormone (TRH)	3	C
Thymosin α-1	28	SP
Thymopentin (TP-5)	5	C
Cyclosporin	11	E
Integrilin	7	C

[a] Manufacturing methods: C = classical (solution-phase) chemical synthesis; E = extraction from natural sources; R = recombinant; S = semisynthesis; SP = solid-phase chemical synthesis.

lution-phase syntheses usually preclude the consideration of this technique for the manufacture of products for early-phase clinical studies, when the need for rapid production is essential, in order to confirm the feasibility of proceeding further with the development of the product. On the other hand, while solid-phase methods are usually employed for the manufacture of supplies during the early stages of a product's development, scale-up of the method is commonly thought to be difficult or impossible, especially if the original Merrifield method, using Boc strategy, is employed, because of the use of strongly acidic reagents, such as liquid hydrogen fluoride, to detach the peptide from the resin. However, with the introduction of new resins, such as the 2-chlorotrityl chloride[8] and SASRIN[9] resins, it is now possible to synthesize protected peptides and fragments that can be detached from the resin with the protecting groups intact. This has opened up the interesting possibility of a "hybrid" approach, in which the manufacture of complex sequences is approached through the solid-phase synthesis of relatively large, protected fragments, which are subsequently assembled either by solution-phase or solid-phase methods. While still not extensively utilized, this approach offers considerable promise for the commercial-scale production of large peptides, which would otherwise present

significant challenges when manufactured by either conventional solution-phase or solid-phase methods.

Scale-Up Development Strategy

Because of the need for rapid production, usually of relatively small quantities for toxicological and early phase clinical studies, expediency often dictates that products be manufactured by solid-phase methods during early stages of development. Once the product progresses past Phase 2, however, when the dose in humans is defined, a scale-up strategy must be in place. If the requirements for the product exceed the capabilities of the solid-phase approach and a change in the manufacturing method is required, it is imperative that the strategy includes both consideration of the requirements imposed by the regulatory agencies consideration, as well as the technical issues associated with scale-up. Therefore, the strategy must accommodate not only the projected requirements for the bulk drug substance at product launch and beyond, but also the regulatory requirement to demonstrate that the product manufactured by the two methods is chemically and biologically equivalent, and meets the safety profile established in toxicological and clinical studies. If these criteria are not met, the consequences can be extremely costly, both in terms of delays in launching the drug, as well as the cost of repeating toxicology studies and even clinical trials. Since the demonstration of chemical equivalence is most often based on comparison of impurity profiles in the bulk drug substance produced by the different methods, we propose that, as far as possible, the two manufacturing methods should be designed to use similar, or if possible, identical side-chain protecting groups on the individual amino acids in the sequence. In this way, the final, protected precursor of the product will be very similar, or preferably even identical, from both methods, and the same methods can be used for the final steps in the process, i.e. deprotection, purification, and final isolation. Using this approach, we believe that it is possible to predict, with a high degree of assurance, that expectations for demonstration of equivalence can be met.

OVERVIEW OF THE SOLUTION-PHASE APPROACH

Background

In the almost 50 years since the solution-phase approach was first demonstrated,[1] numerous advances have been made, especially in the areas of protecting groups and coupling methods, which increase the options available to the peptide chemist for the design of a solution-phase synthesis.[6] When such a large-scale synthesis is first contemplated, therefore, consideration must first be given to the *strategy* of the approach, i.e., the general chemistry, especially the choice of linear or convergent synthesis, the selection of segments and their assembly, the (orthogonal) protecting groups used, etc. As the method is developed, the specific *tactics* of the approach must be considered, i.e., the problems that are of critical importance when manufacturing on an industrial scale, including chemical, technical, economic, and safety aspects of the process, as well as the demands imposed by regulatory agencies, all of which must be considered in pharmaceutical production. While all of these aspects may be more or less neglected on a laboratory (development) scale, any one may become limiting as the process is scaled-up to an industrial level.

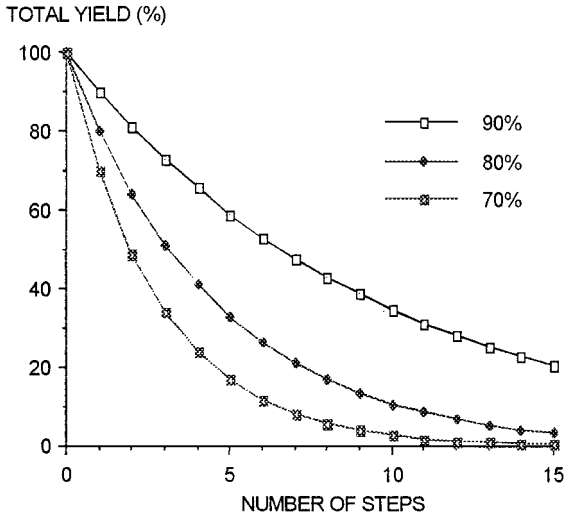


FIGURE 1 Effect of yield per step on overall yield of a synthesis (the "arithmetic demon"[6]).

Strategy

Linear vs Convergent Synthesis. The size of the peptide usually determines the strategy, and peptides up to five amino acids in length are generally manufactured by a linear strategy (i.e., stepwise addition of each amino acid in the sequence until the entire sequence has been assembled), while for longer peptides a convergent strategy (i.e., synthesis of small segments or fragments that are subsequently assembled to give the final sequence) is more suitable. This is mainly due to "the arithmetic demon,"[10] by which

the overall yield in a multistep process is critically dependent on the yield per step (see discussion under the chemical and technical aspects section below and Figure 1). In addition, the choice of solvent is more limited for longer peptides, which, in turn, limits the methods available for the workup of reaction mixtures.

If a convergent strategy is utilized, the selection of fragments is crucial, and as a guide, the following factors should be taken into consideration:

- Optimally, fragments should contain C-terminal Gly or Pro residues, since this minimizes the risk for racemization.[11]
- If it is not possible to select fragments containing C-terminal Gly or Pro, the C-terminal residue should be an amino acid that is less prone to racemize, such as Ala or Arg.[11]
- Fragments should preferably be no longer than five amino acids.

Before embarking on a large-scale synthesis, it is clearly prudent to test several possible strategies on a smaller scale. With the increased availability of resins that can be cleaved under mild conditions, with side-chain protecting groups intact, a very convenient way to accomplish this is to synthesize segments on a 0.5–1.0 g scale, using solid-phase technology, and subsequently couple them in solution. Besides giving information about the extent of racemization, this provides a hint about the solubility of the segments. Since the solid-phase method is very expedient, synthesis of the corresponding isomer, containing the C-terminal D-amino acid, is also recommended. Using this diastereomer of the desired peptide, analysis by HPLC of the extent of racemizaton during the coupling of the fragments is very facile.

Protecting Groups

α-Amino Group Protection. For large-scale solution synthesis, the most frequently used protecting groups are the Boc and benzyloxycarbonyl (Z) groups, because of the volatile by-products formed during the deprotection step.[6,11] Use of the Fmoc group is less attractive, because of the lack of volatility and the reactivity of the dibenzofulvene by-product. The Boc group is removed by acids, and for large-scale production, the most common reagents are neat trifluoroacetic acid (TFA) and HCl/ethyl acetate mixtures. The HCl salts are often solids rather than oils, which is an advantage if they are to be isolated. The Z group is removed by reduction, usually by catalytic hydrogenolysis with H_2/catalyst, but also by reduction with sodium in liquid ammonia. In the past, HBr in acetic acid was also frequently used for this purpose. However, on a large scale, the fact that the hydrobromide salts, which were isolated as precipitates from dry diethyl ether, were hygroscopic was often a disadvantage of this method.

Carboxyl Group Protection. The most suitable carboxyl "protecting group" for large-scale solution synthesis is the free acid itself. This, of course, limits the choice of coupling agents (see below). Many peptides occur as C-terminal amides, however, making protection unnecessary. If protection is required, either the benzyl or *t*-butyl esters are preferred. These are removed in the same manner as the Z group and Boc group respectively (see discussion above), so they must be orthogonal to the α-amino protecting group. If an aliphatic ester (e.g., methyl or ethyl) is used, saponification, using NaOH or KOH, is the method of choice for deprotection, although this poses a risk for racemization, or in the case of hindered amino acids such as proline, the reaction rate could be very slow (sometimes even zero). However, an advantage of these simple esters is that they can be transformed into hydrazides simply by treatment with hydrazine, which can then be used directly for coupling via the azide method (see below).

Side-Chain Protection. In general, it is desirable to use the minimal side-chain protection approach for large-scale synthesis, since this will minimize the number of steps. However it is not possible to dispense with protection completely:

- The δ- and ϵ-amino functions of ornithine and lysine, respectively, must, of course, always be protected, and as for the α-amino function, the Z and Boc groups are recommended.[6,11,12]
- The carboxyl functions of aspartic and glutamic acids must also be protected, preferably by the same esters as those mentioned above for α-carboxyl protection, i.e., benzyl or *t*-butyl esters,[6,11,12] taking orthogonality into account when choosing the side-chain protecting groups.
- The thiol function on cysteine may be protected by the Acm group or as a benzyl sulfide, the former group being removed by iodine with concomitant disulfide formation, and the latter preferably being removed by reduction with sodium in liquid ammonia.[6,12] (Note: trityl is another possible protecting group, and the combination of Acm and trityl groups can be useful to facilitate site-directed cyclizations.)

A disadvantage of the minimal side-chain protection approach is that the peptide is necessarily more hydrophilic, which often complicates extractive workup procedures. In this case, global protection could be an alternate approach, provided that the protecting groups have been chosen in such a way that they can be removed simultaneously, or at most, in two steps.

Coupling Techniques

Coupling of Individual Amino Acids. Today, the peptide chemist has a veritable arsenal of coupling methods at his disposal, the most commonly used of which are mixed carboxylic–carbonic anhydrides,[13] carbodiimides (e.g., dicyclohexylcarbodiimide (DCC), water-soluble carbodiimide (WSCDI)),[14] and uronium reagents (e.g., o-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU), o-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU)).[15,16] In addition to these methods, commercially available preactivated amino acid derivatives, such as active esters (e.g., hydroxysuccinimide (HOSu) and p-nitrophenol (HONp) esters),[17] and N-carboxyanhydrides (NCAs),[18,19] may be used. These derivatives have the added benefits of not generating any by-products from the activating agent and also of being compatible with an unprotected C-terminal amino acid residue in the amino component. The latter is also the case for the azide coupling method.[20] For the carbodiimide and TBTU methods, however, C-terminal protection is mandatory.

Coupling of Segments. When coupling segments, the risk for racemizaton is increased, due to the possibility of oxazolone formation. However, several methods are available for this type of coupling. One of the most common is the carbodiimide method (e.g., DCC or WSCDI) with an added auxiliary nucleophile (e.g., 1-hydroxy-benzotriazole (HOBt), 1-hydroxy-azabenzotriazole (HOAt), HOSu).[21,22] Another reagent that has become popular in recent years, due to increased commercial availability, is TBTU.[15] The mixed anhydride method, using isobutyl chloroformate, with or without an added auxiliary nucleophile, is also used frequently, as is the azide method, due to the low racemizaton associated with it. However, the N_3^- anion, produced as a by-product with the latter method, gives rise to safety concerns.

Tactics—Scale-Up and Technical Considerations

The discussion of strategy (above), which focused on the chemical aspects of peptide synthesis, is, of course, of a general nature and applies to both small-scale syntheses as well as to large-scale production. However, there are many technical factors that must be considered when a laboratory-scale synthesis is transferred into the manufacturing plant. These aspects—the "tactics" of scale-up—are discussed below.

The term "large-scale synthesis" is, obviously, quite subjective. When performed in a research laboratory, a one-kilogram batch can be considered to be very large scale, while the same quantity in a pharmaceutical plant usually is not. For the purpose of this article, large-scale is defined as a range of kilograms to metric tons. While the chemistry of any large-scale manufacturing process does not differ from that used on a small scale, a number of comments must be made about practical matters. Mostly these concern a combination of chemistry and technique. The final goal is to develop as efficient, cheap, and fast a procedure as possible, but also equally important is fulfilling the requirements of the regulatory authorities. Although many of these factors seem obvious, they are seldom discussed explicitly. Therefore, a short discussion of some of the more important factors is given below, with special emphasis on peptide synthesis.

Chemical and Technical Aspects. In any multistep process, it is clear that the number of steps should be kept at a minimum, in order to increase the total yield, as required by "the arithmetic demon."[10] Obviously, a fifteen-step synthesis will be more expensive and laborious than a ten-step synthesis, if the yields are in the same range. However, if alternative synthetic routes are available, a longer reaction sequence with higher yields may be advantageous. Thus, a fifteen-step synthesis with a 90% average yield can give a higher overall yield than a five-step synthesis with a 70% average yield, or an equal yield to a seven-step synthesis with an 80% average yield (see Figure 1). On the other hand, much more work and time is presumably required for a longer synthesis and, therefore, careful evaluation of the overall economics of the process is required.

Closely related to the previous discussion is the question of whether to use a convergent or a linear strategy. Typically, the synthesis of all longer industrial peptides is performed by segment condensations, i.e., by the convergent strategy. The reason for this choice is, of course, the same as in the previous discussion. It should also be noted that using a convergent synthesis is advantageous from a "safety" aspect. In all chemical production, there is always a possibility of mistakes or failures, which may mean that the batch has to be discarded. Obviously, in such

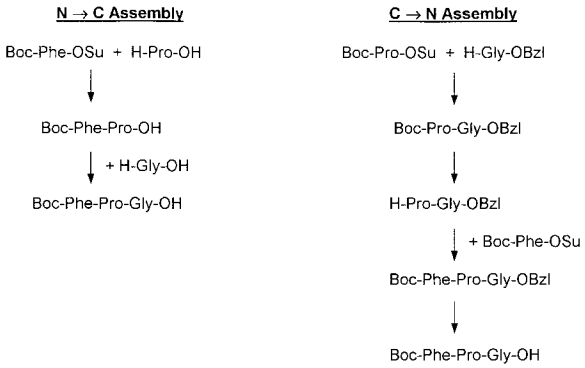


FIGURE 2 Comparison of strategies for assembling the tripeptide, Boc–Phe–Pro–Gly–OH.

a case, it is better to resynthesize a shorter segment in a convergent synthesis than a longer sequence in a linear synthesis. As mentioned in the section on strategy, peptides of up to five amino acids in length are usually conveniently assembled and isolated by conventional methods, using extractions and precipitations. However, from a practical point of view it may be advantageous to use even smaller segments of two or three amino acids in length. The reason for this is that it is often easier to obtain purer segments by precipitation or even crystallization, which can be used to advantage in segment condensations. Second, use of smaller segments may shorten the production time because of the possibility of using parallel production equipment.

It should also be noted that small segments often can be assembled in two ways: either the normal, C → N direction, with both N- and C-terminal protection, or the other possibility, i.e., the N → C direction, as illustrated schematically in Figure 2 for the tripeptide segment, Boc–Phe–Pro–Gly–OH. As shown in this hypothetical example, it is possible, by assembling the peptide in the N → C direction and avoiding C-terminal protection, to shorten the synthesis by two steps, which can be very important on a large scale. However, the risk of racemization and the conditions for performing the salt couplings in a practical manner must be evaluated critically.

The use of extreme reaction conditions should be avoided in large-scale manufacturing processes. While it is easy and quite common to perform a reaction in the research laboratory at −30°C or even −70°C, such conditions may cause problems on a scale-up, because the necessary equipment is expensive and also because longer cooling and heating times are necessary, which restricts access to the equipment for other purposes. Fortunately, such conditions are seldom encountered in peptide synthesis, where reaction temperatures generally range between −20°C and +100°C. Other extreme reaction conditions, such as high pressure, very dry conditions, very long reaction times, highly exothermic reactions, or the use of very specialized equipment, are also seldom necessary in peptide chemistry. However, it may be necessary to use toxic or hazardous chemicals such as TFA or hydrogen, which require special precautions. The high concentrations that are often required for segment condensations can also give rise to stirring problems, while the low solubility of fragments may restrict the scale of production because of limitations on reactor volumes.

As noted above, low solubility can create problems, especially as the peptide chain increases in length. For shorter peptides, ethyl acetate is the solvents of choice, due to its low toxicity, low boiling point, and lack of miscibility with water, making it well-suited for extractive workup procedures. For longer peptides, DMF is recommended, which requires the use of alternate workup methods, such as precipitation, for example with ethanol, rather than extractive procedures. *N*-methylpyrrolidinone (NMP) and dimethylsulfoxide (DMSO) also have potential for use with longer peptides, but they remain to be explored as solvents for large-scale solution synthesis.

An important consideration during scale-up is the technical feasibility of large-scale reaction and isolation procedures. This is a heterogeneous group of unit operations and other procedures that can be used conveniently on a laboratory scale but occasionally will create serious problems in large-scale syntheses. Examples of such apparently trivial problems are as follows:

- use of extreme reaction conditions (see discussion above);
- use of steel reaction vessels on a large scale (instead of the glassware typically used on a laboratory scale) that makes it difficult to see, for example, color changes or separations in two-phase systems;
- use of solids, such as drying agents (e.g., $MgSO_4$), which requires unnecessary filtrations and handling of solvents;
- precipitates or concentrated products that cannot be removed as easily from a reaction vessel as from a glass flask;
- concentration under reduced pressure of aqueous solutions from chromatographic purifications, which may be very time-consuming (an example of a problem that is more easily handled on a large scale by the use of reverse osmosis, a technique that is not as easily applied on a small scale); and

- because most unit operations on a large scale are much more time demanding than the same operation on a laboratory scale (for example, concentration on a rotary evaporator at elevated temperatures can be carried out in minutes on a laboratory scale, while the same procedure on a large scale requires many hours), side reactions not observed previously on a small scale may occur to a significant extent during such prolonged operations.

A specific problem that is encountered on scale-up is the preferred requirement for isolated intermediates during the synthesis to be in the form of solids, rather than oils, which are difficult to handle and make in-process control complicated. This can be achieved in two different ways: by precipitation methods or by chromatography. Precipitation, or possibly crystallization for small segments, is the preferred method, because it is generally quick and simple to perform and easy to scale-up linearly, although it can sometimes require a painstaking amount of work to find suitable methods. On the other hand, chromatography, as a general technique, is demanding of time and resources on a multikilogram scale, with the currently available technology, at least, and is not possible to scale up in a linear manner. Nevertheless, a considerable degree of purification is usually obtained, which is sometimes difficult to achieve by other methods, especially for longer segments and "difficult couplings," which makes it a viable alternative to consider in such cases. For the final product, alternative purification methods to chromatography seldom exist at present.

Commercial and Economic Aspects. A considerable number of different protected and activated amino acid derivatives and coupling agents are now available commercially. However, due to the high cost and limited availability of most of these in large quantities, their use in industrial syntheses is often prohibited. For the same reasons, the use of minimum protection strategies is also preferred. For example, the use of side-chain protected arginine derivatives, such as the costly 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) or 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sufonyl (Pbf) derivatives, may be preferred from a chemical point of view, but because of their high cost, the inexpensive HCl salt is the preferred alternative. Similar considerations can affect the choice of coupling additives, such as HOBt, 3-hydroxy-3,4-dihydro-1,2,3-benzotriazine-4-one (HOOBt), and HOAt. Even if the latter is currently the preferred reagent, especially for demanding couplings,[23] its cost is prohibitive on a large scale, and therefore the least expensive reagent (HOBt) is normally used. The choice of whether to use preactivated or nonactivated amino acids is not so straightforward, however. While preactivated amino acids are more expensive, in situ activation procedures may be more time-consuming and may give significantly lower yields and purities, which can offset the higher cost of the preactivated derivatives. Therefore, the choice between the two types of starting materials can only be made after the necessary development work has been completed.

It is not uncommon in published literature procedures to use large excesses—for example, two or more equivalents—of the activated amino acid to complete reactions and increase the purity of the products. This practice is unacceptable, obviously, from an economic standpoint on a large scale, and therefore reagents and reactants should be used in as close to stoichiometric amounts as possible, in order to minimize raw material costs, even if the purity may be adversely affected as a result. Nevertheless, quality demands for final products are equally important and the key challenge for manufacturers today is to deliver a product both economical and of consistently high quality.

Safety and Environmental Aspects. The reagents, chemicals, and solvents used in industrial processes must not only be cheap and commercially available but also safe to use for both humans and the environment. These considerations can often cause difficulties and add considerable costs to projects at the development stage because of problems such as the following:

- elimination of diethyl ether, which is often used as a convenient precipitation agent on a laboratory scale, in large-scale processes, due to the high risk of explosions and/or fires;
- the need to find a substitute for dichloromethane, which is an ozone-destroying solvent;
- substitution of benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as coupling agent, because of formation of the known carcinogen, hexamethylphosphoramide (HMPA), as a by-product;
- substitution of TFA and hydrogen fluoride (HF), both of which are highly hazardous;
- safe use of hydrogenation reactors for deprotection reactions;
- elimination of HN_3, a highly toxic by-product of azide couplings; and
- control of NH_3 emissions because of environmental pollution concerns.

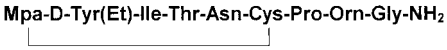


FIGURE 3 Structure of Atosiban drug substance.

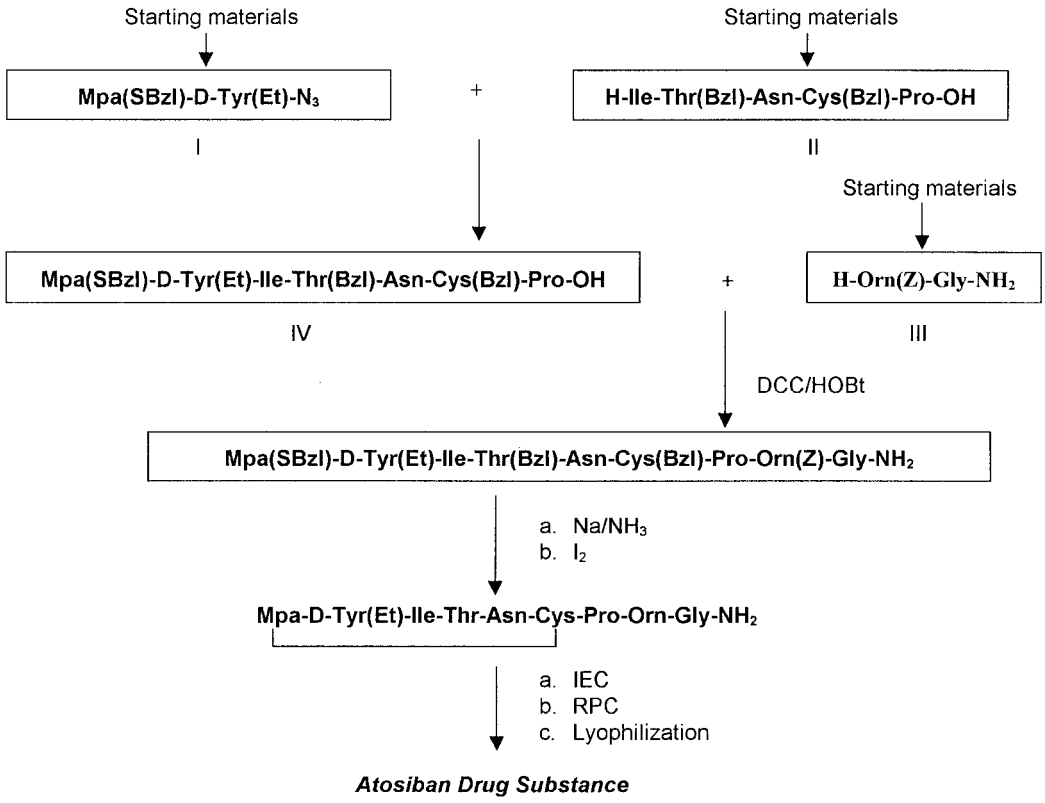
In addition, the potential for runaway reactions must be minimized, although, fortunately, these are seldom encountered in peptide chemistry. In general, because of these concerns, when a choice of reagents, solvents, or reaction conditions exists, the less dangerous alternative must be utilized, even at the expense of the yield or the purity of the product.

The minimization of hazardous waste, because of environmental concerns and high disposal costs, must also be taken in account. Although it is usually not considered in these terms, the manufacture of peptides, as well as many other drugs, is, in reality, the production of large amounts of waste with the drug as a small side product, and the manufacture of one kilogram of peptide can easily result in 1000 L or more of waste products.[24]

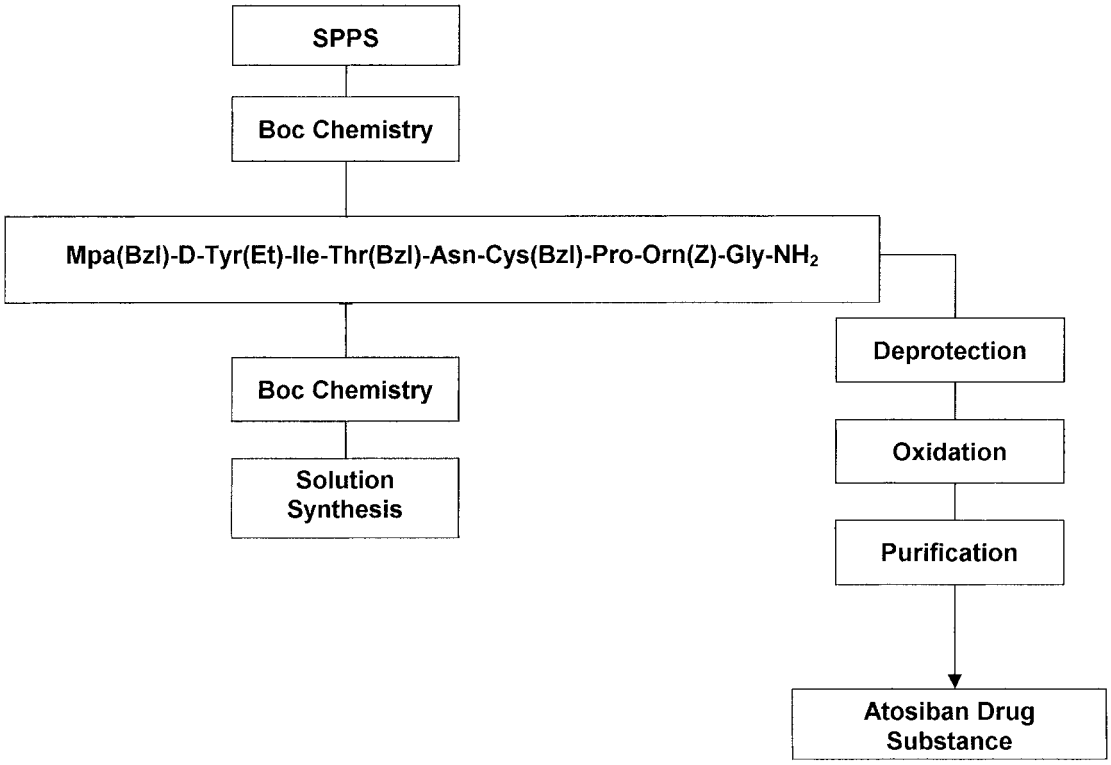
Examples of Large-Scale Solution-Phase Peptide Synthesis

Two processes for industrial-scale production of peptides will be discussed. The first of these relates to the multikilogram scale manufacture of Atosiban drug substance, an oxytocin antagonist developed as a therapeutic product for treatment of preterm labor and delivery.[25] The other process relates to the multikilogram scale manufacture of Desmopressin drug substance (l-deamino-8-D-arginine-vasopressin), an important antidiuretic for treatment of diuresis, such as that associated with diabetes insipidus, nocturnal enuresis, and urinary incontinence.[26]

The Atosiban Process. The structure of Atosiban drug substance is shown in Figure 3 and the synthesis strategy developed for the large-scale production process for manufacturing the product is outlined in Scheme 1. It should be noted that, during the early stages of development, the drug substance for the first clinical trials was manufactured by a method based on SPPS. Subsequently, in order to meet the increased demands for drug substance, a change in manufacturing method from SPPS to solution-phase was considered necessary. However, such a production change requires that the impurity profiles of the old and new methods are at least equivalent. In order to meet this requirement, it was decided to develop the solution-phase synthesis in such a manner that it shared a common intermediate with the SPPS procedure, pref-



SCHEME 1 Overview of the solution-phase synthesis of Atosiban drug substance.

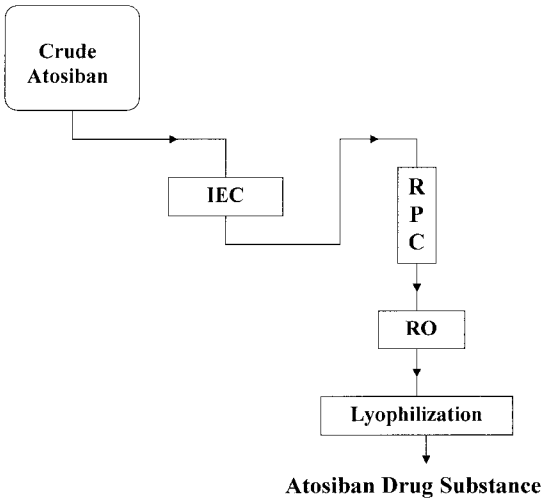


SCHEME 2 Illustration of the development approach for Atosiban drug substance, utilizing a common intermediate for solution- and solid-phase syntheses.

erably the key intermediate of the SPPS process. Scheme 2 illustrates this approach, which has been applied successfully to the development of the production method for Atosiban, in which the common intermediate of the two processes is the protected nonapeptide. The reaction steps following production of the nonapeptide are common for both processes. In this context, it should be noted that the critical impurities of the two processes are formed in the subsequent chemical steps, i.e., in the reduction and disulfide bond formation, yielding an identical impurity profile for both methods. As noted above, this strategy, utilizing a common intermediate, together with the benefits of both SPPS and solution-phase synthesis, should be attractive to apply to the manufacture of other drug substances, when requirements dictate the need to change production methods from SPPS to solution-phase.

Synthesis Strategy for Atosiban. The manufacturing process utilizes N^{α}-Boc protected amino acids and a global protection strategy, with only a single amino acid containing an unprotected side chain, i.e., that of asparagine.[27] The peptide chain is assembled by convergent synthesis, requiring, as indicated in Scheme 1, the synthesis of three separate segments—two dipeptides (I and III) and one pentapeptide (II)—which are subsequently coupled sequentially to yield the protected key nonapeptide intermediate (V). The dipeptides are synthesized using the mixed anhydride coupling procedure,[13] in which, prior to coupling, the amino acid derivatives are preactivated by treatment with isobutyl chloroformate. Fragment II is synthesized by stepwise coupling of preactivated Boc-amino acid derivatives. Starting with unprotected proline, successive *N*-hydroxysuccinimide ester couplings are performed to yield the tetrapeptide. The pentapeptide is obtained by coupling Boc–Ile–NCA to the Boc deprotected tetrapeptide rapidly (approximately 2 h reaction time) and in high yield. In this context, it should be noted that we have successfully applied Boc protected *N*-carboxyanhydrides, which are now commercially available, to the large-scale manufacture of peptides other than Atosiban. In spite of their relatively high cost, such activated amino acid derivatives have proven to be highly efficient, since they react cleanly and rapidly with nucleophiles such as the α-amino group of amino acids and peptides.[17,18]

Segment Condensations. The three segments (I–III) are assembled in such a manner that the risk of racemization is minimized. To this end, the dipeptide Mpa(Bzl)–D–Tyr(Et)–N_3 (I) (Mpa = mercaptopropi-



SCHEME 3 Overview of the purification process for Atosiban drug substance

onic acid) is coupled to the pentapeptide (II) through use of an azide condensation, conditions known to yield low racemization in segment condensations.[11] Likewise, the point of attachment of the heptapeptide (IV), produced in the previous segment coupling step, is a C-terminal proline, a residue less prone to racemize in the DCC-HOBt mediated segment coupling utilized to yield the desired, protected nonapeptide intermediate (V).[11]

Deprotection and Cyclization. The protecting groups of the nonapeptide are removed by reduction using sodium in liquid ammonia[28]. This deprotection method for benzyl protecting groups was applied by du Vigneaud in his pioneering work on the synthesis of oxytocin[1]. It should be noted that it is possible to produce kilogram batches of reduced peptide routinely by this deprotection procedure, provided that the progress of the reduction is monitored carefully. The reduced peptide is oxidized to give crude Atosiban by treatment with iodine at a relatively high peptide concentration (10 – 20 g peptide/liter) in aqueous acetic acid. Under these conditions, the oxidation reaction process takes place instantaneously, affording a high yield of cyclic peptide without significant formation of polymers.

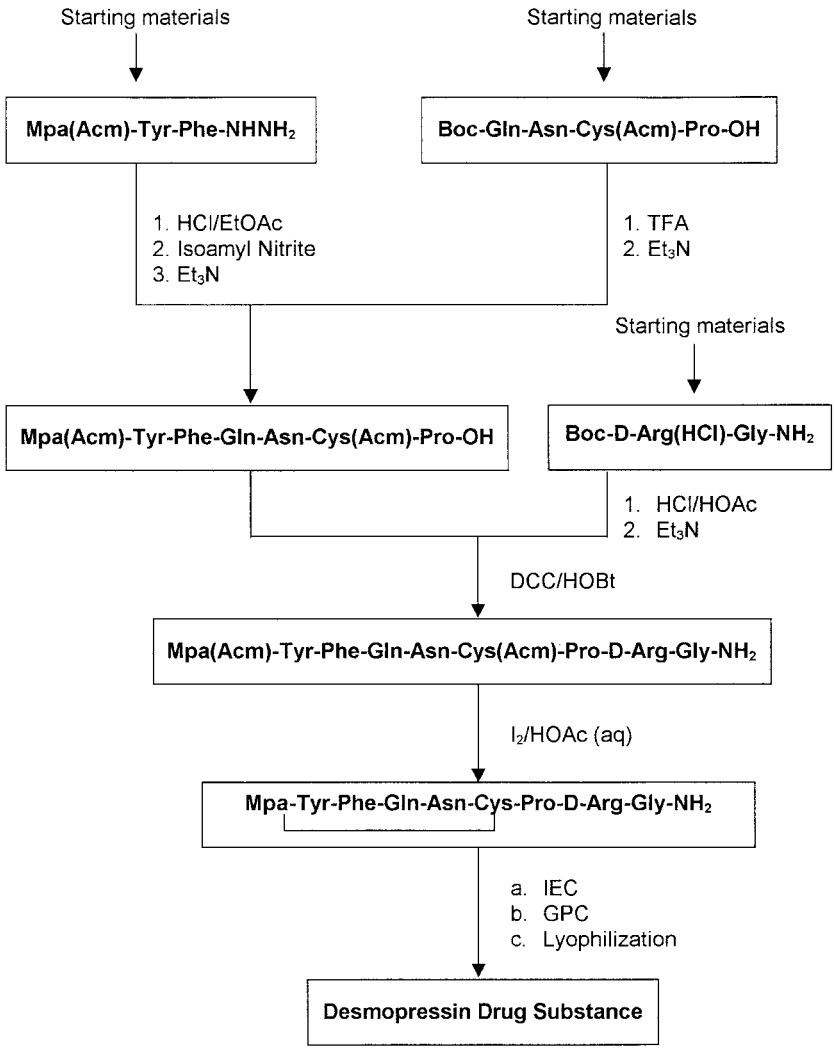
Purification and Isolation. The crude Atosiban peptide is purified in a two-step chromatographic procedure employing ion exchange chromatography (IEC) and reverse-phase chromatography (RPC), which is depicted schematically in Scheme 3. In the IEC step on SP-Sepharose FF, the HPLC purity of Atosiban is increased from about 90% to about 96%. Further purification by RPC chromatography on C-18 derivatized silica, using a gradient of ethanol in acetic acid, enhances the purity to 98% or higher. The purification data obtained by this method is summarized in Table II. It should be noted that the overall purification yield of Atosiban drug substance after both chromatographic steps is greater than 80% and that no reprocessing of side fractions is necessary during the purification process. After the RPC step, the pool containing pure peptide is concentrated by reverse osmosis (RO) and subjected to diafiltration, to remove ethanol, prior to the lyophilization step, which affords the acetate salt of Atosiban. The product has been characterized extensively and the impurity profile established, with identification of all impurities present in drug substance batches at levels greater than 0.1% (HPLC area %). Currently, this industrial-scale manufacturing method, which requires no chromatographic purification of peptide intermediates, is used to produce approximately 20 kg of drug substance per year, with future annual production estimated to be 50–100 kg.

The Desmopressin Process. The large-scale solution-phase process developed for Desmopressin drug substance (Scheme 4) resembles that described above for Atosiban drug substance. The structure of the drug substance is shown in Figure 4. Both compounds are cyclic nonapeptides containing a disulfide bond, formed during the cyclization reaction. In addition, both substances are manufactured by coupling preformed segments, i.e., by the convergent synthesis approach. More specifically, Desmopressin is synthesized via a 3 + 4+ 2 segment coupling strategy, while Atosiban is assembled by coupling the segments in a 2 + 5 + 2 mode.

In contrast to the strategy applied to manufacture the Atosiban molecule, Desmopressin drug substance is synthesized via a linear nonapeptide, utilizing *N*-α-Boc-protected amino acids and the minimal side-chain protection approach. Thus it proved possible to develop a successful Desmopressin production process by utilizing protection of the sulfhydryl groups on Cys^6 and Mpa^1 only. These functional groups are blocked by the acetamidomethyl (Acm group),[2] which is removed, with concomitant disulfide forma-

Table II Summary of Data Obtained for the Purification of Atosiban Drug Substance

Purification Step	Yield (%)	HPLC Purity (Area %)
Crude peptide	100	~90
Ion exchange	>90	>96
Reverse-phase HPLC	>90	>98



SCHEME 4 Overview of the solution synthesis of Desmopressin drug substance.

tion, by oxidation with iodine in aqueous acetic acid. This protection group strategy, in combination with segment synthesis, using mainly *p*-nitrophenyl ester and DCC/HOBt mediated couplings, yields an economical and efficient process for the manufacture of high-purity Desmopressin.[30–33] Furthermore, peptide intermediates produced in the Desmopressin process are more hydrophilic in nature than those obtained in the production of Atosiban drug substance, primarily due to the minimal protection group strategy applied. Therefore, intermediates isolated during the synthesis of Desmopressin are predominantly purified and isolated by precipitation, rather than via extraction with, for example, ethyl acetate, as normally applied to the less polar segments isolated during the Atosiban process.

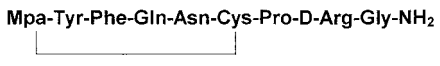


FIGURE 4 Structure of Desmopressin drug substance.

In the synthesis of Desmopressin drug substance, there are two additional features that are interesting to comment on:

- The tripeptide hydrazide, Mpa(Acm)–Tyr–Phe–$NHNH_2$, is obtained by reacting Mpa(Acm)– Tyr–OEt with H–Phe–$NHNH_2$ in DMF/H_2O in the presence of α-chymotrypsin. Using this approach, employing enzyme-catalyzed coupling of commercially available phenylalanine hydrazide, use of toxic hydrazine to form the hydrazide from the peptide ester could be avoided. In addition, the enzyme-assisted coupling is highly efficient, affording the tripeptide hydrazide di-

rectly from the peptide ester in a single step in 90% yield.

- Crude Desmopressin peptide obtained in the oxidative cyclization reaction is purified first by cation exchange chromatography on S-Sepharose FF, which has a dual function. Its primary function is to increase the HPLC purity of the product to a level of greater than 98%, while its secondary function is to eliminate the excess iodine used to form the disulfide bond. Thus, an additional step to remove excess iodine at the end of the oxidation reaction (for example, by the addition of a reducing agent such as sodium thiosulphate or dithionite or ascorbic acid) is avoided. Finally, an additional purification by gel permeation chromatography on Sephadex G-25 is carried out to enhance the purity of the peptide to 99% or higher, prior to lyophilization to give the acetate salt of Desmopressin.

OVERVIEW OF THE SOLID-PHASE APPROACH

Background

From its inception, the solid-phase method has proven to be an extremely useful tool that has been instrumental in the development of numerous different peptides for research, and several excellent reviews have been published on the method.[7,34,35] Despite this, the method has only been used in a limited number of cases for the commercial manufacture of peptide drug substances (Table I). As noted above, the solid-phase strategy is most frequently used to manufacture drug substance during the early stages of development of peptide pharmaceuticals, principally because little development of the method is necessary, and therefore the drug substance required for preclinical and early phase clinical studies, at least, can be manufactured relatively rapidly. Frequently, though, the manufacturing method is changed to the solution-phase approach when larger amounts of drug substance are required, because, for many years, the solid-phase synthesis approach was thought to be unsuitable for the large-scale manufacture of peptides for reasons that include the following:

- the high cost of the process and its lack of scalability;
- lack of adequate in-process controls during the assembly of the protected peptide on the resin support used for the synthesis; and
- the generally low purity of the final products.

The relatively high cost associated with solid-phase syntheses is attributable to the nature of the chemistry on polymers, which, traditionally, uses large excesses of protected amino acid derivatives and reagents. The excess reagents effectively drive reactions to completion and reduce the formation of undesirable side products that can occur at each step of the process. In addition, the volumes of the solvents used for the multiple cycles at the various steps during the assembly of the peptide are larger than those used in classical, solution-phase chemistry. The original expectation that intensive washing of the peptide-resin after each step would be equivalent to the purification methods (such as crystallization), which are used in solution-phase synthesis methods, has proven to be unrealistic. While it is quite certain that such repeated washing cycles cannot achieve the same purification efficiency, this lower efficiency is offset, to some extent, by the use of large excesses of solvents and reagents, which generally leads to high *chemical* efficiencies.

With the advent of new coupling methodologies and resins for solid-phase synthesis, the overall efficiency of the method has generally increased. In addition, the use of more sensitive in-process monitoring techniques for reactions on the polymeric matrix can further increase the efficiency of the process (see discussion below). Furthermore, the use of reverse-phase HPLC methodology for purification of the complex mixtures that often result from solid-phase syntheses, especially when used in combination with an orthogonal method, such as ion exchange chromatography, has proven to be extremely efficient, and has, for the most part, offset the concern about the purity of crude products resulting from the synthesis. Therefore, most, if not all, of the original concerns about the suitability of the SPPS method for scale-up are no longer valid.

General Considerations

Despite the introduction of many new protecting group strategies, the most common solid-phase methods in use today are those based on the original Boc/benzyl strategy, first introduced by Merrifield,[2,36] and the Fmoc/*t*-butyl strategy.[7,35] Each strategy has its own advantages and drawbacks, and during the development phase of a project, the two methods are often compared. Regardless of which method is ultimately chosen, though, considerable effort must be expended, as with any other project, on the optimization of the individual steps in the process. While the extent of optimization will depend on the project, and

particularly on the scale, the following general synthetic steps must be considered:

- attachment of the first protected amino acid derivative to the polymeric carrier;
- cleavage of the temporary protecting group;
- coupling of the remaining amino acid derivatives in the desired peptide sequence;
- multiple washes of the resin after each step;
- detachment of the peptide (either protected or deprotected) from the polymeric carrier;
- cleavage of the temporary and permanent protecting groups on the resin or in solution;
- isolation of the crude peptide;
- purification of the peptide; and
- isolation of the final product.

Resin Supports. The choice of the resin for a particular synthesis (i.e., type of resin, loading, handle modification, etc.) and the chemistry used for attaching the first (i.e., C-terminal) amino acid in the sequence are mainly determined by the peptide sequence and the protecting group strategy to be used, and all of these factors can have a profound effect on the success of a synthesis. A variety of different polymeric matrices are now available for use,[6] with an even greater selection of chemistries for attachment, although the original crosslinked polystyrene matrix, first proposed by Merrifield, is still the most widely used. However, in all strategies, it is critical that the method for attaching the first amino acid should be both high yield and free of racemization. Furthermore, compatibility of the solvent or solvent mixtures with both the polymeric carrier and also the protected peptide chain being assembled is of critical importance at every step of the process, since solvation of the reaction centers on the polymer influences their reactivity. Therefore, the choice of the solvent or the solvent mixture plays an important role, particularly in coupling steps. If a coupling is repeated, the solvent may be changed, while, in other steps (e.g., cleavage or neutralization), the reaction medium is determined by the reaction step itself and is not normally changed subsequently.

Cleavage of Temporary Protecting Groups. Cleavage of temporary (i.e., N-α) protecting groups during the peptide assembly stage is an important step in all peptide synthetic processes, and the orthogonality principle[34] should be applied, whenever possible, as in the case of the Fmoc/*t*-butyl strategy. The N-α protecting groups are generally cleaved by more uniform methods in the Fmoc/*t*-butyl strategy (e.g., by a solution of piperidine in DMF) than in the Boc/Bzl strategy, where many suitable acidic reagents can be used (see discussion below). Despite its high cost and environmental concerns, TFA is still the most frequently used reagent for acidolytic removal of the Boc group.

Coupling Reagents. As with the solution-phase approach (see discussion above), a wide range of coupling reagents and methods are available for use in the solid-phase approach, and the choice is often influenced both by the type of the peptide sequence and also any "difficult couplings," which may be predicted in advance.[37,38] Carbodiimides [i.e. DCC and *N*,*N*-diisopropylcarbodiimide (DIC)], mediated by 1-hydroxybenzotriazole, are often the preferred reagents for this purpose, because of their low cost compared with alternate reagents, such as TBTU[15] and HBTU,[16] although protected NCAs (UNCAs) have been shown to be extremely effective recently.[18,39]

Cleavage/Deprotection. In most solid-phase strategies, the side-chain deprotection step and detachment from the resin are carried out in a single step. The method for detachment of the peptide from the matrix is determined by both the C-terminal modification of the peptide (if any) and the synthetic strategy employed. Careful optimization of this step is usually critical, especially in the Boc/Bzl strategy, since the outcome can have profound impact on the total yield, impurity profile, and the overall economy of the process. Both the Boc/Bzl and the Fmoc/*t*-butyl strategies require acidic conditions for this step, and the choice of scavengers is critical for both methods. The former strategy requires very strong acids, such as anhydrous HF or trifluoromethanesulfonic acid, however, which can present serious concerns on scale-up. On the other hand, the milder acidolytic deprotection conditions, enhanced safety, and decreased environmental concerns of the Fmoc/*t*-butyl strategy typically can influence both the development strategy and the final choice of manufacturing method.

Purification/Isolation. The purification of the final product is, obviously, one of the most critical steps in the process. The choice of methods is dependent on the nature and complexity of the contaminants in the crude product. As noted above, because of the fact that no intermediate purifications are performed in the solid-phase method, the crude products often contain complex mixtures of contaminants, such as deletion sequences, truncated sequences, and diastereomers, which can present a considerable challenge on purification. While preparative reverse-phase HPLC can

often be used on a laboratory scale, a combination of two "orthogonal" methods, such as ion-exchange and reverse-phase HPLC, is often employed on a large scale (see discussion below). However, it is important to reiterate that, since purification steps are often demanding of time and resources, minimization of the extent of side products through careful optimization of all synthetic steps, as well as the cleavage step, can be of critical importance to the overall success of a large-scale solid-phase process (see discussion below).

Economic and Environmental Considerations. As noted above, in the discussion of the solution-phase approach, a number of other factors must be considered when processes are scaled up. These include the following:

- the economy of the process;
- the purity of the final product;
- the safety of the process; and
- the treatment of waste and its impact on the environment.

The economy of a process is, obviously, primarily determined by its efficiency (i.e., the cost of raw materials, the processing time, and the overall yield and purity of the final product), and also by its robustness and reliability. However, waste disposal costs and the costs associated with complying with safety and environmental regulations are also critical factors that must be taken into account during the final choice of the method. The Fmoc/*tert*-butyl strategy is generally considered to be more "environmentally friendly" than the Boc/Bzl strategy, and environmental considerations may, in fact, be the determining factor in the event that the use of particularly hazardous chemicals, such as hydrogen fluoride, which is often used in the Boc/Bzl strategy, is not permitted. In the absence of such considerations, both approaches are normally compared during the screening phase of development. The final choice of method may then be made on the basis of the total yield of peptide obtained in the desired quality, vs the raw material and manufacturing costs, together with the expected total manufacturing requirements for the product. While the raw materials for the Boc/Bzl strategy are generally considered to be less expensive than those for the Fmoc/*t*-butyl approach, the difference may be offset by lower waste disposal costs and/or processing costs for the latter approach.

A critical factor, which can strongly influence the yield of the final product regardless of the protecting group strategy used, relates to the spectrum of impurities contaminating the product. Frequently, the presence of contaminants, such as deletion sequences and diastereomers, can create significant problems during purification, since the relative retention times of the peaks corresponding to these contaminants are often very similar to that of the main peak. Removal of such contaminants can therefore be very difficult, leading to substantial reductions in yield and corresponding increases in cost. It is thus critical to use a sensitive in-process method for monitoring coupling reactions (see discussion below), and that end-capping, for example, by acetylation, be performed in order to minimize the formation of deletion sequences. Similarly, the coupling reagent should be chosen carefully, so as to minimize the possibility of racemization. If purification difficulties are encountered in the early development stage, however, it is may be prudent to reconsider the synthetic approach rather than to search for a more efficient purification method.

Application of Novel Methodologies to Problems Encountered on Scale-Up

After almost forty years' of use, solid-phase synthesis on a laboratory scale is now considered to be relatively routine. However, when a process is first scaled up for commercial use, invariably substantial problems are encountered, which can be both technical in nature, as well as economic and environmental, as discussed above. The challenges that these problems present often require the development of novel solutions, some examples of which are discussed below.

Cleavage of Temporary Protecting Groups in the Boc/Bzl Strategy. In the Boc/Bzl strategy, in which the temporary (i.e. , *N*-α) Boc-protecting group is cleaved under acidic conditions, there is rarely complete orthogonality with the conditions for removal of permanent (i.e., side-chain) protecting groups, which are almost all cleaved to a small, but significant extent under acidic condition. Under the cleavage condition used for the Boc removal (TFA, HCl solutions), for example, the Z and Bzl protecting groups show significant instability, and even the tosyl group, commonly used for protection of arginine, has limited stability. While this may not pose problems in laboratory-scale syntheses, even minor instabilities of side-chain protecting groups can cause significant problems during large-scale manufacturing, because of significantly longer mixing and draining times, and the resulting formation of small amounts of side products can complicate the validation of critical steps.

The reagent which is most commonly used for Boc removal is 20–50% TFA in dichloromethane

(DCM),[40] the excellent swelling properties of which are a significant advantage. However, the high cost of this reagent, coupled with significant safety and waste disposal issues, has prompted the investigation of alternate, less expensive cleavage reagents.[41] These include the following:

- 10% sulfuric acid in dioxane;
- 0.5M methanesulfonic acid in 1:9 dioxane/DCM;
- 4 N HCl in dioxane, or ethyl acetate;
- 1 N HCl in acetic acid; and
- HCl in mixtures of solvents (e.g., acetic acid/DCM, acetic acid/toluene, etc.).

While many of these reagents have found utility in the solution-phase approach, all of them cause significantly less swelling of peptide-resins than TFA-containing mixtures, leading to reduced accessibility to the reaction centers. Therefore, efficient methods for in-process control are essential (see discussion below). This "disadvantage" can, however, be used to advantage in large-scale processes, when larger amounts of peptide can be synthesized on highly loaded resins in reactors of smaller volume, compared to the classical method of assembly, in which TFA solutions are used during the cleavage step.

HCl-containing mixtures[42,43] are particularly attractive, because of the low cost of HCl gas and the lack of serious environmental concerns. However, the fact that such mixtures are best prepared immediately before use, or even in situ, can cause problems, especially from the standpoint of cGMP compliance issues and process validation. A particularly critical factor, which must be taken into consideration with HCl-containing mixtures, is the relative rates of cleavage of the Boc group and the benzyl ester linkage to the resin, in the case of sequences containing a C-terminal carboxylic acid. For this reason, it is particularly important to monitor carefully the decrease of polymer loading during the assembly stage, due to the limited stability of the benzyl ester bond toward these reagents. A technical problem associated with the use of HCl-containing reagents—their highly corrosive nature—requires that all exposed metallic parts of equipment must be protected by coating with an inert plastic material such as polyethylene or, preferably, by more resistant Teflon.

We have investigated the suitability of an industrial process using HCl in DCM, prepared in situ, as a reagent for removal of the Boc group during solid-phase synthesis.[44] At 15°C, it is possible to prepare an approximately 1% solution of HCl in DCM, while at lower temperatures, the concentration increases considerably. Since the rate of cleavage is determined by both the concentration of HCl in DCM and the temperature, it is clearly important to control the temperature during the addition of the HCl gas. The reagent may be prepared directly in the solid-phase reactor by introducing a gentle stream of gaseous HCl, via the fritted disk at the bottom of the reactor, into the suspension of the Boc-peptide-resin in DCM, without additional cooling of the reaction mixture. The temperature should be maintained between 25 and 30°C, by controlling the rate of addition of the HCl gas, throughout the time of reaction (30– 60 min). If the rate of addition of the HCl gas is too high, the temperature of the reaction mixture decreases, due to evaporation of DCM, resulting in a considerable increase in the HCl concentration. Despite the increased concentration of HCl, the rate of cleavage of the Boc group decreases at lower temperatures since, apparently, the higher concentration of HCl is less important in the first-order kinetics of this cleavage reaction.

Using this technique, the Boc group can be removed completely, without significant cleavage of the benzyl ester linkage to the resin, throughout the sequence of peptide assembly, and the technique has been applied successfully to a number of peptides up to 10–15 amino acids in length. Since the in situ saturation of DCM by HCl does not increase the swelling of peptide-polymer during the cleavage steps, it is possible to work with a larger quantity of highly loaded (1–1.3 mmol/g) resin, compared with syntheses in which 50%TFA/DCM is used as cleavage reagent. Furthermore, the technique is more "environmentally friendly," which makes it attractive to use for large-scale manufacturing. Further optimization is necessary, however, because of the need to eliminate water after each coupling, as well as the control of temperature increases during exothermic deprotection reactions, both of which can affect the reproducibility of the method.

Detachment of the Peptide from the Resin. Detachment of the peptide from the resin after assembly, often with simultaneous removal of side-chain protecting groups, is a critical step, during which many side products can be formed. Optimization of this step is therefore critical to the yield and purity of the final product and to the economy of the process. While most of the peptides produced by the solid phase are those containing either C-terminal carboxylic acid or amide groups, C-terminal esters and alcohols can also be important. Methods of detachment leading to these types of compounds are discussed below.

Table III Use of Gaseous Ammonia for the Production of Peptide Amides

Peptide	Peptide-Resin (g)	Detached Peptide (g)	Yield (%)	Purity (%)
Desmopressin	2417	1350	85	86
Terlipressin	3615	2201	86	70
Oxytocin	2150	1150	95	78
Carbetocin	1095	510	85	82
LH-RH	750	390	80	90

Formation of C-Terminal Amides by Ammonolysis.[36,45] Ammonolysis is used for the detachment of many important peptides, including neurohypophyseal hormones and analogs, as well as analogues of LH-RH. Traditionally, the detachment has been carried out by suspending the resin-peptide in methanol or in mixtures of solvents, such as DMF:MeOH 4:1 or dioxane/MeOH 9:1, to which dry gaseous ammonia was added to give an ammonia concentration of about 20%. The reaction proceeds through the methyl ester intermediate and this is also the most common side product, together with the carboxylic acid, which is formed as a result of ester hydrolysis. The requirement to keep the methanol and ammonia dry can cause problems when the technique is used for large-scale manufacturing, however.

We have studied the ammonolytic reaction in a variety of solvents in which the solubility of the protected peptide amide varied widely. It was found from these studies that the choice of solvent is of importance and, surprisingly, the purity of the product was the highest in a solvent (toluene) in which the solubility of peptide was very limited. This observation, together with environmental concerns and efforts to improve the economy of the process, suggested that gaseous ammonia, in the absence of any solvent, could be used for the ammonolysis step.[46–48] A similar method, using slightly different reaction conditions, had been described previously for multiple peptide synthesis applications and in the detachment of the libraries. In this very simple and industrially suitable process, dry peptide-resin is placed in a pressure vessel, gaseous ammonia is introduced to a pressure of 2–5 bars, the vessel sealed, and the peptide-resin maintained under ammonia pressure for 2–3 days. After this time, the ammonia is removed under reduced pressure and the peptide extracted with a suitable solvent (preferably DMF).

The results obtained for several peptides synthesized on Merrifield resin and cleaved by ammonolysis by this method are summarized in Table III. These results demonstrate that gaseous ammonolysis is a suitable method for both the laboratory and production scale synthesis of peptide amides, which can be used with a variety of different types of carriers and in simultaneous multiple peptide synthesis.[47,48] The yield is usually dependent on the C-terminal amino acid in the sequence and the process must be optimized for each individual peptide. The method appears to be relatively mild, however, and also more efficient than the methanol-ammonia approach.

Formation of C-Terminal Carboxylic Acids by Acidolysis. Carboxylic acid derivatives of peptides can be obtained by direct cleavage of peptide-resins. In the Boc/Bzl strategy, HF is frequently used for this purpose, often in the so called low–high modification,[49] in which a solution of HF in dimethylsulfide is used to suppress alkylation side reactions, because of the change of reaction mechanism from SN1 to SN2. On a large scale, however, technical problems can be encountered, because of the need for reactions to be carried out in special Teflon equipment and also because HF is very toxic and highly hazardous. Substitution of HF by trifloromethanesulphonic acid (TFMSA) can have certain advantages.[50,51] Simultaneous deprotection and detachment has been performed using a cocktail consisting of an approximately 10% solution of TFMSA in trifluoroacetic acid containing dimethylsulfide and anisole. The TFMSA must be from a freshly opened container that has been stored under argon.

The cleavage cocktail described above has been used in the manufacturing of Somatostatin. The synthesis was performed using the Boc/Bzl strategy on a Merrifield resin, with the following protecting groups: Cys(Acm), Trp(For), Ser(Bzl), Thr(Bzl), and Lys(2-Cl-Z). The cleavage reaction was carried out on a batch of 500 g of protected peptide-resin, in a special 10 L reactor, equipped with a polypropylene filter, air driven stirrer, and an efficient cooling system. The cleavage reaction was performed twice (for 5 and 3 h) under an inert atmosphere at 10°C. Using this method, it was possible to cleave the peptide from the resin as its C-terminal carboxylic acid in good yield and purity. Isolation was accomplished by combining the acidic filtrate, with efficient cooling, directly with the DMF extract of the resin. The product was surprisingly stable in this very acidic mixture and it was

possible to concentrate the solution by evaporation. After redissolving the concentrate in a large volume of DMF, the Acm groups were removed and the disulfide bridge formed with the aid of iodine in a very rapid (15 min) reaction. After neutralization in water–DMF, the Somatostatin was isolated by ion exchange chromatography. The procedure was reproducible, and could be validated without any of the technical problems that could be expected with the use of HF.

Formation of C-terminal Esters by Transesterification. Detachment of the peptide from the resin as the ester is a base-catalyzed reaction, for which the so-called Beyermann Reagent [1*M* triethylamine (TEA) in MeOH] is frequently employed. Due to low swelling of the peptide-resin in MeOH alone, mixtures of solvents are often employed, in order to improve the accessibility of the alcohol-containing solvent mixture to the reaction sites on the resin. Alternatively, 2-(dimethylamino)-ethanol may be used as a 50% solution in DMF.[52] This autocatalytic transesterification reaction, unfortunately, can be negatively influenced by the low stability of DMF in this mixture, and yields are often relatively low. We have found that the most efficient reagent for transesterification, which is also applicable to industrial-scale syntheses, is a 2–5% solution of 1,8-diazabicyclo[5,4,0]undec-7-ene in MeOH. This method has been used to cleave protected fragments of calcitonin and LH-RH in a relatively short time (30–60 min) with high yields (85–95%). Since the major side reaction in this type of transesterification reaction is racemization, the method is preferred for sequences in which the C-terminal amino acid is glycine or proline, otherwise the extent of the racemization should be monitored carefully.

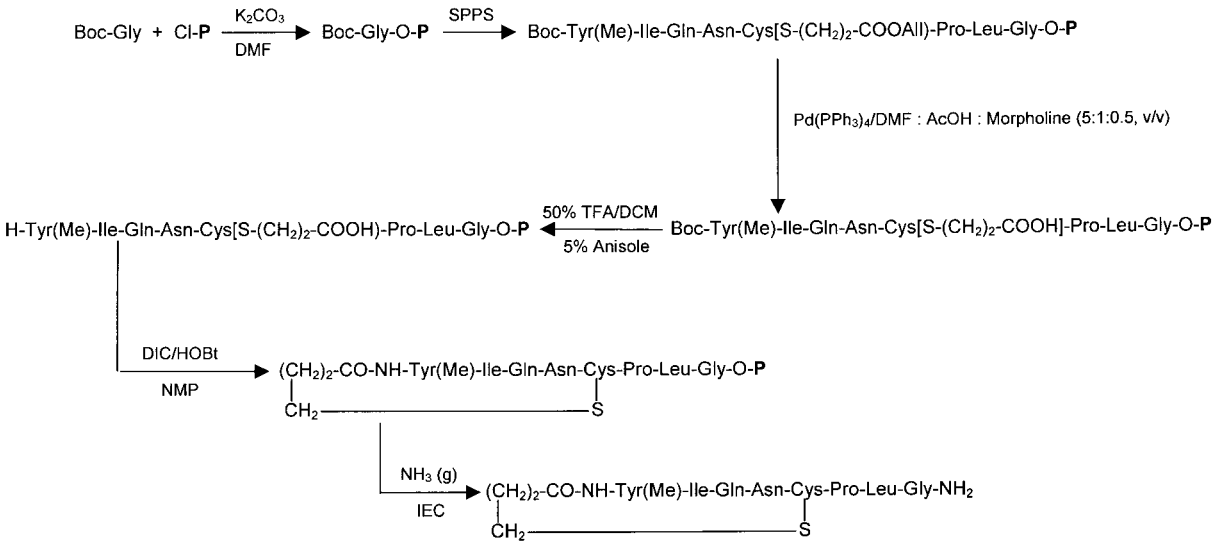
Formation of Peptide Alcohols by Reductive Cleavage. Peptides containing C-terminal alcohol functions are usually stable toward the action of exopeptidases and can, therefore, have prolonged duration of actions in vivo. An example of such a peptide, which is manufactured for pharmaceutical use, is Octreotide. We have developed a synthesis for this product in which the protected peptide is assembled on a Merrifield resin as Boc–D-Phe–Cys(Acm)–Phe–D-Trp–Lys(2-Cl-Z)–Thr(Bzl)–Cys(Acm)–Thr(Bzl)–O–resin. Treatment of the peptide-resin with $NaBH_4$ in a mixture of THF and water at elevated temperatures (50°C) for 2 h results in cleavage of the protected peptide as its C-terminal Thr(Bzl)-ol derivative in 70–75% yield.

Monitoring of Coupling Reactions. A weakness of the solid-phase method in general, especially from the point of view of large-scale manufacturing and cGMP requirements, is in-process control during the peptide assembly stage. Since the growing peptide chain is bound to the insoluble carrier during all steps, i.e., during deprotections and couplings, the possibilities to perform reliable analytical evaluation of the process, in the terms of peptide properties and state-of-the-art analytical chemistry, are very limited. The methods used for monitoring the completeness of cleavage of temporary protecting groups and completeness of the coupling reactions can be divided into several groups according to the purpose or method. Most of the tests in use currently are carried out with small (a few mg) disposable samples of peptide-resin, which are removed from the reactor during the assembly stage. Other "nondestructive" methods exist, which provide the required information by a change in color of the entire contents of the reactor, or by the titration either of the washings or the resin in the reactor. The presence of the free or blocked amino group is the control for the test and both the Boc/Bzl and Fmoc/*tert*-butyl strategies can use the same tests. In addition, the Fmoc/*t*-butyl approach can take advantage of the uv and fluorescence properties of the Fmoc group itself and its cleavage product, dibenzofulvene. The same is true for the less commonly-used Ddz group. Such uv monitoring has been used in continuous flow systems and in some automated solid phase synthesizers.

The following tests are the most frequently used for in-process control:

- the ninhydrin (Kaiser) test[53]; sensitivity: 0.02 meq/g;
- the chloranil (*p*-tetrachlorbenzoquinone) test[54,55]; sensitivity: 0.02 meq/g (suitable for proline);
- the picric acid test[56]; sensitivity: 0.01 meq/g;
- the bromphenol-blue (3′,3,5′,5″-tetrabromosulphophthalein) test[57,58]; sensitivity: 0.015 meq/g;
- the pyridine-hydrobromide test[59]; sensitivity: 0.02meq/g; and
- self-indicating active esters (e.g. 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-bezotriazine esters of Fmoc protected amino acid derivatives)[35,60]; sensitivity: 0.03 meq/g.

The sensitivity of the individual tests can vary and depend considerably on the peptide sequence, due to the difference in the accessibility and reactivity of the amino groups on the polymer. It should be emphasized that, for secondary amines, the ninhydrin test can provide false results and, therefore, the chloranil test is recommended.



SCHEME 5 Solid-phase synthesis of Carbetocin drug substance.

Bromophenol blue is a sensitive indicator, with the ability to monitor the course of couplings in a similar manner to the self-indicating active esters of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine.[60] Both reagents can be added to the reaction mixture after the deprotection (neutralization) step and the resulting color changes monitored qualitatively or quantitatively. However, this test can provide false results if used during the synthesis of longer peptides, especially, when basic amino acids, including histidine, are present in the sequence.

Titration of the entire content of amino groups in the reactor with perchloric acid[61] was used many years ago in Syn1, one of the first automatic synthesizers of Danish origin. However, the method has not found widespread use because of problems with the stability of the peptide on the resin under the acidic condition used during the titration.

Hydrobromide exchange between the weakly basic pyridine (pyridine · HBr) and the more basic primary amino group on the resin-peptide forms the basis of another monitoring method.[59] After neutralization with tertiary base, an aliquot of the washings containing HBr can be titrated argentometrically to determine the loading of the resin after deprotection steps.

We have used the picric acid test effectively in our laboratory to determine residual free amino groups after coupling reactions. Couplings were considered as complete if less than 0.01 meq/g of free amine was found, although frequently this value was as low as 5 eq/g. Alternatively, the same test can be run after each deprotection step, when decreasing substitution values at each step corresponds to the increasing amount of peptide bound to the resin. Deviations from linearity can be an indication of chain termination or loss of peptide (i.e., premature detachment of peptide from the resin). The test sample of the peptide-resin removed after the deprotection step can also be acetylated and the treatment with deprotecting reagent and the picric test repeated. Any change in the result is an indication of a deprotection problem.

Direct monitoring of the peptide assembly can be achieved through the detachment of the peptide from small samples of the peptide-resin by ethylaminolysis or gaseous ammonolysis, both of which are rapid enough to detach small quantities of peptide which are suitable for an in-process test using HPLC analysis of purity. In practice, a combination of different monitoring approaches is recommended, especially during the development stage. Once the process is defined, a single, routine test is usually sufficient and the ninhydrin or picric tests are frequently chosen for this purpose.

Cyclization On-Resin—An Unusual Side Reaction. Cyclization of peptides on the resin, prior to cleavage, has been proposed as a method of achieving the high dilution necessary for such a reaction, because of the relatively large distance between reaction sites on the resin, which results in a "pseudodilution" effect. We have investigated this approach for the synthesis of Carbetocin (deamino-carba-1,2-*O*-methyltyrosine-oxytocin), an analogue of oxytocin used in veterinary indications (Scheme 5). An allyl protecting group was used in the synthesis of the cysteine derivative [*N*-*t*-butyloxycarbonyl-S-(3-allyloxycarbonylpropyl)cysteine],[62] which served as the key derivative during the synthesis of the peptide-resin. After cleavage of the

allyl and Boc groups, the carba-bridge was formed on-resin with the aid of DIC/HOBt coupling. Subsequently, the crude carbetocin was detached from the resin by gaseous ammonolysis in high yield.

During purification, the side product, D-Asn^5-carbetocin,[63] was identified, although it was not clear in which step this diastereomeric peptide was formed. While the extent of formation of this side product depended on the conditions used for cyclization, this was not the only factor. It was found that, not surprisingly, D-Asn^5-carbetocin is formed when either the resin-peptide or the peptide in solution (during isolation) is exposed to basic pH (pH >7.5). Under these conditions, the change of the L-Asn configuration was selective and the mechanism of the reaction was not conventional racemization, but rather inversion of configuration. By simply exposing carbetocin to basic pH, it was possible to obtain D-Asn^5-carbetocin in good yield. However, the reverse reaction (i.e., formation of carbetocin from D-Asn^5-carbetocin) was not observed. The biological activity of this diastereomeric peptide is known to be negligible, and all changes in position 5 of neurohypophyseal hormone peptides usually lead to the almost total abolition of the hormonal effects. This fact and the observed sensitivity of the Asn residue to the inversion of its configuration lead us to the conclusion that similar natural mechanisms can occur in vivo as a means of inactivating neurohypophyseal hormones and elimination of their activities.

OVERVIEW OF THE HYBRID APPROACH

Background

As noted above, the availability of new resins and resin handles has opened up the possibility of synthesizing fully protected peptide segments rapidly by the solid-phase technique, which can be assembled subsequently by solid-phase[64] or solution-phase[9,65] methods. While this approach is not new, it has not been widely exploited, although it is used extensively as a development tool for solution-phase projects, as noted above. The approach is particularly attractive for the manufacture of large molecules, since it combines the advantages of both the solid-phase and the solution-phase methods. In particular, the solid-phase synthesis of fragments can be developed and scaled-up relatively rapidly, and avoids many of the solubility problems often encountered in solution-phase syntheses of relatively long segments. Production cycle times are short, compared with solution-phase methodologies, and yields and purities are often higher, because of the use of excess reagents, especially during coupling reactions, often resulting in intermediates that do not require purification. Through optimization of the sequences of the fragments, especially on the basis of their solubility characteristics, the final stages of the process, i.e., the fragment assembly and deprotection, can be scaled-up by conventional, solution-phase methodologies. Thus, the advantages of both the solid-phase method, i.e., rapid synthesis of fragments with high purities, and also the solution-phase method, i.e., full monitoring of coupling reactions, with isolation, purification, if necessary, and full characterization of intermediates, can be exploited.

Ac-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-NH_2

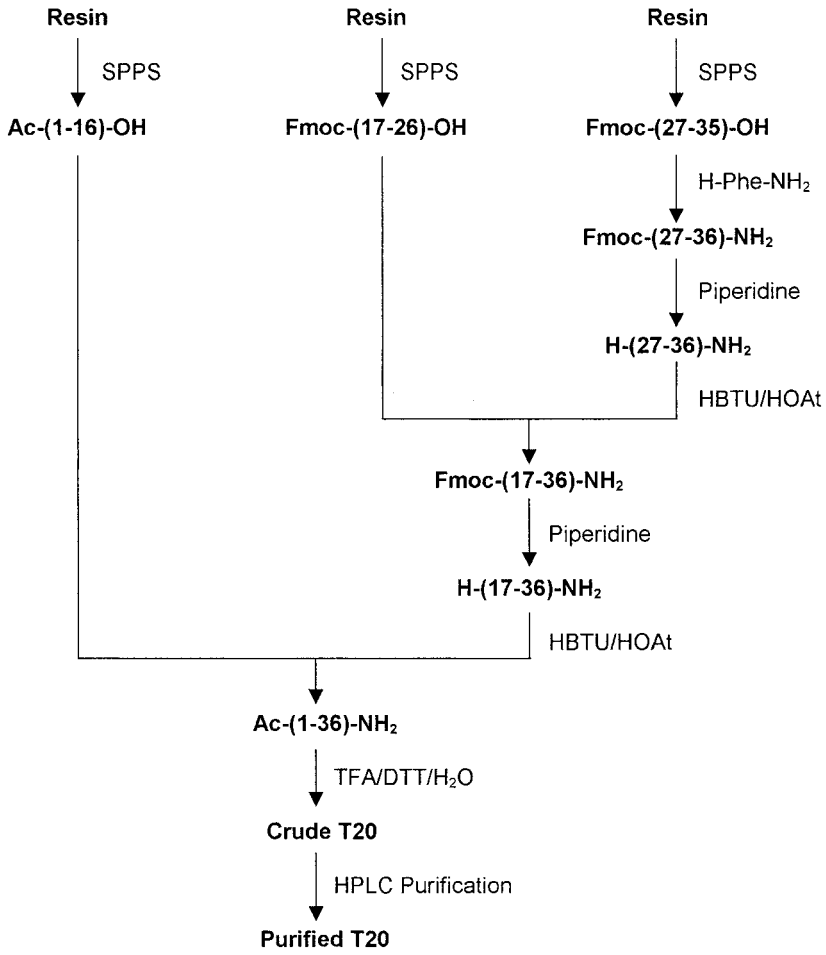
FIGURE 5 Structure of T20.

Example of the Hybrid Approach

Such a hybrid approach is being applied currently to the synthesis of T20,[66] a 36-amino acid peptide, derived from the ectodomain of HIV-1 gp41, whose structure is shown in Figure 5. The product, which is the first member of a novel class of antiretroviral agents that inhibit membrane fusion, is being developed for the treatment of HIV and is currently in Phase 2 clinical trials. During the early development phase of the project, the product was manufactured by conventional solid-phase strategy, using the Fmoc strategy. In order to meet the projected requirements for metric tons of the product on commercialization, however, the strategy was changed, at a relatively early stage, to a three-fragment hybrid approach, which is outlined in Scheme 6.

The three fragments—(1–16), (17–26), and (27–35)—are assembled on 2-chlorotrityl resins,[8] via the Fmoc strategy, using HBTU-mediated couplings and the following side-chain protecting groups: Boc on lysine and tryptophan; trityl on asparagine, glutamine and histidine; and *t*-butyl on serine, threonine, tyrosine, aspartic acid, and glutamic acid. After assembly, the protected fragments are cleaved from the resin by multiple treatments with dilute (1%, v/v) TFA in methylene chloride, with neutralization of the TFA with pyridine. After concentration of the solutions, the products are isolated by precipitation in high yield and high purity and are used without further purification for the solution-phase fragment assembly.

In the first step of the fragment assembly, Fmoc–(27–35)–OH is coupled with Phe–NH_2, using HBTU



SCHEME 6 Outline of the synthesis of T20 drug substance.

in the presence of HOAt, to give the (27–36) fragment. After removal of the N-terminal Fmoc group, H–(27–36)–NH_2 is coupled with Fmoc–(17–26)–OH in the same manner. Finally, after removal of the Fmoc group, H–(17–36)–NH_2 is coupled with Ac–(1–16)–OH, by the same coupling technique, to give the fully protected final molecule. Deprotection is accomplished using a "cocktail" containing TFA, dithiothreitol, and water to give the final crude product. In all cases, intermediates are isolated by simple precipitation techniques and purified by recrystallization or trituration. The yields of all reactions are uniformly high and the HPLC purity of the final crude product is >70%. The crude product is purified by preparative, reverse-phase HPLC techniques and isolated by lyophilization.

The synthesis of T20 is a rather remarkable example of the application of modern peptide synthesis techniques to a very challenging scale-up problem. Even though the process has not yet been scaled up fully, T20 is already being manufactured in batches of >10 kg. It is certainly the most complex peptide to be manufactured on such a large scale, and based on the level of success achieved in the project to date, it is likely that the ultimate goal of the project will be achieved, i.e., manufacture of metric tons of the product on an annual basis.

PURIFICATION AND ISOLATION TECHNIQUES

While optimization of every step in the manufacture of a peptide drug substance is important, in order to ensure that the product can be manufactured as economically and reproducibly as possible, the final steps in the process, i.e., purification and isolation, are critical to any process, regardless of the synthetic approach used. In principle, a variety of methods is potentially available for the purification of peptide drug substances, of which the following have historically been considered:

- crystallization;
- countercurrent distribution;
- partition chromatography;
- gel permeation chromatography;
- low-pressure, hydrophobic interaction chromatography;
- ion exchange chromatography; and
- reverse-phase, high performance liquid chromatography.

Since peptides are, in general, difficult to crystallize, crystallization is rarely attempted for final purification steps, except in the case of small (i.e., five amino acids or less) molecules. While countercurrent distribution and partition chromatography were used for purification of many of the early peptide drug substances, they have largely been replaced by more powerful techniques, such as those based on preparative, reverse-phase HPLC.

Today, therefore, in contrast to most other drugs, pharmaceutical peptides are normally isolated and purified by chromatographic procedures, which are more or less mandatory, due to the complexity of the products and the strict purity demands from regulatory agencies. The most common chromatographic techniques in use to today are gel permeation, low pressure hydrophobic, ion exchange and reverse-phase HPLC chromatographies. Obviously, wide ranges of chromatographic media are available for each type of chromatography, and the choice of the combination of purification steps and individual media used will be determined by the nature of the peptide and impurities it contains. Since chromatographic procedures, in general, are demanding of time and resources, the purification of the final product should be performed in as few steps as possible. Ideally, a single purification step should be utilized, since every chromatographic step potentially decreases the total yield, although this is often difficult to achieve in practice. In a typical purification scheme, the crude product from the synthesis is first subjected to a step—such as ion exchange, gel permeation, or hydrophobic interaction chromatography—which is designed to remove by-products from the final deprotection step(s), most of which are low molecular weight and uncharged, and may also afford substantial purification of the product. If further purification is required, a final "polishing" step may be performed using a complementary technique, such as reverse-phase HPLC. Finally, the organic modifier from the reverse-phase HPLC step (e.g., ethanol or acetonitrile) is evaporated and the product isolated by lyophilization, which, preferably, provides the final product in the correct salt form (typically acetate) without the need for a separate step. Such a purification scheme, using two complementary techniques, is often more efficient than, for example, the use of several reverse-phase HPLC steps in different mobile phases.

Chromatographic purification and lyophilization are both expensive steps, which, together, may actually contribute the major part of the cost of manufacturing a particular peptide on a large scale. Furthermore, limitations may be encountered, particularly with reverse-phase HPLC purification and lyophilization steps, when manufacturing on a very large scale (i.e., metric tons). In such cases, it is, obviously, important to optimize the process, if possible, to the point where the requirements of the final purification step are less demanding. If this can be achieved, techniques such as ion exchange chromatography and/or low-pressure hydrophobic interaction chromatography, both of which can be scaled-up with few limitations, may be adequate for production of the final product at the required level of purity. Furthermore, alternate techniques for final isolation of the product must be considered in order to accommodate the needs for larger-scale manufacturing. In this context, diafiltration/ultrafiltration may be considered as a useful adjunct to any final isolation step, since it provides a simple and scalable method of removing low molecular weight salts and organic solvents, as well as concentrating solutions prior to, for example, lyophilization. Alternate methods of isolation, such as spray drying or precipitation, which are more easily scaled-up than lyophilization, should also be considered.

REGULATORY CONSIDERATIONS

The ultimate manufacturing goal for any peptide pharmaceutical is the development of a process that is both economical and also meets the requirements of the regulatory authorities. The latter consideration is every bit as crucial as the technical aspects of the process itself, since failure to meet regulatory requirements can result in the failure to obtain approval to market the product. The ability to meet the expectations of the authorities requires similar planning to that required for the technical phases of the project, in that a "quality system" of procedures, documentation, and test methods for raw materials and finished products, must be established that guarantees that the process is highly reproducible and will reliably result in final product of consistent quality. This can be a particularly difficult challenge for manufacturing pro-

cesses for peptides, because of the large number of often complex steps involved in their production.

Synthetic procedures intended for use in industrial scale production must be reliable in several ways. A synthesis for large-scale purposes is developed in a laboratory by skilled chemists. However, it is most likely reproduced routinely in an environment with personnel less competent in chemistry but with higher quality and safety demands. Thus, the tolerance against variations in reaction parameters, such as temperature, reaction time, or reagent amounts, must be known and acceptably high. Ultimately, the process—including the in-process analytical methods, and those for testing and release of the finished product—must undergo formal validation, in order to confirm its reproducibility, both in terms of yields of intermediates, the yield of the final product and the consistency of its impurity profile.[67]

Prior to approval of a product for sale, the manufacturer of the bulk drug substance must be subjected to an inspection by one or more of the regulatory agencies, such as the U.S. Food and Drug Administration, for compliance with the applicable regulations.[68] At that time, the documentation of virtually every single aspect of the company's operations, from the ordering procedures for raw materials, to the final shipment of the finished bulk drug substance, must be able to stand up to in-depth scrutiny. Preparing for such a "Pre-Approval Inspection" can require considerable effort and expense on the part of the manufacture, but such preparation should be a part of the planning virtually from the beginning of the technical development phase of the project. While the overall effort to meet the final goal of approval is still the same, proper planning can assist by spreading the effort over most of the life of the project. The positive aspect of these regulatory requirements, of course, is that the safety of the final customer, i.e., the patient, is virtually guaranteed, because of the requirements for consistency of the manufacturing process, coupled with complete traceability from raw materials through the finished product.

CONCLUSIONS

It is clear that, almost 50 years since the first published synthesis of a biologically active peptide, by du Vigneaud and co-workers, the technology now exists to manufacture complex peptides on an extremely large scale. Furthermore, recent advances in the area of drug delivery have provided practical solutions to the problem of lack of oral bioavailability that plagued peptides for may years, and limited their use as pharmaceuticals mostly to life-threatening diseases. As a result, there has been a resurgence of interest in peptides for therapeutic applications, which is likely to result in numerous new peptides reaching the marketplace as drugs. This increased demand for production of peptides as pharmaceuticals will certainly provide a considerable manufacturing challenge. However, based on the current status of the technology, it is likely that this challenge will be met.

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REFERENCES

1. du Vigneaud, V.; Ressler, C.; Swan, J. M.; Roberts, C. W.; Katsoyannis, P. G.; Gordon, S. J Am Chem Soc 1953, 75, 4879–4880.
2. Merrifield, R.B. J Am Chem Soc 1963, 85, 2149–2154.
3. Itakura, K.; Hirose, T.; Crea, R.; Riggs, A. D.; Heyneker, H. L.; Bolivar, F.; Boyer, H. W. Science 1977, 198, 1056–1063.
4. Wright, G.; Carver, A.; Cottom, D.; Reeves, D.; Scott, A.; Simons, P.; Wilmut, I.; Garner, I.; Colman, A. BioTechnology 1991, 9, 830–834.
5. Cunningham, C.; Porter, A. J. R., Eds.; Methods in Biotechnology, Vol. 3: Recombinant Proteins from Plants—Production and Isolation of Clinically Useful Compounds; Humana Press: Totowa, NJ, 1997.
6. Lloyd-Williams, P.; Albericio, F.; Giralt, E. Chemical Approaches to the Synthesis of Peptides and Proteins; CRC Press: Boca Raton and New York, 1997.
7. Fields, G. B.; Noble, R. L. Int J Peptide Protein Res 1990, 35**,** 161–214.
8. Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Wenqing, Y.; Schäfer, W. Tetrahedron Lett 1989, 30, 3943–3946.
9. Mergler, M.; Nyfeler, R.; Tanner, R.; Gosteli, J.; Grogg, P. Tetrahedron Lett 1988, 29, 4009–4012.
10. Ireland, R. E. Organic Synthesis; Prentice-Hall: Englewood Cliffs, NJ, 1969; p 29.
11. Bodanszky, M. Principles of Peptide Synthesis; Springer-Verlag: Berlin, 1993.
12. Jakubke, H.-D. Peptide Chemie und Biologie, Spektrum Akademischer; Verlag: Heidelberg, Berlin, Oxford, 1996.
13. Vaughan, R. R., Jr. J Am Chem Soc 1951, 73, 3547–3548.
14. Rich, D. H.; Singh, J. In The Peptides: Analysis, Synthesis, Biology; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol 1, pp 241–261.
15. Dourtoglou, V.; Gross, B. Synthesis 1983, 572–574.
16. Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillesen, D. Tetrahedron Lett 1989, 30, 1927–1930.

17. Bodanszky, M. In The Peptides: Analysis, Synthesis, Biology; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol 1, pp 105–196.
18. Fuller, D. W.; Cohen, M. P.; Shabankareh, M.; Blair, R. K.; Goodman, M.; Naider, F. R. J Am Chem Soc 1990, 112, 7414–7416.
19. Fuller, W. D.; Goodman, M.; Naider, F. R.; Zhu, Y.-F. Biopolymers (Pept Sci) 1996, 40**,** 183–205.
20. Meienhofer, J. In The Peptides: Analysis, Synthesis, Biology; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol 1, pp 197–239.
21. König, W.; Geiger, R. Chem Ber 1970, 103, 788–798.
22. Carpino, L. A. J Am Chem Soc 1993, 115, 4397–4398.
23. Carpino, L. A.; Elfaham, A.; Albericio, F. Tetrahedron Lett 1994, 35, 2279–2282.
24. Johansson, C. PolyPeptide Laboratories AB, personal communication.
25. Melin, P. Bailliéres Clin Obstet Gynaecol 1993, 7**,** 577–600.
26. Andersson, K.-E.; Bengtsson, B.; Paulsen, O. Drugs of Today 1988, 24, 509–528.
27. Johansson, C.; Blomberg, L.; Hlebowicz., E.; Nicklasson, H.; Nilsson, B.; Andersson, L. In Peptides 1994. Proceedings of the 23rd European Peptide Symposium; Maia, H. L. S., Ed.; ESCOM: Leiden, 1995; pp 34–35.
28. Schön, I. Chem Rev 1984, 84, 287–297.
29. Veber, D. F.; Milkowski, J. D.; Varga, S.; Denkewalter, R. G.; Hirschmann, R. J Am Chem Soc 1972, 94, 5456–5461.
30. Larsson, K.; Mellbrand, T.; Mörnstam, B.; Roschester, J.; Sköldbäck J.-Å U.S. Patent No. 5,500,413, 1996.
31. Larsson, K.; Mellbrand, T.; Mörnstam, B.; Roschester, J.; Sköldbäck, J.-Å. U.S. Patent No. 5,674,850, 1997.
32. Larsson, K.; Mellbrand, T.; Mörnstam, B.; Roschester, J.; Sköldbäck, J.-Å. U.S. Patent No. 5,763,407, 1999.
33. Andersson, L. H. H.; Sköldbäck, J.-Å. U.S. Patent No. 5,990,273, 1999.
34. Merrifield, R. B. Peptides, Synthesis Structure and Application; Academic Press: New York, 1995; Chap 3, pp 94–159.
35. Atherton, E.; Sheppard, R.C. Solid Phase Peptide Synthesis: A Practical Approach; IRL Press at Oxford University Press, 1989.
36. Stewart, J. M.; Young, D. Solid Phase Peptide Synthesis (2nd ed.); Pierce Chemical Company: Rockford, IL, 1984.
37. Lebl, M. Peptide Companion (PC program); CSPS: San Diego, CA, 1994.
38. Pennington, M. W.; Byrnes, M. E. Methods Mol Biol 1994, 35, 1–16.
39. Loffet, A.; Zhang, H. X. In Innovation and Perspectives in Solid Phase Peptide Synthesis; Epton, R., Ed.; Mayflower, 1994; pp 301–308.
40. Lundt, B. F.; Johansen, N. C.; Voelund, A.; Markussen, J. Int J Peptide Protein Res 1978, 12, 258–268.
41. Houghten R. A.; Beckman, A.; Ostresh, J. M. Int J Peptide Protein Res 1986, 27, 653–658.
42. Stewart, J. M.; Wooley, D. W. Nature 1965, 206, 619–620.
43. Ohno, M.; Tsukamoto, S. Bull Chem Soc Jpn 1972, 45**,** 2852–2855.
44. Krchnak, V.; Krojidlo, M.; Lepsa, L. Czechoslovak Patent No. 255500.
45. Kiso, Y.; Yajima, H. In Peptides, Synthesis Structure and Application; Gutte, Ed.; Academic Press: New York, 1995; pp 40–87.
46. Flegel, M.; Rinnova, M.; Panek, Z.; Lepsa, L.; Blaha, I. In Peptides. Chemistry, Synthesis, Biology. Proceedings of the 14th American Peptide Symposium; Kaumaya, P., Hodges, R. S., Eds.; Mayflower Scientific, 1996; pp 119–120.
47. Bray, M. A.; Maeji, N. J.; Jhingran, G. A.; Valerio, R. M. Tetrahedron Lett 1991, 32, 6163–6166.
48. Bray, M. A; Jhingran, G. A.; Valerio, R. M.; Maeji, N. J. J Org Chem 1994, 59, 2197–2201.
49. Tam, J. P.; Heath, W. F.; Merrifield, R. B. J Am Chem Soc 1983, 105, 6442–6455.
50. Tam, J. P.; Heath, W. F.; Merrifield, R. B. J Am Chem Soc 1986, 108, 5242–5251.
51. Pennington, M. W. Methods Mol Biol 1994, 35, 41–62.
52. Barton, M. A.; Lemieux, R. U.; Savoie, J. Y. J Am Chem Soc 1973, 95, 4501–4506.
53. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. Anal Biochem 1970, 34, 595–598.
54. Christensen, T. Acta Chem Scand 1979, 33b, 763–766.
55. Vojkovsky, T. Peptide Res 1995, 8(4), 236–237.
56. Gisin, B. F. Anal Chim Acta 1972, 58, 248–249.
57. Krchnak, V.; Vagner, J.; Safar, P.; Lebl, M. Coll Czech Chem Commun 20 1989, 53, 2542–2548.
58. Krchnak, V.; Vagner, J. Peptide Res 1990, 3, 182–193.
59. Dorman, L. C. Tetrahedron Lett 1969, 2319–2321.
60. Flegel, M.; Johnson, T.; Sheppard, R. C. In Innovation and Perspectives in Solid Phase Peptide Synthesis; Epton, R., Ed.; SPCC (UK): Birmingham, 1990; pp 307–324.
61. Brunfeldt, K.; Roepstorff, P.; Thomsen, J. Acta Chem Scand 1969, 23, 2906–2907.
62. Panek, Z.; Flegel, M.; Pavlik, M.; Lepsa L. Czech Republic Patent Application No. PV 2807-99.
63. Panek, Z.; Pavlik, M.; Flegel, M. In Innovation and Perspectives in Solid Phase Peptide Synthesis and Combinatorial Libraries; Epton, R., Ed.; Mayflower, 1998; pp 365–366.
64. Nyfeler, R. Methods Mol Biol 1994, 35, 303–316.
65. Riniker, B.; Fretz, H.; Kamber, B. In Peptides 1992. Proceedings of the 22nd European Peptide Symposium; Schneider, C. H., Eberle, E. N., Eds.; ESCOM: Leiden, 1993; pp 34–45.
66. Bray, B.; Friedrich, P.; Kang, M.C.; Lichty, M.; Mader, C.; Merutka, G. Proceedings of the 26th European Peptide Symposium, September 2000, in press.
67. Draft Guidance, Guidance For Industry. Manufacturing, Processing or Holding Active Pharmaceutical Ingredients; U.S. Department of Health and Human Services, Food and Drug Administration, 1998.
68. U.S. Code of Federal Regulations, Parts 210 and 211.