Through your organic chemistry tenure you have been taught the underlying principles necessary to construct simple organic molecules in the laboratory. All of the laboratory work you have completed thus far this semester will culminate in today’s experiment. In this lab you will see how the different branches of science can overlap as you strive to make one of life’s most important structures, a peptide.

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 often composed of a peptide backbone. The general features 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; and with the exception of glycine, R is defined as any organic 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 represent a molecules chirality, where Rectus signifies clockwise rotation and Sinister signifies counterclockwise rotation. In biological systems, the terms Dexter, (D-), and Laevus, (L-) are used, in replace of Rectus and Sinister, to denote the chirality of an amino acid by describing how a molecule rotates plane polarized light (Figure 2).

Biological systems exist in a chiral environment, and all naturally occurring amino acids exist as the L- isomer. Diasteriomers are very much different molecules despite their similar appearance on paper. How a molecule is oriented in space dramatically affects that molecules function. Speaking 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. Naturally occurring amino acids are chiral, and when coupled together to form peptides (small proteins), this chirality must be preserved. One methodology used to accomplish this feat is to use carbodiimine coupling agents. 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 preserves the amino acid’s stereochemistry while also greatly increasing the overall yield of the peptide product.
can be formed from acid chlorides and amines. Unfortunately, the process of forming an acid chloride from a chiral carboxylic acid often results in a loss of enantiopurity. Consequently, carbodiimides are the most commonly used reagent for forming peptide bonds, though there are large current research efforts directed towards the development of “greener” technologies (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 protecting groups are usually easily removed in acid/base workups in order to generate even longer peptide chains once the primary coupling has completed. It should be easy to envision the concept of the amino protecting group being removed from your dipeptide so that it may attack an activated carboxylic terminus of another amino acid. This would in essence create a tripeptide. In the reaction above we see the amino terminus side of valine joining the carboxylic terminus of alanine. This creates the alanylvaline dipeptide. Without protecting groups, the likelihood of generating undesired byproducts increasingly grows. For example, the amino terminus of alanine has an equal probability of attacking the carboxyl terminus of valine, creating the valylalanine dipeptide, if protecting groups are not utilized.

Day 1 - Acyl Protection Of Phenylalanine:

![Reaction Diagram]


Data Table: Have a copy of this in your notebook prior to the beginning of lab. It is your responsibility to find the molecular weights and densities, if needed, before you arrive.

<table>
<thead>
<tr>
<th></th>
<th>Phenylalanine</th>
<th>Acetic Anhydride</th>
<th>2 N NaOH</th>
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<tbody>
<tr>
<td>Molecular Weight</td>
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<tr>
<td>Density</td>
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<tr>
<td>Amount</td>
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<td>Mmmol</td>
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<tr>
<td>Mole Ratio</td>
<td>1</td>
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Procedure:

Obtain a 20 mL vial and a screw cap. Charge it with 1.00 g of phenylalanine, and 10.00 mL of 1.00 N NaOH. Shake the contents of your vial until phenylalanine is fully dissolved; then add 1.00 mL of acetic anhydride. Vigorously shake your vial periodically for 30 minutes. You do not need to shake it continuously for 30 minutes. During this time you will observe a sudden
increase in the solutions temperature. At the end of the 30 minutes, you will need to acidify your solution. Test your vial with pH paper using a glass TLC pipette and acidify with 6 N hydrochloric acid until you achieve a pH between 1 and 2. Be sure to do this very carefully and to shake your vial between HCl additions to ensure proper mixing.

Once you have achieved the desired pH, you will notice crystals beginning to crash out of your solution. Pour the contents of you vial into a separatory funnel and add an additional 15 mL of water; use the additional water to properly rinse out your vial. Extract your product into EtOAc by washing the aqueous solution 3x’s with 20 mL portions of EtOAc. Combine all 3 organic layers into an Erlenmeyer flask and, using magnesium sulfate, dry the EtOAc. Collect the mother liquor by vacuum filtering off your magnesium sulfate and pour it into a preweighed round bottom flask. Remove the solvent via rotary evaporation and obtain a percent yield. Place a small amount of crystals into a vial to be used in the future for TLC analysis.

Synthesis Of The Dipeptide:

\[
\begin{align*}
\text{Isoleucine} & : & \text{181.66 g/Mol} \\
\text{Alanine} & : & \text{139.58 g/Mol} \\
\text{Valine} & : & \text{167.64 g/Mol} \\
\text{Phenylalanine} & : & \text{215.68 g/Mol}
\end{align*}
\]

Your TA will give you a second amino acid that is already protected on the carboxyl terminus; this is the “R-OMe” section of your data table. The molecular weight of this amino acid is provided above. The molecular weight corresponds to the hydrochloride salt derivative of the protected amino acid. When you are determining your molar equivalents, you are going to assume you achieved a 100% yield of your Ac-amino acid, and it will serve as your limiting reagent. DCC (MW = 206.33 g/mol), N,N’-dicyclohexylcarbodiimide, is serving as your carboxylic acid activator. HOBt (1-hydroxybenzotriazole, MW = 135.15) is another activator that often increases the yields of peptide coupling reactions.

<table>
<thead>
<tr>
<th></th>
<th>Ac-Phe</th>
<th>R-OMe</th>
<th>DCC</th>
<th>HOBt</th>
<th>Diisopropylethylamine</th>
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<tbody>
<tr>
<td>MW</td>
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<tr>
<td>Mole Ratio</td>
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<td>1</td>
<td>1.1</td>
<td>1.1</td>
<td>3</td>
</tr>
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</table>

Add the methyl ester, carbodiimine, HOBt and base to the round bottom flask containing your Acetyl protected amino acid. 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 stopper for this step; rubber septa have a tendency to contaminate reactions with
phthalates (plasticizers that often leach out of synthetic materials). Once fully dissolved, place your stoppered, round bottom flask in your drawers and allow them to sit for approximately 48 hours.

**Day 2 – Dipeptide Purification**

After the reaction is complete a small amount of unreacted DCC will still be present in your reaction along with its urea byproduct. If you observe any undissolved material in your round bottom, this is the DCC byproduct. Perform a quick vacuum filtration to remove the solids before using the mother liquor to continue on to the next step. You will be able to isolate the dipeptide and any unreacted amino acid starting material from DMF by performing an extraction. Pour the mother liquor (if filtered, or just the reaction mixture, if no filtered) into a separatory funnel. 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 dissolved in the EtOAc, perform TLC analysis to find an appropriate solvent system for flash purification using hexanes and EtOAc. You should observe 3 or 4 spots and it will be up to you to determine which spot corresponds to your peptide. Start with a 1:1 mixture (10 mL total) and observe where your spots travel. Remember to co-spot your TLC plate with your acylated amino acid, methylated amino acid, DCC, HOBT and your extracted product. Adjust your solvent system accordingly by increasing or decreasing your EtOAc concentration while remembering to keep the overall volume of the solvent system at 10 mL (i.e. if you decrease EtOAc by 1.0 mL be sure to add an additional 1.0 mL of hexanes). Once the appropriate solvent system has been determined, show your TA your TLC plate so he/she may confirm.

Transfer your solution into a pre-weighed round bottom and rotavap off the EtOAc and record the mass of the dry product. Scrape out approximately 300 mg of product from your round bottom into a glass vial; if your product does not exist in crystal form, do not worry. If your product is an oil, complete the following: place an empty vial on the scale and zero it, pipet the oil from your round bottom flask into the vial until you achieve a weight of 300 mg. If you overshoot this weight, do not try and remove oil from your vial, just record the weight and continue on to the next step.

Once you have a vial containing 300 mg of your crude product, dissolve the product in your recently determined solvent system (remember to use as little solvent as possible for this step, between 1.0 and 2.0 mL). Load your sample into your flash column (remembering to saturate the column in your solvent system prior to sample loading) and isolate your desired spot. Should you have issues with getting your peptide to dissolve in your solvent system, you may add an additional mL of EtOAc to your vial. If all of your peptide is unable to be dissolved this is okay. Try holding your vial in the heated rotavap bath, shaking your vial gently to coax your product into solution. If this does not work, simply charge your column with whatever amount of peptide was able to dissolve, leaving behind any non-dissolved material. Your grade for this experiment will be based more on purity than yield.
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 3: Purification Assessment

After allowing your sample to dry, weigh your round bottom flask and determine your percent recovery (from the 300 mg used to run your column). Make an NMR sample using deuterated DMSO and give the NMR tube to your TA. Your TA email when your NMR sample becomes available for your analysis.

** The above itinerary is an idealized situation. We realize that time may be an issue, which is why this lab has been allotted 4 days. If at any point you feel as though you will not have enough time to complete the next step please STOP and continue where you left off at your next laboratory meeting.

Notes To Students:

This lab is serving as a final, and as a result you will NOT be permitted to pose questions to your TA or your peers during the experiment. Your TA will be allowed to answer any question about the lab up until 4:00 PM the day before your final begins. This means that if any e-mail or message is received with a timestamp of 4:00 PM or later the TA will NOT respond. TAs outside of the CHM 226 lab around the department will also be instructed that they are not allowed to answer any questions for this specific lab. If you feel the necessity to ask a question during the experiment that is contingent on you progressing, the TA may answer but will assess a 10% penalty. As with the midterm, laboratory technique and preparedness are essential in organic chemistry. If for some reason you need to restart the experiment as a result of procedural or careless error you will be permitted to, however, a -50% penalty will be imposed.

You will be allowed to write down any notes regarding procedures for conducting techniques, i.e. you may write a step-by-step procedure for how to run a flash column. However, you will NOT be permitted to bring any handout into lab. All procedures must be written in your laboratory notebook and approved by your TA prior to the beginning of the experiment.

Techniques To Be Familiar With:
- Extractions
- Flash Column
- Rotavaps
- TLC (both methodology and theory)
- Testing pH

** 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.