Your final project in this laboratory is to make a tripeptide. This is a multistep synthetic sequence that will build on your previous training in organic chemistry laboratory and lectures.

Thinking back to general biology, you will recall that amino acids serve as the building blocks for life. Understanding amino acid structure and function is pivotal for the construction of vaccines and other pharmaceuticals, both of which are typically composed of a peptide backbone. The defining feature of an amino acid is a central carbon atom that is covalently bound to an amino group, a carboxyl group, a hydrogen atom, and a R-group (Figure 1). It is also important to note that stereochemistry plays an important role in peptide chain synthesis. You should take note that the central carbon atom present in all amino acids, with the exception of glycine, is chiral. However, the nomenclature used to describe biological species is different than that used to describe non-biological organic compounds. You will remember from Organic I that the terms Rectus, (R-), and Sinister, (S-), were used to denote how a molecule rotates plane polarized light, where Rectus signifies clockwise rotation and Sinister signifies counter-clockwise rotation. In biological systems, the terms Dexter, (D-), and Laevus, (L-), are used, in replace of Rectus and Sinister, to represent a molecules chirality (Figure 2).

Biological systems exist in a chiral environment, and all naturally occurring amino acids exist as the L- isomer. The chirality of a molecule dramatically impacts its biological function. From a pharmaceutical standpoint, a drug’s spatial configuration can be the difference between a cough remedy or a potentially lethal chemical. With that in mind, chemists and pharmacists alike always seek to create enantiopure compounds. In the synthesis of peptides, this desire for enantiopurity means that we must preserve the stereochemistry at each alpha-carbon as we add new peptide bonds.

To help peptide bonds form under mild conditions, we can use carbodiimide for carboxylic acid activation. The function of a carbodiimine is to activate the carboxyl terminus of an amino acid so that it may more readily undergo nucleophilic attack from the free amino terminus of a second amino acid. This carboxylic activation will dramatically preserve the amino acids stereochemistry while also greatly increasing the overall yield of your peptide. Carboxyl activation has proven to be essential for traditional liquid-phase peptide synthesis. Without the use of an activator, the desired reaction will be sluggish and low yielding. This is attributed to the fact that once the peptide bond is formed, water is simultaneously produced, and has the ability to hydrolyze the amide bond and reverse the reaction (Scheme 1).

Directing your attention again to Scheme 1, you will see that the amino terminus of one amino acid and the carboxyl terminus of the second amino acid are bound to “PG,” or a protecting group. Protecting groups are used to deactivate one side of an amino acid in order to provide selective coupling. These
Day 1 - Boc Protection Of Primary Amino Acid:

Reference: Latli, Bachir; Hrapchak, Matt; Xu, Yibo; Qui, Fenghe; Krishnamurthy, Dhileepkumar; and Senanayake, Chris. J. Label Compd. Radiopharm 2011, 54, 799-808.

Procedure:

Take a 50 mL vial and charge it with 1.00 g of the amino acid assigned to you by your TA. Dissolve the amino acid in 40.0 mL of methanol, add 2 equivalents of triethylamine, and let the contents of your flask stir for 5 minutes. It would be a good idea to begin heating your sand bath at this time. Assemble a reflux apparatus and turn on your chiller. After 5 minutes has passed, add 1.1 equivalents of Boc₂O to your round-bottom flask via a syringe and bring the reaction mixture to reflux. Allow the mixture to stir, uninterrupted for 30 minutes. While your reaction is under reflux, run a TLC on your starting material to find a general solvent system using hexanes and ethyl acetate. Once your solvent system has been determined, run TLCs on your reaction mixture to observe starting material transformation. Record these Rf's.

At the end of the 30 minutes remove your reaction flask from heat and allow it to cool slightly. Rotavap off your solvent and then perform an extraction by redissolving your reaction mixture in ~ 20 mL of EtOAc and washing with water (3x). Your desired product will be found in the organic layer. Dry this layer with MgSO₄, remove the solids via vacuum filtration, and rotavap down the mother liquor in order to obtain a crude yield.

Day 2: Synthesis Of The Dipeptide:

Your TA will give you a second amino acid that is already protected on the carboxyl terminus. The molecular weight of this amino acid methyl ester is provided above. When you are determining your molar equivalents, you are going to use your Boc protected amino acid as your limiting reagent. DCC
(MW = 206.33 g/Mol) N,N'-Dicyclohexylcarbodiimide and is serving as your carboxylic acid activator. HOBt (1-hydroxybenzotriazole, MW = 135.15 g/mol) is another activator.

Charge the methyl ester (1 equivalent), carbodiimide (1.1 equivalents), HOBt (1.1 equivalents) and diisopropylethylamine (3 equivalents) to the round bottom flask containing your Boc protected amino acid (1 equivalent). Add 30 mL of DMF to your round bottom and shake the contents of the flask vigorously until all reagents are fully dissolved. It is important that you use a glass septum for this step. Once fully dissolved, place your stoppered, round bottom flask in your drawers and allow them to sit for approximately 48 hours.

Day 3: Dipeptide Purification

HOBt is a beneficial peptide coupling agent because it creates water-soluble byproducts. You will be able to isolate the dipeptide and any unreacted amino acid starting material from DMF by performing an extraction. However, unreacted DCC can only be removed through flash purification, but it’s byproducts can be filtered off. You may notice undissolved crystals in your round bottom when you return for today’s experiment. This is a byproduct of DCC and can be removed by vacuum filtration. If this is the case, simply perform a vacuum filtration and continue on to the next step using only the mother liquor. If no vacuum filtration was required then proceed using your reaction mixture. Using 75 mL of water, quench the reaction mixture and wash with 25 mL of EtOAc. Place the organic layer in a flask and wash the aqueous layer two more times using an additional 25 mL of EtOAc each time. Combine all three portions of EtOAc into your separatory funnel and perform a final rinsing using an additional 75 mL of clean water.

With your peptide now suspended in EtOAc, perform TLC analysis to find an appropriate solvent system for flash purification using hexanes and EtOAc. Start with a 1:1 mixture (10 mL total) and observe where your spots travel. Remember to co-spot your TLC plate with your Boc protected amino acid and methylated amino acid. Adjust your solvent system accordingly by increasing or decreasing your EtOAc concentration. Once the appropriate solvent system has been determined, rotavap off the EtOAc and leave your reaction in your drawer until the next class.

Day 4: Dipeptide Purification Continued

Dissolve the peptide in the recently determined solvent system (remember to use as little solvent as possible for this step, between 1.0 and 2.0 mL). If sample solubility becomes an issue, talk to your TA for troubleshooting. Once fully dissolved, load your sample into a 10g flash column and isolate your desired spot.

Combine all of the test tubes that contain your isolated dipeptide into a dry, preweighed round bottom flask and rotavap off the solvent. Allow your sample to dry in your drawer for 48 hours.

Day 5: Dipeptide Deprotection

Obtain a weight of your purified dipeptide. In order to add a third amino acid to your peptide you must remove one of the protecting groups on either terminus. For today, you will remove the protecting group located on the amino terminus end, Boc. This is very simple, and straightforward, and it also has the benefit of creating easily removed side products, CO₂ and ethylene. You will first dissolve your amino acid in 10 mL of methanol; this is done to insure the protecting group on the carboxyl terminus is not also removed in the process. After this is done, make a 3 M solution of HCl from concentrated HCl (12.00 M). This part will require a little bit of extra thinking because we need to account for the 10 mL of methanol already present. In order to remove Boc from a peptide in this quantity, we will need a 30 mL solution. Add 7.5 mL of concentrated HCl and 32.5 mL of EtOAc to a beaker. Pour the contents of your beaker into your reaction flask slowly and allow the mixture to stir for approximately 30 minutes. Follow
Day 6: Tripeptide Synthesis

After the reaction has been allowed to go to completion a third amino acid is now ready to be added to your peptide chain. Follow the same procedure utilized on day 1 for synthesizing the dipeptide. Fill out the following chart in your notebook. Once again, your TA will provide you with a Boc protected amino acid once you are ready to synthesize the tripeptide.

Day 7: Tripeptide Purification

Perform an extraction using the same procedure described on day 2. After you have collected your organic layer, perform TLC analysis to find the spot corresponding to your tripeptide. If you cannot achieve an adequate Rf using hexanes and EtOAc, then methanol and chloroform will be provided for you. Should this need arise, the TA will instruct you on how to properly make solvent systems using these solvents. Once you have obtained an appropriate solvent system, show your TA your TLC plate and, if approved, perform the final flash chromatography of the year. Collect all test tubes pertaining to your product spot, combine them into a 100 mL round bottom flask, and rotavap off the solvent. Leave your product in your drawers until the next class.

Day 8: Purification Assessment

After allowing your sample to dry, take a weight of your round bottom flask and determine your percent yield. Make an NMR sample using deuterated DMSO and give your NMR tube to your TA. Your TA will send out emails when NMR samples become available for your analysis.

Notes To Students:

This lab is your final project. Your TA will be watching your techniques closely. You ARE allowed to ask questions, but please understand that you are expected to do some of your own troubleshooting before asking your TA for step by step analysis.

Like all labs, the only document allowed in this lab will be your lab notebook.

At the conclusion of this final project, you will need to write a formal lab report in a journal style format. Guidelines for this lab report will be distributed in a separate document.

Techniques To Be Familiar With:

- Extractions
- Flash Column
- Rotavaps
- TLC (both methodology and theory)

** The above techniques are suggested but do not necessarily reflect all information you will be responsible for. The general rule of thumb to follow should be, if you’re unsure, then write it down.

NOTE RE: DAY ASSIGNMENT: I have divided up these experiments into 8 separate days. There are 7 days allotted for this lab. It is your responsibility to work efficiently and proceed at least as quickly as the suggested time table. If you feel like you can proceed to the next step of the experiment, then you should feel free to do so in consultation with your TA. According to the day assignments listed here, you will be responsible for preparing an NMR sample during an additional time that will be arranged with your TA. If we can avoid that scenario, that would be preferable.