Chapter 15

Peptide Synthesis via Fragment Condensation

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1. Introduction

In the classical solution synthesis, fragment condensation has always been the way to build up peptide chains with more than approximately five amino acids' length. Since the early achievements in this field, like the synthesis of glucagon and secretin *(1)*, little has changed from a strategic point of view. For a recent example, see the synthesis of human epidermal growth factor *(2)*. However, taking the classical approach to synthesize larger peptides is cumbersome, and needs experience and time.

With recent developments of appropriate linker resin combinations in solid-phase peptide synthesis, protected peptide fragments have become readily available (*see* Chapter 14), usually in good yield and of high purity. These developments in solid-phase peptide synthesis opened up the way to new strategies: the combination of solid-phase and solution synthesis, and the fragment coupling onto resin. In the former approach, protected peptide fragments are synthesized on solid support, cleaved from the resin with full preservation of protecting groups, purified, and characterized. The fragments are then assembled in solution. In such a way, the advantages of solid phase are combined with the advantages of solution synthesis, allowing for the key steps, i.e., the fragment couplings, full control and monitoring, as well as isolation, purification, and characterization of the intermediates *(3,4)*.

The other strategy uses the protected fragments for coupling onto resin; the growing peptide chain is assembled on the polymer support. This

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approach, if successful, allows very rapid preparation of larger peptides even compared to stepwise solid phase. The strategy is usually named convergent (*see* refs. *5,6* and literature cited therein). Assembling a peptide this way, using purified peptide fragments, is advantageous to the stepwise approach: side products from incomplete couplings are more easily removed, since they differ not by just one amino acid, as may be the case in stepwise solid-phase synthesis. However, truncated and deletion sequences cannot be fully excluded in the final product unless their absence in the fragments (obtained from stepwise solid-phase synthesis) can be shown. Fragment purity therefore is essential.

Whereas fragment coupling in solution is a very well-established procedure, fragment coupling to a resin-bound peptide chain constitutes a rather recent development, as can be deduced from the growing number of papers on the subject. However, the approach was discussed and compared to the combination approach quite some time ago, in 1981 *(7)*.

Both fragment coupling strategies require some experience because—unlike amino acid derivative coupling—no standard protocol can be given, and therefore, such couplings are usually not performed in an automated way, for the following reasons: The solubility of any designed protected fragment is not predictable with accuracy, but if there is insufficient solubility, coupling may become difficult. This holds especially for the convergent approach. Although in solution synthesis numerous fragment couplings in suspension or even in gels have been shown to proceed successfully *(8)*, such couplings will proceed sluggishly if at all in the convergent approach where diffusion of the reactants into the matrix is essential. The structure of the resin as well as the peptide loading on the resin clearly have their impact on the reaction rate of the coupling *(9)*. Low peptide loadings (0.1 mEq/g) usually improve difficult couplings *(10)*. Even so, taking into account that fragment coupling is slower compared to amino acid coupling and also that one rather tends to avoid large excesses of precious fragments, good solubility of a fragment is an important prerequisite for a successful fragment coupling on resin. Even then coupling may be hampered by polymer/peptide chain matrix interactions.

The synthesis of protected peptide fragments is described elsewhere in this book in full detail. There are numerous coupling methods described in the literature *(11–14)*; many of them may be applied for fragment couplings. It is beyond the scope of this chapter to mention or discuss them all. The aim of the following section is rather to give the

reader an overview of all points to consider (*see* Section 3.1.) and to provide the reader with a practical guideline (*see* Section 3.2.).

2. Materials

1. All solvents and reagents mentioned are commercially available, and may be used as such. However, in slow couplings/diluted solutions, a high solvent quality is important. Ethers must be free of peroxides.
2. Fragment couplings on resin may be performed in any vessel recommended for solid-phase synthesis or in standard glass flasks equipped with a stirring device. These are also used for couplings in solution. Precipitated products may either be isolated by centrifugation or filtration.
3. Equipment for in-process analysis, such as TLC and HPLC (*see* Chapter 3, *PAP*) is available on the market in a great variety. HPLC equipment must include a UV detector.

3. Methods

3.1. General Considerations

3.1.1. When or Why to Choose a Fragment Approach

When your stepwise solid-phase synthesis failed to give a reasonable quantity and/or quality of the desired peptide, a fragment approach may be tried, especially in the case where the stepwise approach is hampered by β-sheet formation, which can occur at a peptide chain length of about 6–15 amino acids. These difficult couplings can be overcome by coupling a fragment that bridgespans over the troublemaker sequence. The same holds for any difficult (for whatever reason) coupling regions in individual couplings.

When purification of your peptide is tedious and you assume or know this to be the result of deletion sequences closely related in structure to the desired peptide, a fragment approach may help. The fragments used, however, have to be reasonably pure and should themselves not contain deletion sequences. Peptides from convergent synthesis usually show much less broadening of the main peak in RP-HPLC and are easier to purify. Side products from incomplete couplings differ from the peptide by the length of a fragment and are thus usually easy to remove.

When your peptides to be synthesized either contain repetitive sequences or share common sequences, such sequences have to be synthesized only once, purified, and either coupled several times (for repetitive sequences) or to different peptides. The approach seems especially useful for synthesis of multiple antigenic peptide matrices (MAPs *[15]*) or templates *(16)*.

Whenever you have chosen a classical solution synthesis of your peptide (of let us say more than four to six amino acids), you would use fragment condensation. Depending on structure, even a pentapeptide may be preferentially assembled by a 3 + 2 coupling.

3.1.2. How to Choose the Synthetic Strategy: In Solution vs on Resin

Whether you choose a fragment approach in solution or on resin depends on your experience and on the time available; for large-scale syntheses, economy has also to be taken into consideration. The solution approach needs more experience and time, but is more generally applicable. Numerous examples are described in the literature. Furthermore, C-terminal fragments usually have to be synthesized in solution. This again needs more practical knowledge than making them on resin. Unfortunately, rapid synthesis of fragments protected at their carboxy terminus by *tert.butyl*, or *benzyl*esters via solid phase is not (yet) possible. Protected fragments obtained from solid-phase synthesis may serve as intermediates for this purpose and may be esterified *(17)* in a subsequent step. Special attention has to be paid to the risk of epimerization. Protected amides may be obtained via aminolysis directly from the peptide resin.

Fragment coupling onto resin is more rapid since all components are made via solid phase. However, restrictions, such as the solubility of the fragment and the interaction of the fragment with the peptide–polymer matrix, may slow down the coupling or even prevent it from proceeding. Coupling fragments onto resin may often not be as straightforward as coupling amino acid derivatives.

3.1.3. How to Choose the Coupling Sites

For both approaches, you have to define your coupling sites, whether you use Boc or Fmoc strategy. To define reasonable fragments, consider the following points:

Length of the fragment: Typical fragment length would be from 5–10 and maybe up to 15 amino acids. Even some larger peptides of up to 22 amino acids length (e.g., fragment 1–22 of CRF) have been prepared in the author's laboratories and successfully coupled. However, fragments of this length usually are difficult to purify, and may contain deletion and truncated sequences. These also will couple and thus, unfortunately, the advantage of fragment coupling is partially lost. With fragments of the

recommended length, there is a good chance for sufficient solubility. Hence, purification can be easily achieved and, finally, coupling proceeds rapidly and efficiently.

Epimerization: Whenever possible, Gly is chosen as the C-terminal amino acid for the obvious reason that it cannot racemize. Second choice would be Pro since at least the azlactone mechanism of racemization is impossible. The disadvantage of C-terminal Pro fragments is that their synthesis is somewhat more complicated because of diketopiperazine formation (*see* Chapter 14).

Reaction rate and side reactions: Gly again would be preferred for fast reactions. In case there is no Gly or Pro available at appropriate positions, you should avoid sterically hindered amino acids, such as Ile or Val, as coupling sites (both N- and C-terminal). As N-terminus you should select neither a secondary amino acid (*N*-methylamino acid or Pro) nor Gln. The latter may lead to pyroglutamate formation.

3.1.4. How to Choose the Strategy: Protecting Groups

Both synthetic strategies well known from stepwise solid-phase synthesis can be applied to the fragment approach. Boc strategy would use N-Boc as temporary, Bzl type as (semi) permanent side-chain protection; Fmoc strategy would use Fmoc as temporary protection and *tert.butyl* type protecting for side chains. Details concerning the choice of protecting groups are available elsewhere in this book (*see* Chapter 14). A third possibility, restricted to the coupling in solution approach, is the use of carbobenzoxy (Z) as temporary protecting group and *tert.butyl*-type protection for side chains. However, once the peptide chain contains Met and/or Cys, hydrogenation of Z may not be possible and alternative orthogonal protecting groups, such as Fmoc, Adpoc, Bpoc or the like, have to be used. For a definition of orthogonality, *see* Chapter 14, Note 1.

The three protecting-group strategies mentioned (Boc/Bzl, Fmoc/*tert.butyl*, Z/*tert.butyl*) are the most widely accepted and used ones. Numerous other strategies/protecting-group combinations are possible. The only requirement is orthogonality between temporary and permanent protecting groups.

Those familiar with Fmoc strategy would choose Fmoc-protected fragments, and those familiar with Boc strategy, Boc-protected fragments. For cleavage of the temporary protecting group and also for washing procedures, the standard protocol could be used. Those not familiar with solid-phase synthesis should consult Chapter 14 first and then perform

coupling in solution with the Fmoc-protected fragments obtained according to the general protocol given there. The C-terminal fragment is used as *tert.butyl*ester, unless the peptide to be synthesized is an amide (no protection required).

3.1.5. How to Choose the Appropriate Fragment Coupling Method

Undoubtedly, many of the peptide coupling methods described in the literature up to now may be used for fragment couplings. Usually one prefers either preactivating the carboxylic moiety or a one-pot reaction with carboxylic acids (rather than synthesizing active esters of fragments) with one notable exception: Acyl azides, well known for the low epimerization risk, are prepared from hydrazides, which themselves are usually prepared from methyl esters, but also via hydrazinolysis of appropriate peptide resins, e.g., Sasrin *(18)*. An alternative, however, to this procedure may be the use of diphenylphosphoryl azide (DPPA) and free carboxylic acids. It is beyond the scope of this chapter to enumerate all the suitable methods. Protocols for some of the most useful standard methods, like mixed anhydride or the abovementioned azide coupling, are to be found in the literature *(19)*.

DCC coupling methodology with all its variations in structure of the reagent (water-soluble carbodiimides and the like) or in terms of additives (HOSu, HOBt, 3-hydroxy-1,2,3-benzotriazin-4[3H]-one and the like) still can be considered as generally applicable. It can be used in most of the common solvents for peptide synthesis.

TBTU-type reagents may also be used for fragment couplings, with or without addition of HOBt. For fragment couplings with epimerization risk, use of TPTU is recommended rather than TBTU *(20)*. BOP is known to give very fast coupling reactions, also in fragment couplings. However, these couplings may proceed with concomitant partial epimerization.

3.1.6. How to Handle the Epimerization Risk

If there is no Gly at the C-terminus of the fragment, there always is a possibility (risk) of racemization of the C-terminal amino acid, and no method can be considered absolutely safe. There are a few general rules and a lot of data available from literature; nevertheless, it is absolutely necessary to take appropriate care for each individual case of fragment coupling with respect toward epimerization. Products obtained from such fragment couplings should always be checked for epimerization either

by hydrolysis and subsequent analysis for D/L amino acid, or by preparing the unwanted diastereomer and developing a suitable analytical method capable of separating the pair of diastereomers (such as RP-HPLC). Contamination with a small amount of diastereomer may be acceptable if appropriate purification methods are at hand, such as countercurrent distribution or preparative HPLC (Chapter 4, *PAP*).

In other cases, there is no other way than running a series of experiments not only with various coupling reagents, but also with appropriate variations in reaction conditions, such as solvent, excess of reagent, concentration of components, temperature, the nature of additives, and so on, followed by analysis of the products obtained with regard to epimerization. These experiments can be carried out on a small scale (10–100 mg of peptide fragment or peptide resin). According to our experience, DCC/HOSu and DCC/HOBt are still the methods that—generally speaking—are the most promising ones. Recently, addition of copper(II)chloride was claimed to prevent racemization *(21)*. For couplings in solution in a classical synthesis, azide couplings may still be highly recommended, although some cases of epimerization, especially for C-terminal His, Tyr, and Phe, are known from the literature.

3.2. General Procedures: The Experimental Approach

3.2.1. The Choice of the Solvent

Determine the solubility of the fragment(s) (*see* Note 1). Start with DMF, DMA, or NMP or mixtures of these. HMPA is a good solvent in mixtures. However, it should not be used because of its cancerogenicity. DMSO also is a very good solvent. However, its oxidative potential restricts its general use, especially when Met is present in the peptide sequence. Methylene chloride and/or trifluoroethanol may be other choices. The latter may require laborious optimization of reaction conditions (Note 2). Addition of Li salts, such as LiCl or $LiClO_4$, may enhance solubility of fragments *(21)*. Addition of the coupling reagent and/or the additives may also facilitate dissolution.

Attempt to obtain a concentration of the fragment in the range from 20 to 5 g/100 mL of solvent (Note 3). Below a 5 g/100 mL concentration, coupling still may take place, but rather slowly. If there is no other choice, one may try couplings in the 1–5 g/100 mL range. When performing a coupling onto a peptide resin, the coupling solvent must be capable of swelling that resin. The peptide resin has to be washed with the coupling solvent before coupling.

When performing a coupling in solution, solubility of both fragments has to be checked. If the amine component consists of a salt, an appropriate base, such as DIPEA or NMM, has to be added (1 Eq) to liberate the amine. This also may have an influence on solubility.

3.2.2. The Coupling Conditions

Usually the carboxylic component is used in excess when performing a coupling onto resin. The excess may be chosen from 1.5 Eq to whatever is needed. A good starting point is 2–3 Eq.

In solution couplings, you generally use less excess. A good choice for starting is in the range of 1.0–1.5 Eq of the carboxy component.

Reagents and additives, such as DCC and HOBt, are usually used in equimolar amounts or in slight excess with regard to the carboxy component. A good starting point here is to use 1 Eq of reagent and additive (*see* Note 4).

The simplest way of performing a fragment coupling is the "one-pot" procedure. All components/additives/base are dissolved in the solvent, and the reagent, e.g., DCC or TBTU (*see* Notes 5 and 6), is added last. For couplings onto resin, the resin has to be prewashed with the coupling solvent. For TBTU couplings, at least equimolar amounts of DIPEA have to be added, preferentially 1.5 Eq with regard to TBTU. For DCC/HOBt couplings, the apparent pH should be between 6.5 and 7. Addition of base, such as DIPEA (up to a pH of 8), may speed up reaction. Be careful, however, with elevated pH values when there is a risk of epimerization. Faster reaction rates may be obtained via preactivation of the carboxylic component. The latter is dissolved. The additive (HOBt) and the reagent (DCC or TBTU) are added, and the mixture left for 0.5–2 h. Dicyclohexyl urea may be filtered off in the case of DCC preactivation before addition. The preactivation mixture is then added to the neutralized solution of the amino component. In solid phase, the resin has to be washed with the coupling solvent, which subsequently has to be filtered off. The preactivated carboxylic acid solution is then added.

3.2.3. The Coupling Time: The Monitoring of the Coupling

Coupling time may vary from a few hours to a few days. Usual coupling time for fragments is overnight.

For monitoring coupling reactions in solution, TLC is a very convenient method. Conditions leading to a separation of both starting materi-

als and the product have to be worked out; specific development of TLC plates with ninhydrin (for NH_2[NH]), Greig-Leaback (—CONH—), and UV helps in interpreting the chromatograms.

Monitoring coupling reactions on resin is a more tedious case. Standard tests, such as the Kaiser-test and the TNBS-test (both described in Chapter 14) may only give a first indication. Their results, especially when negative, quite often turn out not to be reliable. The best way to monitor a coupling is to cleave a sample from the resin and analyze it by RP-HPLC. Cleaved samples also have to be prepared from the fragments for reference purpose. RP-HPLC illustrates the coupling both in a qualitative and quantitative manner. Other methods for monitoring are amino acid analyses, peptide sequencing, or mass spectroscopy.

Should the test(s) reveal incomplete coupling, one may add more reagent or, preferentially, more reagent and carboxy component. The amounts to add depend on the turnover of the coupling. One may use up to the same amounts already used in the first instance.

In solution couplings, the components may be added in solid form or in concentrated solutions. In solid-phase couplings, either more reagent is added or, preferentially, the resin is filtered and the coupling repeated all over again with fresh solutions of all components as described above (*see* Note 7). If coupling does not go to completion, but the turnover is acceptable, the amino function may be blocked, e.g., by acetylation, and the synthesis is continued (*see* Note 8).

3.2.4. The Work-Up

Work-up of couplings performed on resin is carried out as described for stepwise solid-phase synthesis elsewhere in this book (*see* Chapter 4). All unused components, reagents, additives, and so on are simply washed out, and the peptide resin is ready either for final cleavage or for cleavage of the temporary N^{α}-protecting group according to the protocols described in the corresponding chapters in this book. Thereafter, synthesis is continued by either stepwise coupling or a further fragment coupling. Work-up of couplings performed in solution follow the general protocols used in solution chemistry. The work-up procedure strongly depends on the properties of the specific peptide synthesized. No standard work-up protocol can therefore be given.

Work-up may proceed via precipitation (*see* Note 9) either by adding water (for lipophilic products) or a hydrophobic solvent, such as ethyl

acetate or ether. If the product does not precipitate, the solution is either diluted with, e.g., ethyl acetate (followed by an extraction procedure), or evaporated. The residue obtained may then be treated in an appropriate manner: dissolution and precipitation, dissolution and extraction, trituration, dissolution followed by purification with countercurrent distribution, or any suitable type of chromatography. *See* the following section for some examples.

3.3. Examples

3.3.1. Fragment Coupling on Resin Using DCC/HOBt

- I Fragment: Fmoc-Ala-Gln(Mtt)-Ser(*t*Bu)-Gly-Leu-Gly-OH
- II Peptide resin: H-Cys(Acm)-Asn(Mtt)-Ser(*t*Bu)-Phe-Arg(Pmc)-Tyr(*t*Bu)-O-Wang

I represents position 17–22, and II position 23–28 of human Atrial Natriuretic Peptide. The loading of the starting Fmoc-Tyr(*t*Bu)-O-Wang resin was 0.6 mEq/g. Compare with Section 3.3.5. for a corresponding coupling in solution.

3.3.1.1. Coupling Conditions

Peptide resin (2.7 g) and 2.2-g fragment (2 Eq) were coupled in 22 mL DMF using 4 Eq of HOBt and 4 Eq of DCC. After 5 min, DIPEA (2 Eq) was added. The reaction mixture was gently agitated. After an overnight coupling, a sample was cleaved with TFA/H_2O/DTT (95:5:5) and analyzed by HPLC. Content of unreacted peptide II was found to be <0.5%. Addition of base accelerates the reaction, but it is not absolutely necessary.

The fragment can also be preactivated using amounts of solvent and reagents as given above for approx 30 min. The resulting solution is then added to the resin previously swollen with DMF.

3.3.2. Fragment Coupling on Resin Using TBTU

- I Fragment: Fmoc-Ser(*t*Bu)-Asn(Trt)-Lys(Boc)-Gly-Ala-Ile-Ile-Gly-OH
- II Peptide resin: H-Leu-Met-Val-Gly-Gly-Val-Val-O-Wang

I represents position 26–33, and II position 34–40 of β-Amyloid protein (1-40). The loading of the starting Fmoc-Val-O-Wang resin was 0.6 mEq/g.

3.3.2.1. Coupling Conditions

Preactivation of fragment I: 3.6 g of the fragment (2 Eq) were dissolved in a 1:1 mixture of DMA and NMP (60 mL), 2 Eq of TBTU and 3 Eq of DIPEA were added, and the mixture was gently agitated for 30 min

and then added to the peptide resin II previously washed with the coupling solvent mixture. Coupling time was overnight, and turnover as determined by HPLC after cleaving was over 96%.

3.3.3. Fragment Coupling in Solution Using DCC/HOSu

- Fragment I: Z-Gly-Val-Val-Lys(Boc)-Asn(Trt)-Asn(Trt)-Phe-Val-Pro-Thr(*t*Bu)-Asn(Trt)-Val-Gly-OH
- Fragment II: H-Ser(*t*Bu)-Lys(Boc)-Ala-Phe-NH_2 HCl

Fragment I represents the sequence 21–33, and fragment II the sequence 34–37 of α-human CGRP; 4 g of fragment I (1.7 mmol) and 1.1 g of fragment II (1 Eq) were dissolved in DMF (40 mL). After addition of NMM (1 Eq), HOSu (3 Eq), and DCC (3 Eq), the reaction mixture was left at room temperature for 2 d. Monitoring by TLC showed disappearance of both starting materials. The product was precipitated with ether, and treated with ethyl acetate and isopropanol. Yield was 75%. Purification was achieved by countercurrent distribution, using the solvent system: MeOH/1N AcOH/1.2-Dichlorethane/$CHCl_3$ (10:3:8:4). After 1300 cycles, fractions containing pure product were pooled, evaporated, and the product precipitated with water. Yield (purification) was 88%.

3.3.4. Fragment Coupling in Solution via Azide

- Fragment I: Z-Ile-Phe-Thr(*t*Bu)-Asn-Ser(*t*Bu)-Tyr(*t*Bu)-$NHNH_2$
- Fragment II: H-Arg(HCl)-Lys(Boc)-Val-Leu-Gly-OH

Fragment I represents the sequence 5–10, and fragment II sequence 11–15 of human GRF; 10 g fragment I (10 mmol) were converted into the azide in DMF/DMA (3:2, 60 mL) using 4 Eq of HCl and 2.5 Eq of *tert.butyl*nitrite at –12°C. After 30 min reaction time, the mixture was neutralized with DIPEA. A solution of 8 g fragment II (1 Eq) in DMSO/DMF/water (50/40/4.5 mL) was added to the azide solution at –10°C, and the pH was adjusted to 8. The mixture was kept at room temperature for 3 d, concentrated *in vacuo*, and the product precipitated with water containing 10 mmol HCl. Further purification was done by dissolution in $CHCl_3$/MeOH (1:1) at 45°C, concentrating *in vacuo* and addition of MeOH. Yield was 70%.

3.3.5. Fragment Coupling in Solution Using DCC/HOBt

- Fragment I: Fmoc-Ala-Gln(Mtt)-Ser(*t*Bu)-Gly-Leu-Gly-OH
- Fragment II: H-Cys(Acm)-Asn-Ser(*t*Bu)-Phe-Arg(HCl)-Tyr(*t*Bu)-O*t*Bu HCl

Fragment I represents the sequence 17–22, and fragment II the sequence 23–28 of human Atrial Natriuretic Peptide. Compare this to Section 3.3.1.

for a corresponding coupling on resin; 2.4 g fragment I (2.2 mmol) and 2.4 g fragment II (1 Eq) were dissolved in DMF/DMA (3:2, 50 mL). HOBt (1 Eq), DCC (1 Eq), and NMM (1 Eq) were added at 0°C and the reaction mixture left at room temperature overnight. Monitoring by TLC showed absence of starting materials. DCU was filtered off and the product precipitated with ethyl acetate/ether (1:1). Yield was 70%.

4. Notes

1. Fragments containing carboxylic acids, such as TFA or acetic acid, either as contaminants or as counterions may not be used for fragment couplings with DCC, since they themselves may couple to the amine. Even if present in low percentage, they may interfere to a great deal because they have a low molecular weight compared to the fragment, and hence, they may be present in quite high a concentration based on molarity. For couplings with TBTU, the presence of TFA may be tolerated as long as the desired reaction proceeds smoothly.
2. Trifluoroethanol is rather acidic and requires some "neutralization" with base. Furthermore trifluorethyl esters may be formed as (by)products *(8)*.
3. Keep in mind when performing these solubility experiments that you may well wish to use an excess of the carboxy component in the coupling. Therefore, requirements for solubility should not be set too low.
4. HOBt and HOSu may be used in excess (1.5–2 Eq with respect to the coupling reagent); sometimes, the presence of basic moieties like unprotected Arg side chain may "consume" additive.
5. For fast reaction, BOP may also be used, usually in the presence of a base, such as NMM, or DIPEA, and of HOBt (optional).
6. For literature references, *see* ref. *23* for DCC/HOBt, ref. *20* for TBTU, and ref. *24* for BOP.
7. It may be worth trying somewhat different conditions for a second coupling in solid phase, e.g., changing the solvent and/or the reagent; furthermore, addition of some structure-breaking or chaotropic agents or cosolvents may be tried ($LiClO_4$, ethylene carbonate, DMSO, trifluoroethanol, HFIP, and the like [*see* Chapter 1]).
8. Acetylation may facilitate final purification of the peptide. Acetylation blocks the amino group forever and prevents any further reaction.
9. If couplings were performed using DCC, the dicyclohexyl urea formed (and precipitated) during the coupling reaction would be filtered off first.

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