Chapter 8

Site-Specific Chemical Modification Procedures

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1. Introduction

Identification of important or essential residues in proteins and peptides is an overall goal in understanding the mechanism of an enzyme or the binding affinity of a peptide ligand to its receptor. Most early attempts to accomplish this relied on chemical modification of amino acid residues in the native peptide or protein *(1)*. Selectivity of chemical modification reagents has always been the largest problem to overcome when this approach is taken. Isolation of the desired product from the heterogeneous mixture of components and determination of the modified residue or residues become very labor-intensive processes.

Peptide synthesis offered a viable alternative to this process by utilizing orthogonal deprotection strategies to position the specific residue or residues to be modified. As solid-phase synthetic procedures have improved, this process has become even easier by minimizing the more labor-intensive solution procedures. Advancement of the automation procedures *(2,3)* now permits routine synthesis of peptides and miniproteins, such as HIV protease *(4,5)* and interleukin-3 *(6)*. Site-directed analogs can be easily synthesized by either incorporation of an orthogonal deprotection strategy, or by simply removing resin aliquots during the total synthesis of the native sequence and completing synthesis of the molecule following replacement of one or more amino acids in the sequence *(7,8)*.

In this chapter, several convenient methods for preparing specifically positioned chemically modified peptides will be described. These include

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solid-phase and some solution procedures. Most of these methods are simple acylations on the N-terminus or another internal primary amine. Some of these procedures have been presented in detail in the literature, but many have not. Additionally, we describe the positioning of specifically modified side chains using a combination of Fmoc/*t*Bu-Bzl strategy. Lastly, a description of several carboxylic acid amidation reactions that have been useful in the preparation of cyclic and fluorescent peptides is presented.

2. Materials

1. All solvents described are purchased from commercial sources, such as Fisher (Fair Lawn, NJ) or Aldrich (Milwaukee, WI), and used as such.
2. 2,4 Dinitrofluorobenzene (DNFB), *N*-hydroxysuccinimide, pyridine–sulfurtrioxide complex, biotin, acetic anhydride, dansyl chloride, *p*-benzoylbenzoic acid (BBA), 7-methoxycoumarin-4-acetic acid, palmitic acid, myristic acid, and stearic acid may be obtained from Aldrich. Biotin *p*-nitrophenyl ester, 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide HCl (EDAC) and 5-(2-aminoethylamino)-naphthalene sulfonic acid (Na salt) (1,5 EDANS) may be purchased from Sigma (St. Louis, MO). 4-Dimethylamino-azobenzene 4'-carboxylic acid (DABCYL) may be obtained from TCI America.
3. Specialized amino acid derivatives are commercially available from Bachem Bioscience (King of Prussia, PA). These derivatives include Fmoc-Bzl and Boc-Fmoc- or Boc-(OFm)-protected residues.

3. Methods

The general strategy presented in this chapter is to exploit the solid-phase principle whenever possible. This greatly reduces difficult extractions and recrystallizations required when solution procedures are carried out. It is the goal of this chapter to present the most common types of chemical modifications of synthetic peptides. Many of these modifications are very similar, but very few procedures have been presented in the literature. Additionally, this chapter will present approaches for the synthesis of branched peptide structures and specifically positioned benzyl-type analogs requiring no special chemical modification protocols.

3.1. Simple N-Terminal Extensions

These modifications involve reaction of the free N-terminus of the completed peptide chain with an alkylating or acylating species. In all cases listed below, the N-terminal-protecting group has been removed. In the case of Boc synthesis, it is necessary also to perform a base wash

with 10% DIEA in DCM to neutralize the TFA salt. All of these modifications will result in a blocked N-terminus, which is refractory to Edman degradation *(9,10)*. As a necessary control, a Kaiser test should be performed as described below in order to ensure coupling of the derivative.

3.1.1. Ninhydrin Analysis (Kaiser Test)

This test, developed by Kaiser et al. *(11)*, provides a clear indication of the amount of free amine present. This test is routinely performed before and after a coupling procedure to ensure completeness.

1. Dissolve 80 g phenol in 20 mL absolute ethanol, and keep in a brown bottle marked A.
2. Dilute 2 mL of 1 m*M* aqueous solution of KCN up to 100 mL with pyridine. Keep this in a brown bottle marked B.
3. Dissolve 500 mg of ninhydrin in 10 mL of absolute ethanol, and keep in a brown glass bottle marked C.
4. Take a small aliquot of peptide resin (approx 5–10 mg) and place in a 10 × 75 mm borosilicate test tube.
5. Add two drops of reagent A followed by two drops of reagent B and, finally, add two drops of reagent C.
6. Place this test tube in a heating block that is maintained at 100°C for 2 min.
7. Remove the test tube, and check the color of the solution. A dark blue result indicates a strong positive reaction for primary amines. A clear yellow solution indicates a strong negative reaction for primary amines. Lighter shades of blue and blue-green indicate an incomplete coupling reaction.
8. Certain amino acid residues do not react strongly with ninhydrin. Proline gives a resin bead with a red color for a strong positive. Asp, Asn, Glu, Gln, His, and Cys do not always react well with ninhydrin and often give a red to red brown color as a positive reaction.

3.1.2. Acetylation

Acetylation places an acetyl group on the N-terminus of the peptide. This will increase the mol wt of the peptide by 42 mass units.

1. Take the deblocked peptide resin, and add a solution of 20% acetic anhydride dissolved in DMF at a ratio of 7 mL/g of resin. Ninhydrin analysis of the deblocked peptide resin should be positive prior to this reaction. Addition of 1.5 Eq of DIEA after 5 min neutralizes the protons that have been generated.
2. Allow this acetylation to proceed for 30 min at room temperature. This is a fairly rapid reaction and is usually complete within 10 min.

3. Wash the resin with two washes of DMF, two washes of DCM, and two washes of MeOH.
4. Ninhydrin analysis at this point should be a strong negative.
5. No special cleavage procedures need to be followed in either Boc or Fmoc chemistries.
6. This procedure can be used to radiolabel the peptide by incorporation of radiolabeled acetic anhydride, which is commercially available.

3.1.3. Biotinylation

For a number of immunological procedures (*see* Chapter 10, *PAP*) as well as for structure–function studies, biotin has been a very useful probe *(12,13)*. For solid-phase biotinylation, we follow the procedure of Lobl et al. *(14)*.

1. Biotin is only moderately soluble in solvents compatible with peptide synthesis. We have found that biotin-*p*-nitrophenylester and biotin-*N*-hydroxysuccinimide ester are very soluble in DMF. We routinely both of these compounds.
2. Dissolve 3 Eq of either biotin-NHS or biotin-*p*-nitrophenylester in 8–10 mL of DMF/g of resin. Stir until all of the biotin is dissolved (usually 2–5 min).
3. Add this solution to the deblocked peptide resin. We have found that this reaction is fairly slow, and requires several hours or overnight reaction. The temperature may be increased up to 40°C to speed up the reaction. Ninhydrin analysis should again be positive prior to addition of the biotin and negative following the successful coupling.
4. No special cleavage procedures are required for either Boc or Fmoc peptides.
5. Biotin is fairly hydrophobic and usually will result in a later retention time for the biotinylated peptide on RP-HPLC (*see* Chapter 3, *PAP*).
6. The mol wt of the final product will be increased by 226 mass units (*see* Chapter 7, *PAP*).

3.1.4. Dinitrophenylation

Addition of the dinitrophenyl group turns the peptide a bright yellow color and increases the mol wt by 166 mass units.

1. The deblocked peptide resin is treated with 4 Eq of dinitrofluorobenzene and 4 Eq of DIEA in DMF for 3 h. We have found that the reaction is complete within 3 h for most peptides. However, some peptides may be less reactive and require a second treatment overnight.
2. The DNP-peptide resin is then thoroughly washed with DMF, followed by DCM, and finally, MeOH.

3. Ninhydrin analysis of the resin should now be a strong negative. If this is not the case, dinitrophenylation is not complete and must be repeated.
4. No special cleavage procedures are required for the peptide resins. Protection from light is recommended for storage of the resin as well as the final product.
5. The DNP-amino acid derivative is stable to acid hydrolysis and will not be detected by standard amino acid analysis methods.

3.1.5. Dansylation

Attachment of the dansyl group to the peptide will give the peptide a pale yellow-green color. This substituent will increase the mol wt by 233.3 mass units.

1. Dissolve 5 Eq of dansyl chloride in 10 mL of DMF/g of deblocked peptide resin. Add 2 Eq of DIEA to this solution to maintain the pH of the reaction.
2. Add this solution to the deblocked peptide resin and allow to react overnight at room temperature or increase the temperature of the reaction up to 35°C for 3–4 h. We have found that elevating the temperature slightly helps speed the reaction to completion.
3. Terminate reaction by draining the reaction vessel and washing thoroughly with two washes of DMF, followed by two washes of DCM, and finally, two washes of MeOH. Ninhydrin analysis of the final resin should now indicate a negative result for free amines.
4. No special cleavage protocols are required for dansylated peptides. These peptides and peptide resin should be stored in the dark.
5. The dansyl-amino acid derivative is stable to standard hydrolysis procedures and will not be detected by standard amino acid analysis.

3.1.6. Dabcylation and Dabsylation

These derivatives will turn the peptide a bright red color. The carboxylic acid form (dabcyl) and the sulfonyl chloride form (dabsyl) both react with the N-terminal amino acid. However the dabsyl derivative will be stable to normal amino acid analysis hydrolysis procedures.

1. For the dabcyl derivative, we routinely use NMP as the solvent of choice. Dabcyl is fairly soluble in DMF, but has much greater solubility in NMP. Dissolve 4 Eq dabcyl in 10 mL NMP/g of resin.
2. Activate the carboxyl group by adding 4.4 Eq of diisopropyl carbodiimide and 8 Eq of hydroxybenzotriazole (HOBt).
3. Add this solution to the deblocked peptide resin. Allow this coupling reaction to continue overnight. We have observed this to be a fairly slow coupling derivative.

4. Terminate the coupling process by washing the peptide resin extensively with NMP until no more red color is seen in the wash. Next, wash the resin with three washes of DCM followed by three washes of MeOH. Ninhydrin analysis should now be negative. If it is not negative, then recouple by performing a base wash of the resin and starting back at step 1.
5. The dabsyl derivative can be coupled directly by dissolving 4 Eq in NMP with 4 Eq of DIEA and coupling overnight at 35°C. Perform step 4, and check for completeness of reaction.
6. These derivatives are stable to both TFA- and HF-deprotection procedures. Addition of these derivatives will increase the mol wt by 251 mass units for the dabcyl and 288 for the dabsyl derivative.

3.1.7. Fatty Acid Acylation

Attachment of a fatty acid, such as myristic, palmitic, or stearic acid, is easily accomplished using standard coupling procedures (*see* Note 1).

1. Dissolve 4 Eq of the fatty acid in 10 mL of DCM or DMF, or mixtures of these two solvents/g of deblocked peptide resin.
2. Activate the fatty acid carboxyl group by addition of 4.4 Eq of diisopropylcarbodiimide and 8 Eq of hydroxybenzotriazole to this solution.
3. Add this solution to the peptide resin, and allow to react over night at 35°C. This acylation is rather slow and may require additional couplings to obtain complete acylation.
4. Terminate the reaction by draining the reaction vessel, and washing with three washes of DCM followed by two washes of DMF, followed by two washes of DCM, and lastly, two washes of MeOH. Ninhydrin analysis should be negative.
5. No special precautions must be followed in the cleavage of these peptides. Solubilization of the fatty acyl-peptide may be hampered by these fatty acid groups and may require slightly more drastic solvents, such as glacial acetic acid or even neat TFA. Molecular weight increases by 209, 238, and 267 mass units for myristic, palmitic, and stearic acids, respectively (*see* Note 2).

3.1.8. Acylation with 7-Methoxycoumarin 4-Acetic Acid (MCA)

Coumarin derivatives are **not stable to HF cleavage** and thus should only be used when employing an Fmoc strategy with a TFA cleavage.

1. Dissolve 4 Eq of the coumarin derivative in 10 mL of DMF/g of deblocked Fmoc-peptide resin.
2. Activate the carboxyl group by adding 4.4 Eq of diisopropylcarbodiimide and 8 Eq of hydroxybenzotriazole to this solution.
3. Add this solution to the deblocked peptide resin, and allow to couple for 4 h.

4. Terminate the reaction by draining the reaction vessel and washing with two washes of DMF, followed by two washes of DCM, followed by two washes of MeOH. Ninhydrin analysis of the resin should be negative. If it is not negative, perform a base wash procedure and recouple from step 1.
5. As mentioned above, these derivatives are not stable to HF. They should only be coupled when an Fmoc strategy has been used. Addition of 7-methoxy-coumarin 4-acetic acid will increase the mol wt of the peptide by 216 mass units.

3.1.9. Reductive Alkylation

This procedure can be used to place an alkyl or aromatic substituent at the N-terminus of the peptide. This procedure is a modification of the Jentoft and Dearborn method *(15)*. A novel method of limiting the number of small alkyl substituents was recently reported at the 22nd European Peptide Symposium where the N-terminal amine was protected with the Dod-protecting group prior to the reductive alkylation *(16)*. This group is then removed by treatment with TFA, and the peptide can be elongated or cleaved.

1. Dissolve 1–4 Eq of the appropriate aldehyde (i.e., benzaldehyde for a benzyl group, acetaldehyde for an ethyl group, and so on) in 10 mL of DMF containing 1% acetic acid/g of deblocked peptide resin. The greater the number of Eq of aldehyde used, the greater the potential to generate disubstituted products.
2. Add this solution to the deblocked peptide resin, and allow to mix for 30 min.
3. Initiate the reduction process by addition of 1–4 Eq of sodium cyanoborohydride (depending on the number of Eq of aldehyde used) to the reaction mixture. Allow reaction to mix for an additional 2 h.
4. Terminate the reaction by draining the reaction vessel and washing the resin as described above.
5. Reductive alkylation with smaller substituents, such as formaldehyde and acetaldehyde, usually results in the formation of dialkyl products.
6. This procedure can be used to incorporate a radiolabel into the N-terminus of the peptide by using a radiolabeled aldehyde.

3.1.10. Anthranilylation

Attachment of this amino acid derivative results in a fluorescent peptide derivative. This fluorescence can be internally quenched by *p*-nitro-Phe and nitro-Tyr derivatives, resulting in a convenient method of preparing internally quenched proteolytic substrates *(17,18)*.

1. Dissolve 4 Eq Boc-anthranilic acid in NMP with 4.4 Eq of DCC and 8 Eq HOBT.
2. We have found that incorporating potassium isothiocyanate to a final concentration of 0.4*M* in this coupling mixture improves this coupling.
3. Add this preactivated solution to the deblocked peptide resin, and allow to couple from 4 h to overnight.
4. Following coupling of the derivative, the Kaiser test will be negative.
5. Remove the final Boc group with 50% TFA in DCM prior to HF cleavage.
6. Addition of this residue will increase the mol wt of the peptide by 121 mass units.

3.1.11. Photoprobe Coupling (Addition of Benzoylbenzoic Acid)

Addition of benzoylbenzoic acid (BBA) results in a peptide that can be conveniently used as a photoprobe and may be covalently linked to its target by UV irradiation *(19,20)*. This derivative results in an addition of 208 mass units to the peptide. Additionally, incorporation of a photoprobe into the side chain of Phe has been reported *(21)*. This protected derivative Boc-Bpa-OH (*p*-benzoyl-phenylalanine) is now commercially available from Bachem Bioscience Inc.

1. Dissolve 4 Eq of BBA in NMP. Add 4.4 Eq of DCC and 8 Eq HOBt to this solution, and allow to preactivate for 30 min.
2. Add this solution to the deblocked peptide resin, and allow to couple for 2–4 h.
3. Following addition of this derivative, Kaiser analysis will be negative.
4. Cleavage of the peptide should proceed as a normal HF cleavage with **no thiol scavengers.**

3.2. Specialized Uses of Boc-Lys(Fmoc)-OH and Boc-Orn(Fmoc)-OH

These amino acid derivatives have the unusual property of being differentially labile while still attached to the solid-phase support. This property allows both Boc and Fmoc chemistries to be performed in an orthogonal deprotection scheme. This allows the chemist to create site-specific modifications and create branched peptide structures very easily.

3.2.1. Placement Specificity for Chemical Modification

The following procedure will refer to all of the reactions described above by incorporation of specialized amino acid derivative Boc-Lys(Fmoc)-OH.

1. This derivative can be positioned during a Boc strategy synthesis so as to incorporate a differentially labile protecting group stable to the TFA-deprotection steps of the Boc synthesis.
2. On completion of the solid-phase assembly of the peptide chain, the final Boc group is not removed. Instead, the Fmoc group on this internal Lys residue is removed by treatment with 20% piperidine in DMF.
3. This results in an internally positioned primary amine that can then be reacted with any one of the abovementioned acylation reactions stable to HF.
4. The resulting peptide resin is then treated to remove the Boc group. Following Boc removal, this resin can then be further reacted with another acylating reagent if desired. The resulting product is subsequently deprotected using standard HF deprotection procedures yielding the monospecific or dispecific adduct.
5. By incorporating multiple residues of the Boc-Lys(Fmoc)-OH sequentially, these can be modified prior to elongating the peptide chain to create multiple derivatization sites. For example, a fatty acid may be linked near the C-terminus, an internal photoprobe can be positioned in the middle of the molecule, and the N-terminus may be biotinylated. This could be easily accomplished by incorporating two Boc-Lys(Fmoc)-OH derivatives and N-terminal acylation. However, synthesis would proceed with coupling the first derivative and immediate removal of the Fmoc group prior to elongation of the peptide chain. The side chain is acylated with the appropriate fatty acid derivative. Extension of the primary chain then resumes by removal of the Boc group and addition of the next portion of the sequence until the second derivatization point is encountered. At this point, the Fmoc group would be removed, and the 4-benzoylbenzoic acid coupling reaction is performed. Following this coupling, the remainder of the sequence would proceed after removal of the Boc group, and lastly, the N-terminus would be derivatized with biotin using biotin nitrophenylester.

3.2.2. Synthesis of Branched Peptide Structures

By incorporating the Boc-Lys(Fmoc)-OH at the appropriate position in the peptide sequence, a branched peptide can be synthesized one of two ways.

1. Following incorporation of this derivative, synthesis continues on the main peptide sequence until it is complete. The main peptide chain is left either protected with the Boc group intact, or the Boc group may be removed and the N-terminus acetylated. Following completion of the main peptide chain, the branched peptide chain can be assembled by deblocking the Fmoc group with 20% piperidine in DMF. Coupling of the branched peptide can either proceed using an Fmoc strategy if the final Boc group was

not removed or with either a Boc or Fmoc strategy if the N-terminus has been acetylated.

2. This method requires the use of Fmoc-Bzl amino acid derivatives (these derivatives are commercially available). If one desires to synthesize the branched peptide chain first, this is accomplished by stopping the Boc strategy immediately following the coupling of the Boc-Lys(Fmoc)-OH. At this point, an Fmoc strategy may be employed, but the Bzl side-chain-protection strategy must be used on all of the side chains of the branched portion. Otherwise the *t*Bu-protecting groups added during the Fmoc portion of the synthesis would be removed when the Boc strategy resumes. On completion of the branched portion of the molecule, the Fmoc can be left on or the branched N-terminus must be acetylated. Now, the Boc synthesis of the main peptide chain resumes until completed. If the Fmoc group has not been removed, deblock with 20% piperidine **prior to HF cleavage.**
3. When following either of these strategies, use of a scavenger, such as DTT and 4-methylindole, in the TFA-deblocking solution during the Boc procedure is strongly recommended if Trp and Met are present. It is more important, especially in the case of Scheme 2, that the Trp is present without the formyl-protecting group in Fmoc synthesis. On repetitive cycles of TFA exposure during the ensuing Boc section following the Fmoc portion of synthesis, the Trp indole side chain will be destroyed if a scavanger is not incorporated.

3.3. Positioning of Benzyl-Protected Side Chains

When an Fmoc strategy is being utilized, specific analogs may be generated by utilizing an Fmoc-Bzl-protected derivative instead of the standard Fmoc-*t*Bu derivative at selected positions. The benzyl group is not cleaved during standard TFA cleavage schemes for Fmoc peptides. This will result in a specifically benzylated derivative following cleavage with TFA. This type of strategy can be used to increase the hydrophobicity of a peptide. This strategy was employed to make the dibenzylated CD_4 receptor fragment, which inhibited HIV-induced cell fusion and infection in vitro *(22)*.

3.4. Carboxylic Acid Modifications

Up to this point, all of the reactions described have been simple manipulations of general coupling protocols using the solid-phase approach. Reactions at carboxyl groups are generally carried out using solution procedures. Recently, with the synthesis of Boc-Glu(OFm)-OH and Boc-Asp(OFm)-OH, some solid-phase carboxyl modifications are

now possible. These include cyclization through a primary amine to the deblocked OFm derivative *(23)* and preparation of a fluorescent Boc-Glu-(EDANS)-OH derivative from the Boc-Glu(OFm)-OH derivative while attached to the solid-phase support (M. W. P., personal communication). This section will describe procedures for the preparation of the EDANS derivative using a solution procedure. For peptides containing only one carboxyl group, where either the C-terminal carboxyl group or the C-terminus is amidated, and only one internal side chain carboxyl exists, a Boc strategy may be used. However, if more than one carboxyl group is present, we recommend using an Fmoc strategy employing Sasrin™ resin (available from Bachem Bioscience Inc.) to prepare the free acid of the protected peptide *(24)* and subsequent solution reaction on this protected peptide to synthesize the C-terminal adduct (*see* Note 3).

3.4.1. Attachment of EDANS to Carboxyl Group

Dabcyl and EDANS are two fluorophores with excellent donor-acceptor qualities for fluorescence energy transfer (FRET) *(25)*. Incorporating them into opposite sides of proteolytic cleavage site on peptide substrates has been exploited to create a continuous fluorescence assay for HIV protease *(26)*. EDANS can be attached to either the C-terminal carboxyl or the α- or β-carboxyls of Asp and Glu, respectively. If the peptide contains only one carboxyl group, a standard Boc synthesis is recommended. The N-terminus of the peptide must be blocked (acetylated, dabcylated, and so forth).

1. Dissolve 0.25 mmol of the cleaved, N-terminally blocked peptide in 100 mL of DMF (*see* Note 4).
2. Add 2.2 Eq of EDAC (1-ethyl 3-[3-dimethylaminopropyl] carbodiimide) HCl and 2.2 Eq of hydroxybenzotriazole to initiate the reaction.
3. Add 0.5 mmol of 1,5-EDANS (sodium salt) to this solution, and mix until fully dissolved.
4. Allow the reaction to proceed for 3 h at room temperature. Follow the reaction by RP-HPLC. Inject a small aliquot (5 µL diluted with three parts water) directly onto an RP-HPLC column. A massive breakthrough peak will be observed from the DMF.
5. We have routinely observed the retention time to shift to earlier values on successful coupling.
6. Terminate the reaction by diluting with five to eight parts of water and load onto preparative RP-HPLC (*see* Chapter 4, *PAP*). (Do not load this solution onto the HPLC if the peptide begins to precipitate. This solution

must be filtered to prevent damage to the HPLC column.) The DMF, all of the coupling agents, and the unreacted EDANS will wash through on a normal C_{18}-HPLC column. More hydrophobic peptides will be retained and can be isolated by a normal gradient elution.

7. This substituent will increase the mol wt by 247 mass units.

3.4.2. Solid-Phase Cyclization Reactions

Cyclization of protected peptides has been accomplished by incorporating specially derivatized amino acid residues, such as Boc-Glu(OFm)-OH and Boc-Asp(OFm)-OH, in combination with Boc-Lys(Fmoc)-OH *(23)*. When these derivatives are used in combination with one another, it is possible to form a cyclized peptide through the amide bond generated by coupling the side-chain amino group of the Lys or the α-amino group to the side-chain carboxyl group of the Glu or Asp. Other cyclic peptides have also exploited this solid-phase cyclization by incorporating a combination of Fmoc-*t*Bu and Fmoc-Bzl amino acids in the preparation of $Asu^{(1,7)}$-calcitonin *(27)*. More recently, a cyclized derivative of endothelin was prepared that resulted in a potent endothelin antagonist *(28)*. Both of these molecules utilized this amide linkage as a disulfide bond surrogate.

1. During synthesis, the positioning of the differentially labile derivatives is crucial. Information about secondary structure can help determine the position for the complementary pair of Boc-Lys(Fmoc)-OH and the Boc-Glu(OFm)-OH or Boc-Asp(OFm)-OH.
2. Once the two derivatives have been incorporated, the peptide resin N-terminal Boc is not removed. Instead, the internal side-chain blocking groups (the Fmoc group and the Fm group) are removed by treatment with 20% piperidine in DMF for 30 min at room temperature.
3. The internally deblocked peptide resin is then sequentially washed with DMF, iPOH, and DCM.
4. The amide bridge is formed by adding 6 Eq of BOP to the peptide resin in 6 mL of NMP/g of resin in the presence of 6 Eq of triethylamine. This coupling is allowed to proceed for 2 h at room temperature with constant mixing (longer reaction time may be required).
5. Following the cyclization, the Kaiser test should be negative, indicating quantitative cyclization of the internal primary amine.
6. Synthesis on the remainder of the molecule by a normal Boc-based procedure can resume following the cyclization. This internal cyclization will result in the loss of the mass of a water molecule (18 mass units) from the final mol wt of the deprotected peptide.

7. Cleavage of the peptide requires no special precautions other than those required by the amino acids present in the entire peptide sequence.
8. It is also possible to cyclize the molecule by positioning only the Boc-Glu(Fm)-OH or Boc-Asp(Fm)-OH in the molecule, and removing the N-terminal Boc group. Treatment of the resin with piperidine to remove the Fm group, followed by BOP cyclization as described above will result in the formation of cyclic molecule involving the α-amino group of the molecule and the internal side-chain carboxyl group. This will result in a peptide refractory to Edman degradation or further elongation.
9. It is also possible to substitute alternative differentially protected amino acids, such as Boc-Asu(Fm)-OH (Asu = aminosuberic acid), instead of the Asp and Glu derivatives.
10. Head-to-tail cyclization is also possible using a special resin where the side chain of Glu or Asp is linked to the resin and the C-terminal carboxyl group is protected with the Fm group. Using the same procedure as described above will result in either a head-to-tail arrangement or a side chain-to-tail orientation.

4. Notes

1. Chemical modification reactions involving fatty acids often result in peptides with solubility problems. Aggregation may create a major solubilization problem that may require more drastic measures, such as neat TFA or DMSO, to achieve satisfactory solubilization.
2. Peptides containing the sulfated Tyr ester may be conveniently prepared by Fmoc strategy using the Fmoc-Tyr(SO_3 Ba salt)-OH available from Bachem Bioscience. Cleavage of the final peptide is carried out in 50% TFA solution in DCM containing 5% 1,2 ethanedithiol and other scavengers as required by the other amino acid residues.
3. Peptides containing the amino acid γ-carboxyglutamic acid can also be prepared by an Fmoc strategy. This derivative Fmoc-Gla($OtBu)_2$-OH is available from Bachem Bioscience. Cleavage of the peptide from the resin is accomplished in 40–50% TFA in DCM containing 5% 1,2 ethanedithiol and other appropriate scavengers *(29,30)* as required by the other amino acids present.
4. Dabcyl-EDANS substrates are very insoluble in most aqueous buffers. We routinely solubilize these compounds in DMF and dilute this into the aqueous buffer we are using.

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