Standard practices for Fmoc-based solid-phase peptide synthesis in the Nowick laboratory (Version 1.7.2)

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Contributions to this guide

Latest revision 1.7.2. 17 March 2020

A.G.K. wrote the majority of protocols, made the majority of chemdraws, and complied the first few word document versions of this guide.
P.J.S. added the Removal of TFA esters by MeOH reflux and Cleavage of linear peptide from Rink Amide resin protocols, in addition to complied the guide as LaTeX document.
H.J.Y. added the removal of alloc protecting group and dde protecting group, corrected the Chemdraws, and hand coupling section. Updated in 2020 by
G.G. updated in 2020 to reflect changes to intermolecular oxidation, purification via Biotage Isolera, and ether precipitation protocols. As well as minor changes to hand coupling.

The majority of these protocols exist due to countless other unmentioned sources passing their knowledge down from student to student, in addition to a number of academic sources, that may be added to this guide in the future.

General

Peptide synthesis is pretty robust and fool proof; however, there are a few things that can really mess up the reproducibility of these protocols. Chief amongst them is the quality of DMF. It is incredibly important to use good quality **amine-free** DMF. This means either getting it off of the solvent system or placing an amine pack into ThermoFisher DMF 24 h prior to use. This is particularly true for steps that fall under the **Solid-phase peptide synthesis** section of this guide.

Solid Supports

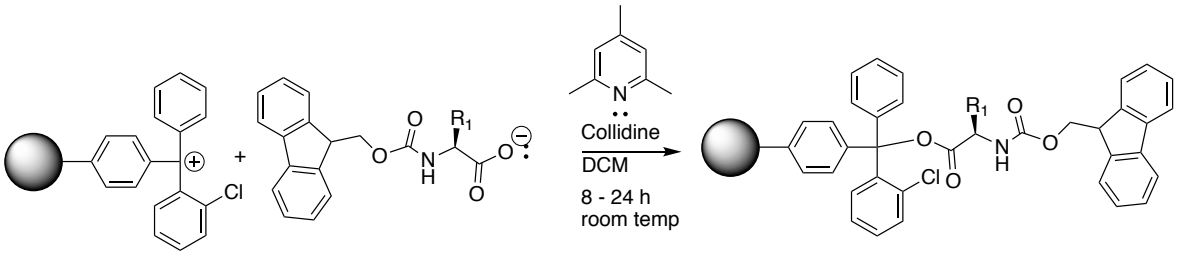
The first step in solid-phase peptide synthesis is choosing what functional group you want your *C* -terminus to be:

If you are making a macrocyclic peptide use 2-chlorotrityl resin.
If you want your *C* -terminus to be a carboxylic acid use 2-chlorotrityl resin or Wang resin.
If you want your *C* -terminus to be an amide use Rink amide resin.
If you are using the CEM, use 100–200 mesh resin for Rink amide and 2-chlorotrityl resin. USING A DIFFERENT MESH will clog the CEM reaction vessel.

Once your choice of resin is made you will need to load your first amino acid onto the resin.

Loading 2-chlorotrityl chloride resin
The purpose of this step is to covalently link the first amino acid onto the resin.

Prep time: 30 min
Reaction time: 8–24 h

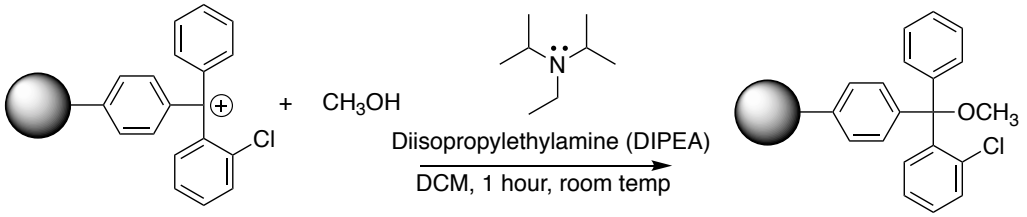


Scheme 1. Loading 2-chlorotrityl chloride resin. The resin is drawn as a carbocation and the amino acid is drawn as a carboxylate to ease explanation of chemistry.

1. Weigh out appropriate amount of resin. Generally, we use 300 mg for a 0.1 mmol scale synthesis. Transfer the resin into a PolyPrep chromatography column (BioRad).
2. Swell resin for at least 30 min (longer is okay) at room temperature in CH_2Cl_2.
3. Weigh out an appropriate amount of the first amino acid and dissolve it in 8 mL CH_2Cl_2 w/ 0.3 ml 2,4,6-collidine. *When making a macrocyclic peptide our first amino acid is almost always Boc-Orn(Fmoc)-OH. Use ca. 100 mg of Boc-Orn(Fmoc)-OH.*
4. Using a flow of nitrogen gas, push out all CH_2Cl_2 from the column that contains the swelled resin and add the amino acid/CH_2Cl_2/collidine mixture.
5. Rock for at least 8 h (no longer than 24 h).
6. Move on to Capping 2-chlorotrityl chloride Resin.

Capping 2-chlorotrityl chloride resin
The purpose of this step is to covalently link a small nucleophile (methanol) to the unreacted carbocations on the 2-chlorotrityl chloride resin.

Prep time: 10 mins
Reaction time: 1 hour



Scheme 2. Capping 2-chlorotrityl chloride resin. The resin is drawn as a carbocation to ease explanation of chemistry.

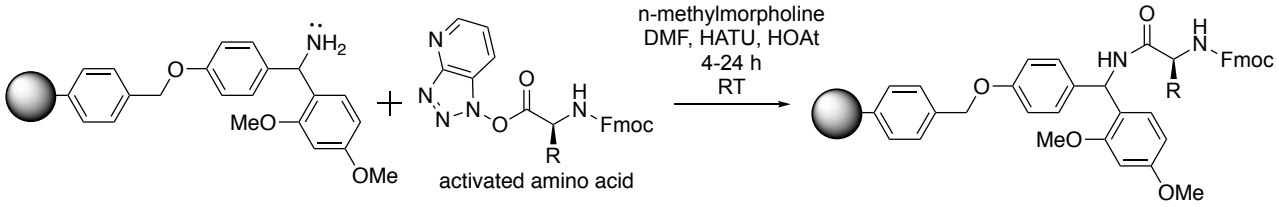
1. Wash the loaded resin 3X with CH_2Cl_2.
2. Make the capping solution. The capping solution is CH_2Cl_2:MeOH:DIPEA (17:2:1). Make this fresh each time by adding 1 mL MeOH and 0.5 mL diisopropylethylamine (DIPEA, or DIEA) to 9 mL of CH_2Cl_2.
3. Dump the capping solution on to the loaded resin and rock for 45–60 minutes at room temperature. It is not recommended to extend the reaction time, as exchange of the loaded amino acid with MeOH is possible.

4. After 1 h, push out the capping solution with nitrogen and wash the resin 4X with CH_2Cl_2 and 4X with DMF. *At this point you can analyze how efficient your resin was loaded. We typically skip this step, though, as loading 2-chlorotrityl resin is VERY reproducible if you do not stray from the protocol detailed above.*
5. Your loaded resin is now ready to go through repeated Fmoc deprotections and amino acid couplings to build the rest of your peptide. These deprotections and couplings can be done manually (hand coupling) or on an automatic synthesizer. Regardless of your preferred method, the order of operation and chemistry is identical.

Loading rink amide resin

The purpose of this step is to covalently link the first amino acid to the resin

Prep time: 1.5 h
Reaction time: 4-24 h



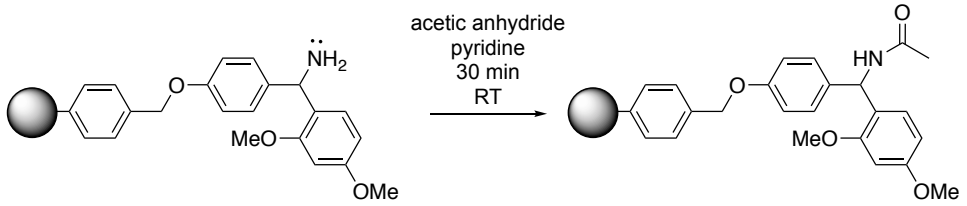
Scheme 3. Loading Rink Amide resin. Note in this scheme the resin has already been Fmoc-deprotected

1. Weigh out appropriate amount of resin. Generally we use 300 mg for a 0.1 mmol scale synthesis. Dump the resin into a PolyPrep chromatography column (BioRad).
2. Swell resin for 1 hour in DMF. After 1 h has passed, use a flow of nitrogen gas to push the DMF out of the column.
3. Cleave Fmoc from the resin by adding 8 mL of 20% (v/v) piperidine in DMF. Allow the resin to rock in the 20% piperidine solution for 1 h. When there is 15 min left start preparing the amino acid you want to load the resin with.
4. Preparing (or activating) the amino acid: weigh out 5 equivalents of the amino relative to achieving 100% loading of the resin. The amount of amino you want to weigh out is most easily figured out using Ryans Excel mass spec calculator. Also weigh out 4.5 equivalents of HATU and 4.5 equivalents of HOAt. Combine the amino acid, HATU, and HOAt in a glass scintillation vial and add 8 ml of 20% *N*-methylmorphiline (v/v) in DMF (alternatively, you can use 20% collidine (v/v) in DMF).
5. After the Fmoc cleavage from the resin is complete, use a flow of nitrogen to push out the 20% piperidine.
6. Wash the resin 3X with DMF.
7. Dump the amino acid/HATU/HOAt solution onto the resin and rock at room temperature for at least 4 h. Do not exceed 24 h.
8. Next, use a flow of nitrogen to push out the amino acid solution.
9. Wash the loaded resin 3X with DMF from the solvent system.
10. Move on to Capping Rink Amide Resin

Capping Rink amide resin

The purpose of this step is to cap unreacted amines on rink amide resin so that the next amino acids you couple are not attached to the resin.

Prep time: 5 min
Reaction time: 30 min



Scheme 4. Capping unreacted sites on Rink amide resin

1. Prepare the capping solution by combining acetic anhydride and pyridine in a 3:2 ratio of acetic anhydride:pyridine. Make this fresh each time. Crudely, by using a Pasteur pipette, combine 3 ”squirts” of acetic anhydride with 2 ”squirts” of pyridine in a scintillation vial.
2. Dump the capping solution on the resin and rock for 30 min at room temperature.
3. After the resin is finished capping, push out the capping solution with nitrogen and wash the resin 4X with DMF.
4. Your loaded resin is now ready to go through repeated Fmoc deprotections and amino acid couplings to build the rest of your peptide. These deprotections and couplings can be done on an automatic synthesizer or manually (hand coupling).

Checking resin loading

The purpose of this step is to determine the mmol of amino acid that are loaded on your resin. The procedure is exactly same regardless of the resin you are using.

Prep time: 20 mins
Reaction time: 5 mins

1. Take a small portion (1–2 mg) of resin and transfer it to a new polyprep column. Dry the resin by blowing nitrogen through it.
2. While the resin drying find a 3-mL quartz cuvette and add exactly 3 mL of 20% piperidine in DMF to it. Blank the UV/Vis with the 20% piperidine at 290 nm.
3. Weigh as close to 1 mg of dried resin as you can and add it to 3 mL 20% piperidine in DMF in the quartz cuvette.
4. Allow the resin to sit in the 20% piperidine for at least 5 min. Longer reaction times are fine.
5. Take a UV/Vis reading at 290 nm against the 20% piperidine blank.
6. Use Ryan’s Excel mass spec calculator to determine your loading percentage (present in our group website under LINKS). This will dictate the mmol of each amino acid you want to use when synthesizing your peptide. Good loading percentages fall between 50–70%.

Solid-phase peptide synthesis

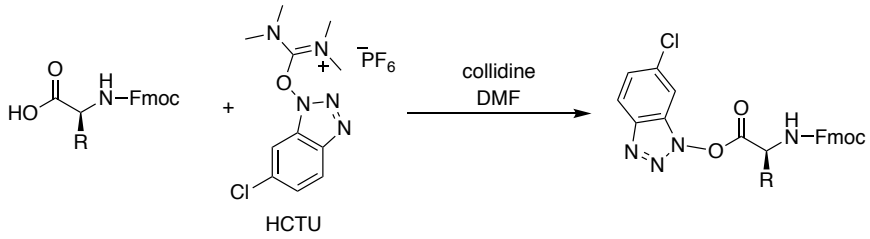
The purpose of this step is to sequentially add amino acids to the resin to build a peptide chain. There are two main steps in coupling an amino to peptide chain. The first step is deprotecting Fmoc from the amino group on the amino acid on the resin to expose an amine. The second step is coupling an activated amino acid to the exposed amine. These steps are done exactly the same on 2-chlorotrityl chloride, Wang, and rink amide resin.

Manual (hand) coupling

You will need stock solutions of 20% piperidine in DMF as well as 20% collidine (or NMM) in DMF for this.

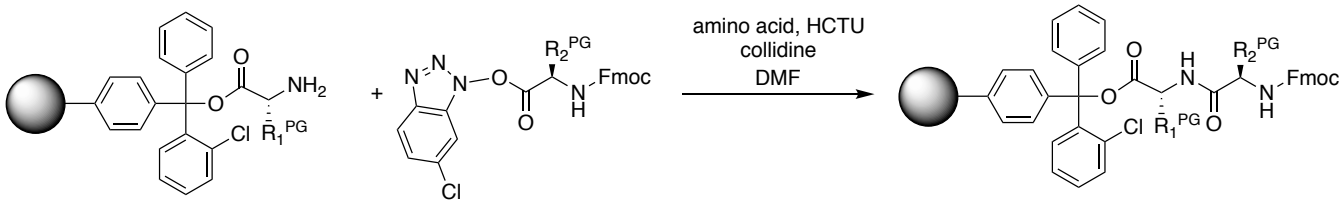
Prep time: variable
Reaction time: variable

1. Transfer your loaded resin to a hand coupling vessel and wash 3X with DMF.
2. Deprotect Fmoc from the amino acid on your resin by adding 5 mL (5 squirts) of 20% piperidine. Mix for 7 min by bubbling in nitrogen gas. Some add a second 5 mL of piperidine, but it is probably not necessary.
3. Wash 5X with DMF, specifically ensuring the sides of the coupling vessel are washed.
4. Prepare the activated amino acid by weighing out an appropriate amount of amino acid and coupling agent (HCTU) and dissolving them in 20% collidine in DMF. *We almost always use HCTU as the coupling agent, unless you are coupling from an N-methyl amino acid other than N-methyl glycine. In that case use HATU and HOAt as the coupling agents.*



Scheme 5. Activating an amino acid, shown using HCTU, although we commonly use HATU or HBTU as well.

5. Dump the solution of amino acid/coupling agent onto the resin taking care to avoid getting the mixture on the sides of the coupling vessel. Mix for *ca.* 15 min. Longer coupling times (1–12 hours) are OK and are recommended as your peptide increases in length.



Scheme 6. Coupling an amino acid, shown using HCTU onto 2-chlorotrityl chloride. This is general to all resin/activating choices.

6. Repeat steps 2–5 until you have added each amino acid in your sequence.
7. After coupling your last amino acid, cleave the final Fmoc group by treating your peptide on

resin with 20% piperidine in DMF.

8. Wash 5X with DMF. Add 5 mL of DMF and bubble nitrogen as you transfer the resin back into a polyprep column. Continue to add DMF to the coupling vessel until all resin is transferred.

   *At this point it is advisable to analyze the success of the synthesis by analytical HPLC and mass spectrometry. We call this "checking the linear." To do this, use the tip of a thin spatula or the tip of a Pasteur pipette to transfer a very small amount of resin to a small glass vial or scintillation vial. Treat the resin with roughly 400 µL of trifluoroacetic acid (TFA) to globally deprotect the peptide. If you have trityl (Trt) protected side chains (Cys, His, Gln, Asn) in your peptide add a single drop of triisopropylsilane (TIPS) to the reaction. Once added, the mixture should change from yellow to clear. This will make the HPLC and mass spec data easier to interpret, as you will not observe Trt-protected side chains. Place the vial in the sonicator for 10 min. After 10 min, filter the reaction through a Pasteur pipette plugged with a small amount of cotton into a new HPLC vial. This separates the peptide from the resin. Dry the eluent by blowing nitrogen or it and dilute the peptide with 500 µL acetonitrile or methanol and take an HPLC and mass spec.

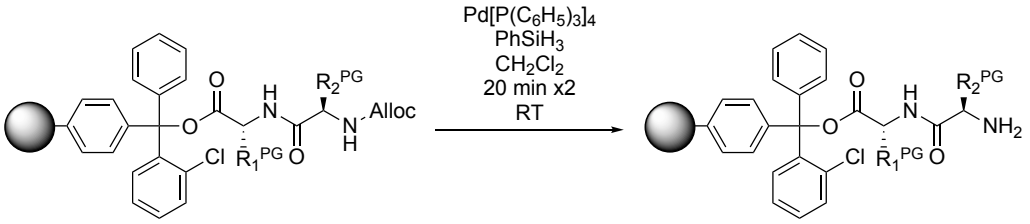
   **Wash the remaining resin in the polyprep 3X with DCM.

9. If you are synthesizing a macrocyclic peptide, proceed to Cleavage of Protected Peptide from 2-chlorotrityl Resin. If you are synthesizing a linear peptide on either 2-chlorotrityl resin or rink amide resin proceed to Cleavage and Deprotection of Linear Peptide.

Alloc deprotection

The purpose of this step is to remove alloc protecting groups during peptide synthesis.

Prep time: 5 min
Reaction time: 40 min

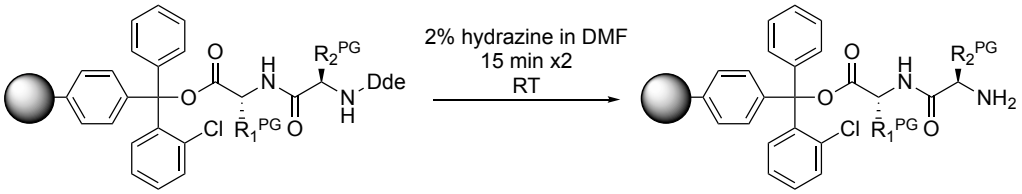


Scheme 7. Alloc deprotection on 2-chlorotrityl resin.

1. Wash the resin in a poly-prep column 3X with CH_2Cl_2.
2. Based on loading, weigh out 0.1 equiv. of tetrakis(triphenylphosphine)palladium(0) and dissolve in CH_2Cl_2 (6 mL).
3. Add 20 equiv. of phenylsilane (density 0.878 g/cm^3) to the resulting solution.
4. Transfer the solution to resin in a poly-prep column.
5. Put it on the rocker for 20 minutes.
6. Repeat steps 1–5.
7. Wash the resin in a poly-prep column 3X with CH_2Cl_2.
8. Check alloc deprotection by cleaving a small portion of the resin with 20% HFIP in CH_2Cl_2 (15 min).
9. Rotovap the cleaved solution and re-dissolve in MeCN.
10. Using the ESI-MS or MALDI-TOF MS, search for the alloc-deprotected mass.

Dde deprotection
The purpose of this step is to remove Dde protecting groups during peptide synthesis.

Prep time: 5 min
Reaction time: 30 min



Scheme 8. Dde deprotection on 2-chlorotrityl resin.

1. Wash the resin in a poly-prep column 3X with DMF.
2. Add 5 mL of 2% hydrazine* in DMF.
3. Put it on the rocker for 15 min.
4. Repeat steps 1–3.
5. Wash the resin in a poly-prep column 3X with DMF.
6. Check Dde deprotection by cleaving a small portion of the resin with 20% HFIP in CH_2Cl_2 (15 min).
7. Rotovap the cleaved solution and re-dissolve in MeCN.
8. Using the ESI-MS or MALDI-TOF MS, search for Dde deprotected mass.

*2% hydrazine in DMF will deprotect Fmoc protecting group, so plan your synthesis before hand.

Automated synthesis – PS3 synthesizer

To use the PS3 synthesizer you must first weigh out 4 equivalents of each amino acid you wish to couple along with 4 equivalents of coupling agent (HCTU or HATU/HOAt) and add the amino acid and coupling agent to amino acid vials. You will also need to book time on the google calendar in advance. Consult with a Nowick lab group member who is in charge of the machine.

Prep time: 1–3 h
Reaction time: variable, typically less than 24 h

1. Transfer your loaded resin to the appropriate reaction vessel, and attach it to the synthesizer. Save the poly-prep column as you can reuse this column when your synthesis is complete.
2. Check the solvent levels and waste levels on the instrument. If the waste is more than halfway full, attach a new waste container.
3. Program your sequence into the PS3. See list of programs taped to wall in the synthesizer room for your options. Generally, use **Program 12** for each amino acid coupling. You must end with **Program 5** in order to remove the final Fmoc group.
4. When your peptide finishes synthesizing, remove the reaction vessel from the synthesizer and transfer the resin to the poly-prep column. If you are making a macrocylic peptide proceed to Cleavage of Protected Peptide from 2-chlorotrityl chloride Resin. If you are synthesizing a linear peptide on either 2-chlorotrityl resin or rink amide resin proceed to Global Deprotection of Acid Labile Protecting Groups.

   *At this point it is advisable to analyze the success of the synthesis by analytical HPLC and mass spectrometry. We call this ”checking the linear.” To do this, use the tip of a thin spatula or the tip of a Pasteur pipette to transfer a very small amount of resin to a small glass vial. Treat the resin with a 400 μL of trifluoroacetic acid (TFA) to globally deprotect

the peptide. If you have trityl (Trt) protected cysteines in your peptide add a single drop of triisopropylsilane (TIPS) to the reaction. This will make the HPLC and mass spec data easier to interpret, as you will not observe Trt-protected cysteines. Place the vial in the sonicator for 20 min. After 10 min, filter the reaction through a Pasteur pipette plugged with a small amount of cotton into a new HPLC vial. This separates the peptide from the resin. Dry the eluent by blowing nitrogen or it and dilute the peptide with 500 µL acetonitrile or methanol and take an HPLC and mass spec.

Automated synthesis – CEM synthesizer

The CEM is not equipped to do "difficult" couplings such as coupling to N-methyl valine, etc. If you have one of these difficult couplings in your sequence, hand couple past it, or use the PS3.

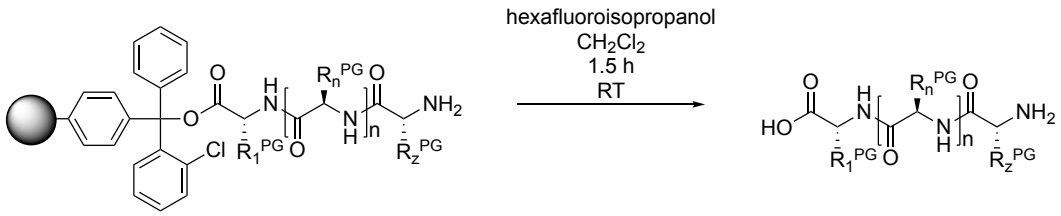
1. Coordinate with the Nowick lab member who is in charge of this machine.

Cleavage from solid support

Cleavage of side chain-protected linear peptide from 2-chlorotrityl chloride resin

The purpose of this step is to cleave the protected peptide from 2-chlorotrityl resin to expose a free carboxy terminus and maintain the protecting groups on the amino acid side chains.

Prep time: 10 min
Reaction time: 1.5 h



Scheme 9. Cleaving a side chain protected peptide form 2-chlorotrityl chloride resin with HFIP.

1. Prepare the cleavage solution. The cleavage solution is 20% hexafluoroisopropanol (HFIP) in CH_2Cl_2. Prepare this fresh each time by adding 3.5 mL HFIP to 11.5 mL CH_2Cl_2 in a small graduated cylinder. Alternatively, 1 squirt of HFIP into 4 squirts of CH_2Cl_2 with a Pasteur pipette.
2. Add about half of the cleavage solution to the resin in the poly-prep column. If you have washed the resin with CH_2Cl_2 before you add the cleavage solution, it will turn red. Rock for 1 h at room temperature, and then drain into a clean round bottom flask.
3. Add the other half of the cleavage solution to the resin and rock for an additional 30 min. After the 30 min is complete, drain into the same round bottom flask.
4. Use the rotovap to evaporate the HFIP and CH_2Cl_2. At this point you may put your peptide on the highvac for extended period of time or proceed to the next step.
5. If you are making a macrocyclic peptide proceed to Macrolactamization if you are making a linear peptide proceed to Global Deprotection of Acid Labile Protecting Groups.

Cleavage and deprotection of linear peptide from Rink Amide resin

The purpose of the step is to cleave the peptide from Rink Amide resin. This will cleave the peptide from the resin, as well as deprotect the side chain protecting groups.

Prep time: 1 h
Reaction time: 1.5 h

1. Wash the completed peptide on resin 3X with CH_2Cl_2, and transfer it into a poly prep column.
2. Dry the resin under a stream on N_2 gas for *ca.* 1 h.
3. Add 10 mL of an 18:1:1 TFA:H_2O:TIPS (prepared by adding 9 mL TFA and 0.5 mL of both H_2O and TIPS) to the resin. Let rock for 1–1.5 hours.
4. Collect solution in a 250-mL round bottom flask.
5. If we have a functioning acid rotovap, evaporate the TFA on this rotovap. If we do not have a functioning acid rotovap, evaporate the TFA under nitrogen or proceed to ether precipitation.

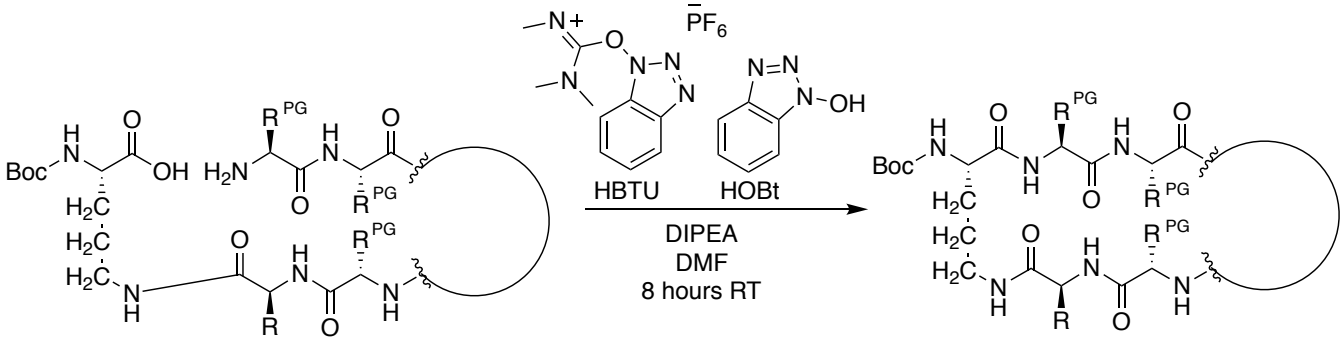
Preparing cyclic peptides

These steps pick up form cleaving the linear-protected peptide form 2-chlorotrityl chloride resin.

Cyclization

The purpose of this step is to covalently link the N- and C-termini of the peptide to create a macrolactam (aka a cyclic peptide, macrocycle, etc.).

Prep time: 5–30 min
Reaction time: 1 day to whenever you get to it



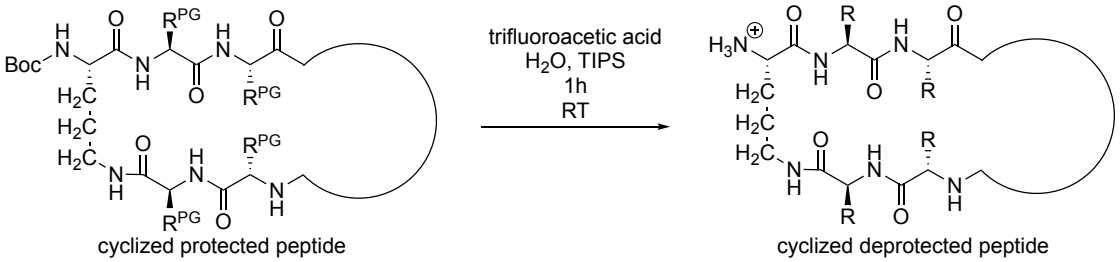
Scheme 10. Cyclizing a peptide with HBTU/HOBt and DIPEA in DMF. NMM can be used or DIPEA.

1. Add DMF from the solvent system to the round bottom flask that contains the linear peptide. *ca.* 125 mL.
2. Add 6 equiv. HBTU (typically 300 mg), 6 equiv. HOBt (typically 150 mg), and 300 μL of N-methylmorpholine (NMM) or DIPEA and let stir at RT under N_2 for at least 24 h, but typically 48 h.
3. Use the rotovap to evaporate the DMF. You will likely have to heat the DMF to 55-70 ºC to achieve a moderate rate of evaporation.
4. After the DMF has evaporated, attach the round bottom flask to the highvac to further dry the peptide. The more DMF you evaporate, the better. Once your peptide is dry, proceed to Global Deprotection of Acid Labile Protecting Groups.

Global deprotection of acid labile protecting groups

The purpose of this step is to remove the acid-labile protecting groups to expose the amino acid side chains. Common acid labile protecting groups we use include: trityl (Trt), O-tert-butyl (OtBu), tert-butyloxycarbonyl (Boc), and pentamethyldihydrobezofuran-5-sulfonyl (Pbf). This step will not remove acetamidomethyl (Acm) from cysteine, alloc protecting group, or Dde protecting groups.

Prep time: 5 min
Reaction time: 1–1.5 h



Scheme 11. Global deprotection of a cyclized peptide. Note this is identical to cleaving a peptide from Rink Amide resin.

1. Remove the round bottom flask from the highvac.
2. Prepare the deprotection solution (aka the deprotection cocktail) by combining 18 ml TFA, 1 mL TIPS, and 1 mL H_2O to the dried cyclized peptide.
    - *If your peptide contains a methionine residue, you can add ~250 mg of ammonium iodide to prevent oxidation of the methionine during the deprotection reaction.*
3. Dump the deprotection solution into the round bottom flask that contains your peptide and stir for 1 h at room temperature under N_2 gas.
4. After 1 h, remove the stir bar. If an acid rotovap is available, use it to evaporate as much TFA as possible. Alternatively, the TFA can be dried under nitrogen or the the peptide can be precipitated out through ether precipitation.
5. Once the TFA is removed, the peptide can be dissolved in a small percentage of acetonitrile (typically 20% CAN in H_2O) and subjected to reverse phase HPLC for purification.

Subsequent chemistries on ”isolated” peptides

This section details a number of reactions you might be interested in doing on a purified peptide, such as disulfide bond formation, or removing TFA esters.

Removing TFA esters with MeOH reflux

The purpose of this step is to remove TFA esters of serine or threonine that may form during global deprotection. TFA esters are seen in mass spec after global deprotection with M+96 species. You ***cannot*** *do this if your peptide contains Q, N, E, D, or a C-terminal amide/acid, as you will trans-esterify with the methanol.*

Prep time: 5–10 min
Reaction time: 0.5–1 h

1. After removing the TFA from a global deprotection, dissolve your peptide in *ca.* 50 mL of

methanol.
2. Equip the round bottom flask with a reflux condenser attached to a 2 gallon water re-circulator. Reflux for 30 min to 1 h.
3. Remove the methanol with a rotovap and proceed to purification.

Ether precipitation

The purpose of this step is to "clean up" your peptide for further chemistry or for removing your peptide from TFA. Right now, the most common chemistry that we do after global deprotection is formation of disulfide bonds, but ether precipitation can be used for any post-deprotection chemistry.

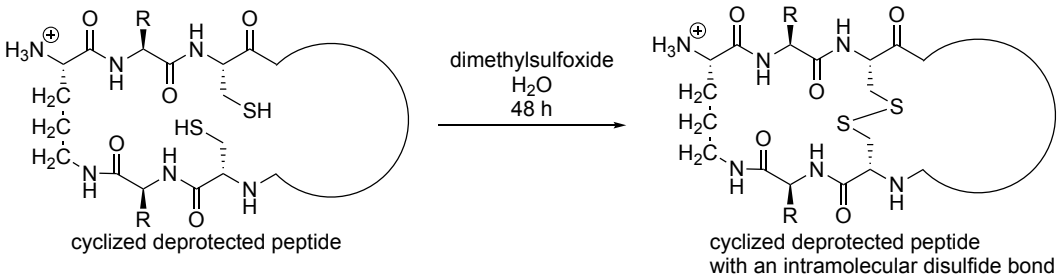
Prep time: 5 min
Reaction time: 20 min

1. Obtain *ca.* 100 mL of ether from the solvent system and cool for 15 min in an ice bath.
2. Aliquot equal volumes (10 mL) of the TFA/peptide to two 50 mL conical tubes.
3. Dump 15 mL of ice-cold ether into each conical. You should see precipitate immediately. Incubate in the ice bath for an additional 5–15 min.
4. Pellet the precipitated peptide by centrifugation. The pellet is your peptide.
5. Decant the ether supernatant into another vessel. Do not discard until you have confirmed that your peptide is in the pellet.
6. Transfer each pellet to a round bottom flask by adding acetonitrile to each pellet. Evaporate on a rotovap then highvac. It is critical to get evaporate ALL of the ether. *Alternatively, the pellet can be dried under nitrogen for 1 h or placed in a vacuum desiccator to dry.*
7. Once all of the ether has evaporated proceed to Purification with RP-HPLC or Oxidation to Form a Disulfide.

Oxidation to form an intramolecular disulfide bond

The purpose of this step is to oxidize two cysteines to form an intramolecular disulfide bond. There are two main methods we use to form disulfide bonds: ***DMSO Oxidation*** *and* ***Acm deprotection****. Only use the Acm deprotection method if your peptide contains Acm protected cysteines. For peptides that contain free thiols on deprotected cysteines, use the DMSO oxidation method.*

DMSO oxidation
Prep time: 5 mins
Reaction time: 48 hours



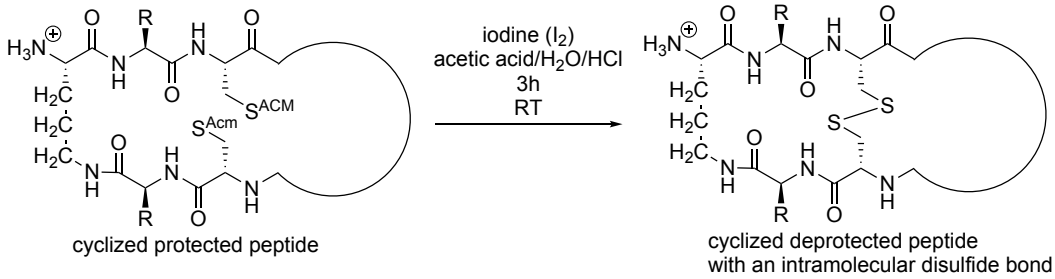
Scheme 12. Formation of a disulfide bond with DMSO.

1. Prepare 100 mL of 10% aq. DMSO.
2. Dump the 10% aq. DMSO into the round bottom flask that contains your peptide and stir for at

least 48 h. Monitor the reaction by analytical HPLC. *Note: if your peptide does not go into solution the reaction will likely not proceed. You may try heating the reaction to 50 °C, or diluting the reaction with H_2O. You can also try to add acetonitrile to reaction mixture.*

3. Confirm the reaction has gone to completion using mass spec. The isotope pattern of your completely oxidized peptide should be the same as your unoxidized peptide. If it is not, then your reaction is not complete.
4. Evaporate all of the H_2O using the rotovap. This will yield a 10 mL puddle of DMSO.
5. To separate your peptide from the DMSO you must use the Biotage or the old C4 column. We try to avoid injecting DMSO on our C18 columns.
6. Inject at 5% MeCN and wait for ALL of the DMSO to come out.
7. Increase the MeCN percentage to 50%.
8. Collect everything peptidic that comes off the Biotage column or the C4 column into a clean round bottom flask and concentrate to 5–10 mL using rotovap.
9. Change the column on the semi-prep HPLC back to the C18 column and do another purification according to the procedure detailed in Purification Using Reverse-Phase HPLC.

ACM deprotection and disulfide bond formation
Prep time: 1 h
Reaction time: 3 h



Scheme 13. Formation of disulfide bond coupled to Acm deprotection.

1. Prepare 0.1 M iodine (I_2) by dissolving 380 mg of I_2 in 15 mL glacial acetic acid (100% acetic acid). Sonicate for at least 30 min. All of the I_2 may not go into solution.
2. Prepare 250 mL 50% aq. acetic by adding 125 mL of glacial acetic acid to 125 mL H_2O.
3. Prepare 25 mL 1 M HCl by adding 2 mL 12 M HCl (conc. HCl) to 23 mL H_2O.
4. Dissolve the peptide in 250 mL 50% aq. acetic acid, and 25 mL 1 M HCl.

Obtain an analytical HPLC trace before adding the I_2.

5. Add 5 mL of 0.1 M I_2, and stir under N_2 for at least 3 h. Monitor your reaction every hour by analytical HPLC.
6. After 3 h, take a mass spec to ensure that your reaction has gone to completion.
7. Quench the unreacted I_2 by adding 1 M sodium thiosulfate drop-wise until the reaction turns clear.
8. Use the rotovap to remove ALL of the acetic acid/H_2O/HCl.
9. Re-dissolve whatever is on the side of your flask in 2 mL 100% TFA and perform an ether precipitation according to the protocol above. The ether precipitation removes most of the I_2.
10. Dissolve the peptide pellets in an appropriate amount of MeCN/H_2O and proceed to Purification Using Reverse-Phase HPLC.

Intermolecular Oxidation to form Trimer from Monomer
The purpose of this step is to oxidize monomeric cyclic peptide into dimer/trimer.
.
DMSO Oxidation:
Prep time: ~ 5 minutes
Reaction time: ~ 48 hours

1. Weigh out monomeric peptide TFA salt. Typically, more successful oxidations require 15-60 mg of peptide.
2. Once the weight of the available peptide is determined, use the available calculator in Ryan's excel spreadsheet to determine the volume for a 6 mM solution of peptide.
3. Once the total volume is determined, calculate 20% of the total volume. This will be made up of DMSO and the remaining 80% is made up of HPLC grade H_2O (pH 7.8). *The pH of the water source has proved to be a relevant factor in this reaction. If errors occur, check the water source pH.*
4. Dissolve your peptide in the calculated volume of HPLC grade H_2O and transfer the solution to a scintillation vial.
5. Add the calculated amount of DMSO to the scintillation vial.
6. Allow the reaction to shake for 48 h and monitor by ion mobility mass spectrometry.
7. After 48 h, purify by RP-HPLC.

Purification of peptides

Reverse-phase HPLC purification on Rainin semi-preparative HPLC

The purpose of this section is to describe the steps involved with purifying a peptide using RP-HPLC. It is not meant to be a definitive guide on how to purify YOUR peptide. You will likely need to experiment to figure out what gradient works best for you. Purifying peptides by RP-HPLC will take lots of practice. Adopt the following to suit your needs

1. Dissolve the deprotected peptide in the round bottom flask in a small amount of MeCN (2 mL) and transfer to a 15-mL conical tube. Wash the round bottom flask with *ca.* 3 mL H_2O to collect the residual peptide in the round bottom flask and transfer to the 15-mL conical. The volume transferred to the 15-mL conical should not exceed 10 mL.
2. To remove particulates, centrifuge the 15-mL conical at 4,000 rpm for 5 min.
3. Pass through a 0.2-µm syringe filter into a new 15-mL falcon tube. Your peptide is now ready to inject on to the preparative RP-HPLC column. *Before you inject, it is advisable to obtain an analytical HPLC trace to gauge what percentage of MeCN your peptide will come out.*
4. Be sure to equilibrate the system to the same percentage of acetonitrile or higher than what your peptide is dissolved in. Injecting a peptide into a lower percentage of acetonitrile will cause it to precipitate out of solution, creating high pressure and potentially clogging the column.

General operation of the Rainin semi-preparative RP-HPLCs

We have two Rainin prep HPLCs in the lab. The general procedure for using each instrument is the same. This is just a rough procedure. Purifying peptides by RP-HPLC will take lots of practice. Adopt the following to suit your needs

1. Make sure that the solvent bottles are full.
2. Turn on lamp and flow (10 mL/min if pressure allows. Pressure should not exceed 2.00 kpsi)

on the HPLC. The absorbance should be set to 214 nm.
3. Wash the column at 95% MeCN for 5 min.
4. Go to 20% MeCN over 2 min at 10mL/min.
5. Inject the peptide while monitoring the pressure and allow the peptide to load onto the column for 10 min. An injection peak should be observed within 10 minutes.
6. Go to 50% MeCN over 55 min at 10 mL/min, or other gradient relevant to your peptide.
7. Collect fractions. Typically count 5 mL for each fraction.
8. After your peptide comes out, wash the column with 95% MeCN for at least 15 min. Then return the MeCN percentage to 50% and run for 10 minutes.
9. The column is stored at 50% MeCN.
10. Turn off the flow by pressing stop on both pumps and turn off the UV lamp.

Reverse-phase HPLC purification on Biotage Preparative HPLC

1. Peptides purified via the Biotage system should be dissolved in a minimal amount of acetonitrile. Cloudy solutions are tolerable on this system and do not need to be filtered.
2. Select "Chemistry". Here you may build a new method or select and existing method. *To select an existing method, go to open->select your user name-> find the method and hit open. Make sure to check each tab is correct and then you can proceed by selecting "run".*
3. If you are building a new method, you will first be asked to determine your gradient. Here you select the percentage of acetonitrile the column should equilibrate at (prior to adding sample), typically 10%, and the number of column volumes that will be used for equilibration, typically 2-3 CV. Secondly, you will need to select the elution gradient. Typically, this is set for the system to run at 10% for 2-3 CV where you will observe coupling agents elute. This is followed by a ramp from 10-60% over 8-14 CV depending on the peptide. Finally, a gradient of 60-100% over 0 CV, a flush at 100% for 2 CV, and a re-equilibration at 50%, by going 100-50% over 0 CV, and 50% for 1 CV.
4. The second tab at the top will request information about the peptide and collection process. Here, enter your user name, the method title, and the sample title. The trays used are 16x130 and will elute in a snake-like fashion. The maximum volume for each elution can be set to 15 mL or lower if you prefer. *You will have the ability to manually change the size of fractions as elution occurs.*
5. Finally, the third tab at the top of the screen is information regarding the detector. This system allows two separate wavelengths to be observed, as well as the 200-400 nm combined trace. The wave lengths are set to 214 nm and 280 nm.
6. You can select the "collect all" button or set up a threshold minimum for collection. A minimum at 50 is sufficient to collect anything of relevant absorbance.
7. Proceed to General Operation instructions.

General operation of the Biotage Preparative RP-HPLC

1. Make sure that the solvent bottles are full.
2. Detach existing column and attach your assigned column by removing the plugs on either end of the column and attaching the solvent lines. *It is important to monitor this during the equilibration process to ensure there are no leaks.*
3. Make sure the column attached matches in the information entered into the second tab. This is important as the flow rate is automatically assigned based on the column attached.
4. Once your method is prepared, select run at the bottom right of the screen. Equilibration will begin.
5. Make sure a gray tray filled with vials is loaded into the collection area.
6. Following equilibration, a button will appear at the bottom right called "load sample". Select load sample and use a 5 mL luer lock syringe (without needle) to transfer your peptide onto the column.

Remove the top solvent line from the column, attach the syringe with 5 mL solution and 1 mL air, and slowing compress the plunger until all peptide has entered the column.

7. Hold the plunger down for 20 seconds to prevent back pressure from pushing your sample back out of the column.
8. Repeat this for up to 15 mL of solution.
9. Once all peptide is loaded, select complete in the dialogue box. You are now ready to select "start gradient". Again, monitor for leaks.
10. The system has a few available buttons that are useful while running.
    - In the bottom left, the "new fraction" feature allows you to collect smaller fractions while important peaks are eluting.
    - The "pause" feature is useful you need to stop the system for any reason. This is useful if you need to load a second tray of vials or notice a leak in your column.
    - The "bypass tray" feature is great once you know you have collected your peaks of interest. You can send the remaining flow straight to waste.
    - A "hold" button is available if you want to run isochratically.
    - The "zoom" and "full" button allow you to zoom in and out on the live trace to analyze your peaks.
11. Once the run is complete, continue to checking fractions. Fractions eluting from the Biotage are significantly more concentrated than those from the Rainin. When running HPLC or mass spec, dilute by adding 3 drops of fraction: 200 μL acetonitrile.

Checking fractions, and lyophilizing

1. To assess the purity of your fractions check them on the analytical HPLC and mass spec (MADLI or direct injection QDA). If there are impurities, an additional preparative HPLC is necessary.
2. Combine the pure fractions and take a "combined HPLC" and a "combined mass spec". It is recommended to save this data now, as opposed to having to recollect it for second-year report, orals, or publication.
3. Evaporate the MeCN/H_2O using a rotovap (if your peptide bumps, isopropanol may be added) *may not be advisable if your planning on doing serious NMR, as some students have noticed IPA sticking around*.
4. Transfer the remaining small puddle to a conical tube and wash the flask with H_2O to ensure all the peptide is transferred.
5. Freeze your peptide in dry ice and then transfer the peptide to a lyophilization vessel. Detach the lid of the 15-mL conical, and use a kimwipe and rubber band to cover the top of the 15-mL conical.
6. Attach the lyophilization vessel to the lyophilizer. 12–48 hours later you will have a dry powder of your peptide as a TFA salt.