1. Introduction

The successful coupling of amino acid derivatives during the synthesis of a peptide by either solution or solid-phase procedures depends on both the reactivity of the carboxyl group of the N-protected amino acid and the steric accessibility of the reactive nucleophile (either a primary or secondary amine). Activation of the carboxyl group is a requisite for the synthesis of an amide bond. Many activation procedures have been developed to accomplish this, and ultimately, the reactivity of the activated species is crucial in determining the coupling yield.

Improvements in solid-phase assembly techniques now permit the routine synthesis of long (>40 residues) complex peptides. However, as the ability to assemble these longer molecules on a solid-phase matrix improved, new problems were encountered. Successful synthesis was hampered by steric factors of the bulky protected derivatives (1), intermolecular aggregation of the protected peptide chain (2,3), formation of hydrogen bonding structures, such as β-sheet (4–7), premature termination, or cyclization on the resin (8–10).

Our laboratory routinely synthesizes large quantities of many peptides. We employ a semiautomated procedure where each individual coupling is monitored for completeness prior to the next deblocking/elongation step. As a result of this type of strategy, we encounter many couplings...
that do not proceed to completeness using either a single carbodiimide/HOBT coupling (11) or double coupling employing the same carbodiimide/HOBT strategy. During the past several years, we have evaluated many of the methods described in the literature to improve the coupling yield. It is important to point out that every peptide presents its own unique set of complications. Thus, it is impossible to give a universal procedure that will work for every peptide. It is the purpose of this chapter to present several of these protocols, which we have found to be very useful.

2. Materials

1. All materials and reagents are purchased from commercial sources and used as such.

2. Synthesis solvents, such as l-methyl-2-pyrrolidinone (NMP), N,N-dimethylformamide (DMF), and dichloromethane (DCM), may be obtained from commercial sources, such as Burdick Jackson (Baxter, McGaw Park, IL), Baker (Phillipsburg, NJ), or Fisher (Fair Lawn, NJ).

3. Coupling agents, such as dicyclohexylcarbodiimide (DCC), diisopropylcarbodimide (DIC), 1-hydroxybenzotriazole (HOBT), and N,N-diisopropylethylamine (DIEA), may be obtained from Chem Impex International (Wood Dale, IL), Aldrich (Milwaukee, WI), or other commercial sources.

4. The following reagents are available from Aldrich, unless otherwise noted: 2,2,2-trifluoroethanol (TFE) 99+% toxic, 1,4-dioxane (anhydrous, 99%), and 4-dimethylaminopyridine (DMAP). Benzotriazol-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate (BOP reagent), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), as well as the related compound 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) may be obtained from Richelieu Biotechnologies (QC. Canada).

5. Chaotrophic salts, such as potassium thiocyanate and sodium perchlorate (anhydrous 99%, oxidizer, hygroscopic, irritant, Aldrich), are also commercially available.

3. Methods

The general strategy of this section is to detail several techniques that promote accessibility of the reactive amino group, increase reactivity of the activated carboxyl group, or both. The following techniques have been reported in the literature and successfully employed in our laboratory where a problematic residue or sequence has been encountered.
3.1. Difficult Couplings

Ideally, the coupling reaction of a deprotected amino group and an activated carboxyl group proceeds to near 100% completion. However, because of the factors mentioned earlier, this is sometimes rather difficult to accomplish. Incomplete couplings quickly destroy the fidelity of the synthesis causing an increase in deletion sequences. Capping protocols (12) help to eliminate these deletion sequences and are essential in longer syntheses. During a long synthesis, each incomplete coupling is magnified sufficiently so as to reduce the yield of the desired product and increase the levels of deletion sequences and capped, truncated peptidyl-sequences.

As a general rule, difficult couplings are usually sequence-dependent and not residue-specific. It has been observed that many difficulties arise in the synthesis as peptides are elongated through residues 12–20 of their sequences (2). This phenomenon has been attributed to the propensity to form β-structure aggregates on the resin (3–7). Examples of this are peptides with known β-structure (M. W. P., personal communication), as well as peptides rich in hydrogen bonding residues, such as Asn and Gln, which in Boc synthesis are generally incorporated with unprotected side chains (13). It is possible to incorporate both Asn and Gln with protected side chains in a Boc strategy using one of the TFA-stable substituted mono- or bisbenzylamides (14). However, these derivatives are not routinely commercially available. When a fluorenyl methoxycarbonyl (Fmoc) strategy is employed, Asn and Gln side chain protection is possible with trityl (15) and methyltrityl (16) groups. These protecting groups help prevent the aggregation phenomenon (16).

An incomplete coupling may be identified by the reaction of a portion of the peptidyl resin with ninhydrin as described by Kaiser et al. (17) and elsewhere in this volume (see Chapter 8). This is a colorimetric reaction that yields a purple, blue, or blue-green color following incubation at an elevated temperature with ninhydrin if any primary amines are present. Secondary amines, such as Pro and N-methyl amino acids, usually are less reactive with ninhydrin and result in a reddish-brown color as a positive reaction. Such a positive result indicates an incomplete coupling reaction. When a manual strategy is employed, a recoupling should be performed.

In automated synthesizers using a Boc strategy, a recoupling protocol may be programmed prior to synthesis, but this may not be practical. In
most cases, a failed synthesis during a Boc scheme will be identified after the peptide has been completed by analysis of resin samples taken by the instrument, such as the ABI 430, during synthesis (18). Many technicians opt to employ a double-coupling scheme routinely throughout a specific region (residues 8–18, for example) or an entire synthesis, even when this is not necessary, so as to avoid having to resynthesize the molecule if it fails during a single coupling strategy.

On-line acylation and Fmoc removal monitoring by UV spectroscopy have significantly increased the appeal of Fmoc synthesis (19). This feature has been exploited mostly by continuous flow synthesizers, which employ a microprocessor that controls the acylation and deblocking steps by directly interpreting the data. This interpretation allows immediate recoupling during the synthesis much like that during a manual synthesis.

### 3.2. Resin Substitution

Use of low-substitution resins (0.1–0.4 mmol/g) may increase α-amine accessibility by decreasing steric interactions as well as interchain aggregation. Many commercial resins are supplied with substitutions of 1 mmol/g or greater. For small peptides of 8–20 residues, this may be acceptable. However, for longer peptides, this high degree of substitution can present difficulties later in the synthesis (20). We routinely lower the substitution in these cases during the first cycle of synthesis. This is easily accomplished by performing the first coupling with a limiting amount of protected amino acid. Following this coupling, the remaining free amino groups are capped, thus eliminating any further reactivity at these sites.

#### 3.2.1. Example Method:

**Reduction of Substitution of mBHA Resin**

1. Place 10 g of mBHA resin (substitution value 1.1 mmol/g) in 125-mL flask. Swell the resin with 100 mL of DCM. Filter the solvent away over a scinttered glass funnel. Repeat this procedure twice.
2. In a separate flask, preactivate 5 mmol of Boc-amino acid with 10 mmol of DCC and 15 mmol of HOBT in 100 mL of NMP for 30 min.
3. Filter the activated amino acid solution over a separate scinttered glass funnel to remove the DCU that has formed during the activation.
4. Add this filtered solution to the swollen mBHA resin, and gently mix for 2 h at room temperature.
5. Terminate the reaction by filtering the activated amino acid solution away from the resin.
6. Wash the resin beads repetitively with 2 × 100 mL DMF, followed by 2 × 100 mL DCM, followed by 2 × 100 mL MeOH, and lastly 2 × 100 mL DCM again.

7. Monitor a sample of the resin by Kaiser analysis (see Chapter 8) for positive amino groups. The beads should still turn very dark blue.

8. Initiate a capping procedure by reacting the unreacted primary amino groups with 100 mL of a 20% solution of acetic anhydride in DMF with 2 Eq of DIEA for 1 h.

9. Repeat steps 6 and 7. The Kaiser test should now give a clear yellow (negative test) solution, indicating all unreacted amino groups have been capped.

10. Following a standard TFA deblocking step and subsequent solvent and base washes, a Kaiser test of the resin beads should show a positive result, either blue or reddish brown color (only for Pro). Accurate determination of the actual substitution can be determined by amino acid analysis. A rough approximation can be determined by performing a quantitative ninhydrin test as described by Sarin et al. (21).

### 3.3. Elevated Temperature

Coupling efficiencies may be increased in a temperature-dependent manner because of thermal disruption of interchain aggregates, although extensive studies on racemization and other peptide modifications must be performed in order to quantify its benefits fully (22,23). **Note:** Coupling reactions maintained above the recommended temperature may result in significant amounts of dehydrated material when performed on peptides containing Asparagine and Glutamine (23).

1. Elevated temperature coupling reactions should be maintained at 35–50°C.
2. Temperature elevation is accomplished by wrapping the reaction vessel in Thermolyne heating tape (Fisher) and regulated with a reostat.
3. The reaction temperature must be checked manually with a thermometer to ensure against variations in temperature.
4. This procedure should be tested experimentally on a small scale until the optimized conditions are found.
5. Alternatively, this procedure may be performed in 5-min intervals every 15 min during a 2-h coupling reaction in order to minimize the deleterious effects of heating.

### 3.4. Carboxyl Activation Procedures

Peptide bond formation is facilitated by activation of the carboxyl group by addition of a condensing agent to a mixture of the amine component of the existing peptide chain and the carboxyl component of the
amino acid being introduced to the synthesis. The earliest procedures, and still today among the most common, incorporated the use of dicyclohexylcarbodiimide (DCC) (24). Also, diisopropylcarbodiimide (DIC) may be substituted in order to allow the formation of diisopropylurea, which is more readily soluble than the dicyclohexylurea formed with DCC use.

The activation procedure may take place in situ. However, reaction of the activating reagent with the amino as well as the carboxyl component is possible. External activation permits activation in a nonpolar medium, as well as avoiding contact of the amino group with the reactive carbodiimide or the coproduct urea. This procedure, however, requires the fresh preparation of solutions before each use.

In situ activation is also possible with the phosphonium (BOP and PyBOP) and the uronium (TBTU and HBTU) type activators. These have the unique advantage of generating the activated species without generating the insoluble urea byproducts (see Section 3.4.3.).

3.4.1. HOBT Active Esters

Although addition of HOBT to DCC-mediated couplings has been reported to improve coupling reactions, the preformed HOBT ester is widely held to be extremely effective (11), and is especially useful for Asn, Gln, Arg, and His derivatives.

1. For a 1.0-mmol synthesis (1.0 mmol of theoretical amino groups), 5 mmol of amino acid, 0.77 g (5 mmol) of HOBT (153 g/mol), and 1.03 g (5 mmol) of DCC (206 g/mol) are dissolved in 25–30 mL of cold DMF.
2. The prepared solution is allowed to warm up to room temperature and stand at room temperature for approx 30 min before adding to the washed peptidyl resin. We routinely protect this solution from moisture by keeping the solution under an N₂ atmosphere.
3. Add this solution to the deblocked peptidyl resin.
4. After approx 30 min of coupling, an additional 20 mL of DMF may be added to the resin in order to facilitate wetting and mixing of the resin.
5. Active esters may racemize slowly in DMF. Therefore, it is advised to recouple after an initial positive ninhydrin test, rather than extend the reaction time (11).
6. NMP or other appropriate solvents may also be used during the coupling reaction. Additionally, DIC (126 g/mol; 0.806 g/mL) may be substituted for DCC. Many automated synthesizers successfully use this type of chemistry for activation and do not use cold DMF.
3.4.2. Symmetric Anhydride Coupling

1. The symmetric anhydride solution is prepared by adding 6 mmol amino acid and 3 mmol DCC (or DIC) in 30 mL of DCM, NMP, or DMF.
2. The solution is allowed to stand for 1 h with occasional mixing.
3. Prior to addition to the resin, the solution is filtered to remove the insoluble DCU. The DCU crystals are washed with NMP to liberate all of the symmetric anhydride.
4. Add this filtered solution to the deblocked peptidyl resin.
5. Do not use the symmetric anhydride method with Boc-Arg(Tos), Boc-Asn, or Boc-Gln; it has been reported to cause double insertion of Arginine residues into the peptide and dehydration of the amides (25). Use either the HOBT ester or one of the following strategies.

3.4.3. Uronium-Type Activation

TBTU (26) and HBTU (27), as well as other uronium-based compounds, have been shown to be ideally suited for solid-phase peptide synthesis (28). The following procedure is an example for a synthesis starting with 5 g of resin with a substitution of 0.6 mmol/g resin. To achieve the appropriate reagent excess, we would use a 10-mmol scale (an approx 3.3-fold excess). This procedure may be scaled according to the need.

1. Dissolve 10 mmol of the protected amino acid derivative in 50 mL of a suitable solvent (either DMF or NMP).
2. To this solution add 10 mmol of HBTU (3.79 g) or 10 mmol of TBTU (3.21 g). Mix until all of the solids are dissolved.
3. Initiate the activation by adding 20 mmol of DIEA (3.47 mL, 2 Eq) and mixing thoroughly. Unlike carbodiimide-mediated activation, no precipitate will form during this activation procedure.
4. Transfer this entire solution to the deblocked peptidyl resin, and allow to couple for 90 min. Although reports in the literature show that coupling completion is very rapid, we have found that slightly longer reaction times eliminate the need for recouplings.
5. Terminate the coupling by filtering the solution away from the resin, and perform a standard washing protocol.
6. Analyze by Kaiser test to determine completeness of the reaction.

3.4.4. Coupling with the BOP Reagent

It has been demonstrated that the BOP reagent proposed by Castro et al. is ideally suited for solid-phase peptide synthesis (29) and that reactions with this reagent are virtually racemization-free (30). All standard
amino acid derivatives may be used with BOP activation, however, we recommend the use of Boc-His(Bom) for Boc strategies so as to avoid detosylation of Boc-His(Tos) by the HOBT that is formed during BOP activation (31). As a general note of safety, BOP generates HMPA (hexamethylphosphoric triamide) as a byproduct. This compound has been the subject of numerous reports concerning its carcinogenicity. Thus, special care must be taken to minimize any physical contact or potential spills.

More recently, several new BOP-type reagents have been developed that have eliminated HMPA as a byproduct following their use, one of which is PyBOP (32). This compound is now routinely used as an effective replacement for BOP.

1. Prepare a solution containing 3 mmol of protected amino acid, 4 mmol of BOP reagent (442.3 mg/mmol), and 6 mmol of DIEA (129 µL/mmol)/mmol of resin-bound amino acid or peptide.
2. Mix this solution thoroughly, add to the deblocked peptide resin, and allow to couple for 2 h.
3. Terminate coupling by filtering away the solution and performing a standard wash protocol.
4. Perform a Kaiser test to determine completeness of the reaction.

We have used the BOP reagent in our laboratory whenever the HOBT ester or symmetric anhydride has been ineffective. This reagent has proven to be a very effective means of successfully completing a difficult coupling or performing a segment condensation onto a resin-bound peptide (see Chapter 15).

### 3.5. In Situ Coupling Additives

We have found that the incorporation of such additives as trifluoroethanol (TFE), tertiary amines, or chaotropic salts into the coupling reaction has greatly reduced the need for subsequent couplings. Coupling may be facilitated by the disruption of secondary structure formation through elimination of hydrogen bonds (2–7). The disruption of hydrogen bonding and interactions between the growing peptide chain and the resin may consequently increase the accessibility of the α-amino group.

#### 3.5.1. Addition of Trifluoroethanol (TFE)

TFE was found to be most effective when used in conjunction with a hindered base, such as DIEA (33). TFE was added so that the final con-
Difficult Couplings

centration of the reaction mixture was 20% TFE in DCM. The favorable
effect of TFE on the resin may be explained by the visible increase in
resin swelling, which may, in turn, increase the resin pore diameter, thus
increasing the accessibility of the activated derivative to the internal sites
of the resin (33). More recently, hexafluoro-2-propanol has been used in
both amino acylation and acetylation (capping) procedures at a final con-
centration of 10% in DCM (34). This solvent system exhibited very simi-
lar swelling profile as that of the TFA/DCM deblocking solution. Note: THF, DMSO, 1,4-Dioxane, and several other solvents may be used
as a substitute, and in the same fashion (35,36). (See Chapter 3).

1. Prepare the activated derivative by the symmetric anhydride procedure
described above using DCM as the solvent. (Use of a small amount of
DMF to help dissolve less-soluble amino acids has been found to be
acceptable.)
2. Take the filtered symmetric anhydride solution, and add TFE to a final
concentration of 20% (vol/vol). Add 1 mmol of DIEA (129 µL/mmol) to
this solution for each mmol of symmetric anhydride.
3. Add this solution to the deblocked peptidyl resin, and mix for 90 min.
4. Terminate coupling by filtering away this solution. Wash the resin as
described above, and monitor completeness of coupling by Kaiser test.

3.5.2. Addition of a Tertiary Amine

Addition of a tertiary amine, such as DIEA, has been found to be most
effective when used in conjunction with other coupling agents, such as
HOBT, BOP, and HBTU (see preceding sections). The tertiary amine
should be added at a 2–3 Eq excess over the theoretical number of amino
groups. The DIEA is added directly to the coupling milieu. Note: There
are some indications that the presence of DIEA may cause racemiza-
tion, especially for sensitive amino acids (12) and in segment conden-
sation (37).

3.5.3. Use of Chaotropic Salts

Chaotropic salts have been found to be most effective when used in
conjunction with normal coupling procedures involving DCC and
HOBT, but may also be used with BOP and HBTU. We have used the
procedure originally described by Klis and Stewart (38), and found that
such salts as potassium thiocyanate (KSCN), and sodium perchlorate
(NaClO₄) are very effective because of their large anions and the pres-
ence of a cation that does not easily form complex compounds (38).
This procedure should be accomplished in a coupling medium that is 0.4M with respect to salt concentration. Also, it has been reported that the effectiveness of these salts improves with an increase in peptide chain length (38). Lithium salts, such as LiCl, have also been used effectively at the same concentration of 0.4M in DMF to break up peptidyl aggregates on the solid-phase support (39).

1. Dissolve the protected amino acid and the appropriate DCC, DCC/HOBT, or BOP/DIEA activators as described earlier in this section.
2. Filter the activated amino acid solution to remove the DCU that has formed in the case of the DCC or DCC/HOBT activation. The BOP solution does not need to be filtered.
3. Prepare the desired salt concentration by dissolving the salt in the filtered solution to yield a final concentration of 0.4M (for example, KSCN 3.88 g/100 mL).
4. Add this solution to the deblocked peptidyl resin, and allow coupling to proceed for approx 2 h.
5. Terminate the reaction by filtering away the amino acid solution and washing the peptide resin using a standard wash protocol.
6. Test for completeness of the reaction using the Kaiser test.

3.5.4. Enhancement by 4-Dimethylaminopyridine (DMAP)

DMAP should be used as an additive for slow and incomplete couplings and not when there is a significant possibility of racemization, as in the case of phenylalanine where the \( \alpha \)-proton is susceptible to abstraction (40-42). For this reason, the routine use of the reagent is not recommended.

1. Preparation of the DMAP solution should be made separate from the DCC solution or the symmetrical anhydride solution (the symmetrical anhydride procedure is preferred to reduce racemization).
2. A solution of 3 mmol of DCC/HOBT or 3 mmol of preformed symmetric anhydride (per mmol peptide resin) should be prepared, and a coupling time of 2 h used.
3. The DMAP reagent is most efficient when employed in small amounts (0.03–0.6 Eq in MeCl\(_2\)) and added to the resin after the coupling reaction has begun (20–30 min). DMAP should not be premixed with DCC or symmetrical anhydride (42).

3.6. Comparison of Coupling Procedures on a Moderately Difficult Peptide

Kaliotoxin (43) is a 37-residue peptide isolated from scorpion venom. This peptide contains three disulfide bonds and is rich in \( \beta \)-pleated sheet
structure. We prepared this molecule in our lab using two similar, manual protocols where every coupling was monitored for completeness. The difference between the two syntheses was that one strategy employed a chaotropic salt in every coupling and the other used a salt recoupling only after the standard HOBT ester failed to give a complete coupling after two couplings. These results are presented in Table 1.

4. Notes

1. There are no simple ways to predict whether a peptide sequence will have difficult residues to couple. As a general rule, peptides with a high propensity to form β-structure can be expected to present difficulties. The difficult residues usually occur in a specific region of the synthesis, usually between residues 12 and 20.

2. Various types of preactivated amino acid derivatives are commercially available. These include UNCA (urethane-protected N-carboxy anhydrides), NHS esters, pentafluorophenyl esters (PFP), and ODHT esters. These may be used without any special activation requirements. Simply dissolve the derivative in the appropriate solvent, and add to the deblocked peptidyl resin. A tertiary base (DIEA) may be added to help speed up the reaction as described in Section 3.5.2.

3. Acyl chlorides (45) and acyl fluorides (46) have been shown to be very effective acylating species. Although these compounds have not been thoroughly tested, blocked amino acyl chlorides have been proposed to be an alternative means to couple within hindered sequences where a symmetric anhydride or an HOBT ester is too bulky (45).

4. In a comparison of couplings utilizing different activated species to sterically hindered amino acids, the PFP and acyl fluorides were found to be ineffective. However, the UNCA, HBTU, and PyBrOP activated species were found to be much more effective in this situation (47).

5. The order in which any one of these procedures may be utilized is relative to your own preference. Generally, we attempt an HOBT ester (via HOBT/DCC) coupling in our initial and repeat couplings. If we enter into a region that appears to require multiple recouplings, we prepare our initial coupling in the presence of a chaotropic salt. Additionally, we may employ different solvent mixtures, such as NMP with THF, DMSO, or TFE in DCM, during the initial coupling and first recoupling. If this fails to improve the coupling result, we switch our activation chemistry to either BOP/DIEA OHBTU/DIEA or TBTU/DIEA. As a last resort, we may employ DMAP or elevated temperature. However, these are more risky and could result in undesirable side reactions. We strongly encourage reducing the substitution of the resin for longer molecules (>30 residues).
### Table 1
Comparison of Coupling Procedures

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a1. Standard DCC/HOBt preactivation in NMP; 2-h coupling
2. First recoupling by DCC/HOBt preactivation in NMP, 2-h coupling.
3. DCC/HOBt preactivation in NMP with 0.4M NaClO₄, 2-h coupling
4. Recoupling with 3 Eq BOP and 5 Eq DIEA in NMP for 90 min
or for peptides rich in β-structural elements to a substitution value of 0.25–0.4 mmol/g of resin.

References


Difficult Couplings


inhibitor of neuronal BK-type Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels characterized from *Androctonus mauretanicus mauretanicus* venom. *J. Biol. Chem.* 267, 1640–1647.


