D3 Lab II: The Combinatorial Synthesis of Acylated Natural Amino Acids: SC2

William L. Scott, Chris Audu, Jordi Alsina, Linda Cook, Eric D. Crockett, Douglas L. Gernert, Lawrence A. Goodwin, Vidyadhar K. Jadhav, Waiping Kam, Jacek Martynow, Judi Smith, Grant Vaught, Timothy J. Weisel, Kirk Wickizer, Eric Woerly, Ziniu Zhou, and Martin J. O'Donnell, IUPUI Department of Chemistry and †Lilly Research Laboratories

Introduction

Goal: Education with a Real-World Application

We have found that undergraduates are eager to learn and apply skills involving practical problems. This lab will show that you are capable of learning and carrying out multiple step combinatorial organic syntheses to create and characterize new potential drug leads.

A. Combinatorial Chemistry

Combinatorial chemistry is an age-old approach to problem solving. It is most powerfully exemplified in nature, where a simple set of 20 amino acids, biochemically arranged in a combinatorial fashion, affords the millions of peptides and protein structures required for life. Organic and peptide chemists are now applying these same approaches in the laboratory. According to Curran and Wipf (Chemical & Engineering News, p. 7, March 17, 1997) “Combinatorial synthesis is the intentional construction of a collection of molecules based on logical design and involving the selective combination of building blocks by means of simultaneous chemical reactions. The collection of molecules resulting from a combinatorial synthesis is a combinatorial library.”

B. Solid-Phase Chemistry

In combinatorial chemistry the need to carry out these multiple reactions on a small scale made the use of solid-phase chemistry attractive. Because of this, there has been much recent work on adapting traditional organic chemistry to the solid phase. Solid-phase chemistry was pioneered by Professor Bruce Merrifield (deceased in 2006) in 1963. For this work he received the Nobel Prize in Chemistry in 1984. He showed that the small scale (10-100 µmol) multistep synthesis of polypeptides could be simply and effectively carried out on a solid support (polystyrene beads). Because the insoluble beads don’t pass through a glass frit, the easy filtration work-ups at each step permitted the use of excess reagents, difficult solvents and, eventually, automation of the complete process. At the end of the synthesis the product is cleaved from the polystyrene beads. The beads are then separated from product by filtration.

C. IUPUI’s Distributed Drug Discovery Project

The need for innovative and inexpensive drugs to treat diseases in the developing world is self-evident. In the developed world, drug discovery has been fueled by the pharmaceutical industry, where economic incentives have financed the expensive
equipment and procedures currently required. Unfortunately, because the world burden of disease is disproportionately focused in poor nations, there is no economic incentive for the pharmaceutical industry to discover drugs for diseases of the developing world.

The premise of IUPUI’s “Distributed Drug Discovery” concept is that by developing simple, inexpensive equipment and procedures for each of the core drug discovery scientific disciplines (computational chemistry, synthetic chemistry and biochemical screening), large research problems can be broken down into manageable smaller units and carried out at multiple academic sites throughout the developing and developed world. The coordinated and recombined results of these “distributed” resources will inexpensively accelerate the identification of leads in the early stages of the drug discovery process. Additionally, this “distributed discovery” effort will provide educational and job opportunities in both the developed and developing worlds while building cultural and economic bridges for the common good.

**D. Amide derivatives of natural and unnatural amino acids as possible drug leads.**

This spring the second semester organic chemistry lab at IUPUI will implement our distributed drug discovery concept at both the chemistry and biological evaluation levels. Using simple, inexpensive equipment, and solid-phase combinatorial chemistry procedures, the students in this lab will make a combinatorial “library” of acylated amino acids. Since amino acids and their derivatives are essential to life, and are often components of biologically active natural products and drugs, there is a possibility that the derivatives made in this laboratory will have biological activity. After further purification and characterization by students at IUPUI these molecules will be sent to the National Institutes of Health (“NIH”) for incorporation into their Small Molecule Repository. This repository is a source of molecules for testing by multiple academic centers in the United States. It is our hope that your research will provide many new compounds, of generic structure A and B, to probe the NIH screening panel, with the potential application of this knowledge to the development of drugs to treat developing world diseases.

![Chemical structures](image)

**A1 to A3**  **B1 to B3**

**E. The Experiment**

This experiment will involve the use of solid-phase combinatorial chemistry. It will employ two amino acids, phenylalanine and tyrosine (with the phenol group of tyrosine temporarily protected as a t-butyl ether). These will be combined with various carboxylic acids R$_2$CO$_2$H as acylating agents, for the synthesis of a combinatorial library of acylated amino acids 4 (see Scheme 1 below). In this lab you will simultaneously carry out six
separate 3-step syntheses on a 50 µmol scale each. The three-step, combinatorial synthetic sequence shown in Scheme 1 involves two variables, R1 (amino acid side chain) in starting resin 1 and R2CO2H (acylating agent), to give the acylated amino acid scaffold 3. This will provide, after cleavage, the six unique derivatives of 4.

**Scheme 1: Preparation of a 2 x 3 Acylated Amino Acid Library**

![Scheme 1](image)

(R1 = PhCH2- or p-OR-PhCH2-
R2 = variety of acyl groups)

R1 will be present as the side chain in the resin-bound Fmoc protected amino acids introduced into Rows A and B. After deprotection to 2, the second (and now “combinatorial”) group R2 will be introduced by an amine acylation with a carboxylic acid, NR2CO2H, catalyzed with HOBT and DIC. After thorough washing, the link of the product to the resin 3 is cleaved with a strong acid (trifluoroacetic acid) and the target molecule 4 is separated from spent resin by filtration.

You will use six positions of the simple “Bill-Board” equipment to carry out these multiple solid-phase reactions. The Bill-Board grid is shown in Scheme 2 below:

**Scheme 2: Bill-Board 6-Pack Showing Positions and Sequence of Reagents**

![Scheme 2](image)
The “combinatorial” nature of this experiment results from the combination, over six reaction vessels, of two different amino acid side chains (R₁) with three different acylating agents (R₂CO₂H) to give six unique products.

**F. The Results**

**At the end of this experiment you will have accomplished the following:**

1. Successfully carried out, with six simultaneous reactions, a three-step organic synthesis on solid-phase.

2. Performed this three-step series of organic reactions on a microscale, beginning with 50 µmols of starting material bound to resin for each of the six reactions, and isolating approximately 10 mg of each crude product.

3. Conducted a combinatorial synthesis, with two points of variation, to make 6 unique molecules.

4. Learned how to do a chromatographic purification of one of the crude products, the control 4-cyanobenzamide derivative of phenylalanine, using silica gel chromatography.

5. Obtained and interpreted an NMR of your purified product.

6. Analyzed LC/MS data on products from all six reactions to determine if you made the desired compound and in what purity, as judged by integrated UV data.

7. While synthesizing the control compound you will also have made 5 new compound analogs. These new molecules, either “as is” or after purification by undergraduates in the coming months, will be delivered to the NIH Small Molecule Repository, for testing in a variety of screens for selective interaction with a range of biological targets.

8. Demonstrated, in the hands of IUPUI undergraduate organic students, the viability of IUPUI’s Distributed Drug Discovery concept, and participated in research to find better treatments for diseases.
Procedure (Gloves must be used and all chemical transfers must be done in the hood):

**Session 1: Deprotection and acylation**

\[
\begin{align*}
\text{Fmoc-Phenylalanine Wang} & \quad \text{Fmoc-Tyrosine (O-tBu) Wang} \\
\text{Distributed to Row A} & \quad \text{Distributed to Row B}
\end{align*}
\]

Scheme 3: Deprotection and Acylation of Resin-bound Amino Acid.

**A. Deprotection of Fmoc group**

1. At the beginning of the first laboratory section the instructor (or assistant) will distribute to each vessel in the Bill-Board 6-packs, 50 µmols of a resin bound Fmoc protected amino acid (I), bound by a Wang linker to polystyrene beads (known as a “Wang resin”). This will be done by pipetting an isopycnic (neutral buoyancy) mixture of the resin to each of the vessels. Note how efficient and simple this procedure is in evenly distributing resin to all 60 vessels used in this laboratory section. Imagine the time it would take to have weighed out the resin into each of the 60 reaction vessels!

   In this experiment the resin used in Row A is Fmoc-Phenylalanine Wang, and in Row B, Fmoc-Tyrosine (O-tBu) Wang. Their structures are shown below (the tertiary butyl group on the phenol oxygen of tyrosine is also a protecting group, and it will be removed by TFA to afford the free phenol at the final resin cleavage step).

2. After the final distribution of the resin allow the isopycnic solvent to drain (with an “air-push” assist if necessary – see below). Using a 1 mL calibrated Beral pipet, wash the resin in your reaction vessels 3 times with approximately 3 mL of NMP. Follow
the general washing procedure below. (See Chart 1 in appendix for a simple way to keep track of repeated operations)

**General Washing Procedure**

_Washes should always be done in the order listed in the experimental._ Place a beaker under the spigot of the drain tray and dispose of waste solvent in the container provided. Wash volumes are approximate and are delivered with a disposable plastic Beral pipet from labeled beakers containing the appropriate wash solvent. Try to wash any resin clinging to the inside of the glass reaction vessels. After the solvent is added, allow to drain by gravity for at least 30 s. Then drain completely using the air push apparatus. The Bill-Board charts shown in the Appendix of this procedural handout are to help you avoid mistakes. If it will help you keep track of things, you might cut out the charts, paste them into your notebook and use them to check off your washing steps as they are done.

**Using the Air “Push” Apparatus**

Place the septum over the mouth of the reaction vessel. Depress the bulb of the Beral (transfer) pipet. To avoid sucking solvent back up, lift the septum off of the mouth of the vessel before refilling the bulb with air. Repeat until all of the solvent has been pushed through the frit. (Often the appearance of the resin will change a bit to a lighter color when the last of the solvent is pushed through).

3. Using a single beral pipet add 2 ml of a solution of 20% piperidine in NMP to each of the reaction vessels. Allow to drain by gravity (No air push). After sitting for 5 minutes add an additional 2 ml of the solution of 20% piperidine in NMP to each of the reaction vessels. Allow to drain by gravity (Again, no air push). After sitting for 5 minutes add, for the third time, 2 ml of the solution of 20% piperidine in NMP to each of the reaction vessels. After standing for a final five-minute period, complete the draining with an air push and then rinse the resin in all six reaction vessels with 3, 2 ml portions of NMP (air push after each two mL wash portion).

**B. Acylation (Each team will be assigned its own set of acylating agents $$^1\text{R}_2\text{CO}_2\text{H}$$)**

**General Capping/Uncapping Procedure**

Before capping, wipe any residual solvent from the bottom threads of the reaction vessel with a Kimwipe. When uncapping, turn the Bill-Board upside-down so that the bottom is facing up. Remove the bottom caps. Then, over the drain tray, turn the Bill-Board right-side up and set it on the drain tray. Give the Bill-Board a few good shakes to release any residual resin from inside of the top caps. Uncap the top of each reaction vessel and place the caps in the used cap beaker for recycling.

4. Obtain 12 clean caps, with six of them cap the bottom of each reaction vessel. (See the general capping and uncapping procedure described above). Add 1.0 mL of the first (standard) acylating agent, 0.25 M $$^1\text{R}_2\text{CO}_2\text{H}$$ in NMP (4-cyanobenzoic acid, 250 µmol, 5 equiv) to the de-protected resin 2 in each of the two vessels _down_ column 1 (e.g. A1 and B1). Then add 1.0 mL of the second assigned acylating agent, 0.25 M
$^{2}$R$_2$-CO$_2$H in NMP (250 µmol, 5 equiv) to the de-protected resin 2 in each of the two vessels down column 2 (e.g. A$_2$ and B$_2$). Finally, add 1.0 ml of the third acylating agent, 0.25 M $^{3}$R$_2$-CO$_2$H in NMP (250 µmol, 5 equiv) to the de-protected resin 2 in each of the two vessels down column 3 (e.g. A$_3$ and B$_3$). This is followed by addition of 0.5 mL of a 0.50 M solution of diisopropylcarbodiimide (“DIC”, 250 µmol, 5 equiv) in NMP, as the coupling catalyst, to all six vessels. (See Chart 2 in the appendix). The tops of the reaction vessels are capped, the Bill-Board wiped dry of any stray solvent and then place in the rotator. The reaction is allowed to proceed for 2 days. *At this point in the experiment the combinatorial distribution of reagents has been completed (first, in Step 1, across rows A and B, for the protected amino acid, R$_1$, and now, in Step 4, down columns 1, 2 and 3 for the acylating agent, R$_2$CO$_2$H).*

**Session 2: Cleavage (and deprotection of t-butyl group on tyrosine derived products).**

5. Remove the Bill-Board from the rotation apparatus and repeat the general uncapping procedure.

6. Filter and wash the resultant acylated resin product (4) two times with 3 mL of NMP, two times with 3 mL of THF, and finally with three times with 3 mL of CH$_2$Cl$_2$. The reason for the extensive washings at this step is to make sure there are no non-resin bound impurities remaining, since they would contaminate the final product, which is cleaved from the resin in the next step. Use the general washing procedure described in Step 2. [See Chart 3 in appendix]

![Scheme 6: Cleavage of product using trifluoroacetic acid.](image)

7. Cap the bottom of each reaction vessel with a clean cap and add 2 mL of TFA-H$_2$O (95:5). *Caution: Trifluoroacetic acid (TFA) is very hazardous. You can receive a severe acid burn if you get TFA on your body. Follow your instructor’s directions for dispensing this acid.* Cap the top of each reaction vessel with a clean cap and rotate the Bill-Board for 30 min.

8. While the cleavage reaction proceeds, clearly label, with a permanent marker, six tool-necked vials and 6 HPLC autosampler vials with notebook “Lot” numbers, following the numbering format given by your instructor. Do not use paper or tape labels here since they will interfere with the fit of the vials in their holders and, if the labels fall off, will change the tare weight. Weigh the labeled, empty collection vials, record the tare mass of each vial in your notebook, and place the vials in the collection tray.

9. The products 5 (*now in solution – be sure to keep the filtrate!*) are collected into the tared tool-necked vials by first inverting the Bill-Board, removing the bottom caps, and placing an inverted collection vial over the reaction vessel. *BE CAREFUL to
match the collection vial information/location with the appropriate reaction vessel (remember that when upside-down, the “A” row is now on the bottom). After all vials are in place, the inverted collection vial rack is placed over the inverted collection vials and the whole apparatus is turned right-side-up. The top caps are now removed and the filtrates from the cleavage reaction are collected into the tared vials. Use the air-push apparatus to finish draining the reaction vessels.

10. Rinse (COLLECT these final rinses) the resin once with 2 mL of TFA/H₂O (95:5) and then once with 2 mL of CH₂Cl₂. Drain completely with air push each time into the collection vial.

11. Remove the Bill-Board from the collection vials (when lifting off the Bill-Board be careful that none of the vials stick to the bottom of the board. If this happens they may unexpectedly fall off and spill the contents of the vial). Carefully swirl the contents of each vial to make sure the cleavage solution and rinses are mixed and the solution is homogeneous. Then, for each product, use the smaller transfer pipets (calibrated with three lines for 0.1 mL, 0.2 mL and 0.3 mL) transfer 100 µL (0.1 mL) samples of product 5 to HPLC autosampler vials (with the same corresponding labels) in the small collection rack for future LC/MS analysis. To avoid cross-contamination, use a different transfer pipet for each new product sample. Give your collection vial rack, with the product containing vials in it, to the instructor (solvent will be removed from them between now and the next lab period) and place in the master autosampler vial rack your HPLC autosampler vials, in the correct order (A1, A2, A3, B1, B2, B3) and the correct row for your team.

12. (Before leaving the lab, rinse the inside of the drain tray with acetone. All washes should be disposed of safely in the appropriate waste container. Place the common equipment: drain tray, wooden blocks and “air-push” apparatus, in the rear of the hood for use by the next lab.)
Session 3: Weigh final products, TLC analysis and chromatographic purification of 4-cyanobenzamide-phenylalanine derivative made in position A1.

13. Weigh each vial containing product and record the weight in your notebook. (Remember that each reaction was done on a 50 µmol scale. Since the molecular weight of your products is typically between 300 and 400 g/mole that means your theoretical yield for each reaction is only 15 to 20 mg. This will be barely visible in your collection vial so don’t be concerned!)

14. The procedure for TLC analysis and chromatography of the 4-cyanobenzamide derivative of phenylalanine (which you made in A1) will be supplied as an addendum for this session.

Session 4: Obtain weight and NMR of purified 4-cyanobenzamide derivative of phenylalanine. Analyze LC/MS results for presence and purity of other five products.
Appendix: Auxiliary Wash and Reagent Addition Charts

If it will help you keep track of repetitive or combinatorial procedures you could cut and paste these diagrams into your notebook and use them to check off in the boxes your washing or reagent addition steps as they are completed.

Chart 1: First Wash (Step 2)

Chart 2: Acylation Step (Step 4)

Acylate down columns 1, 2 & 3 with three different acylating agents $^1R_2$-CO$_2$H, $^2R_2$-CO$_2$H, and $^3R_2$-CO$_2$H
Chart 3: Fourth Washes (Step 6)

A
1. NMP
2. THF
3. CH₂Cl₂

B
1. NMP
2. THF
3. CH₂Cl₂

1. NMP
2. THF
3. CH₂Cl₂

1. NMP
2. THF
3. CH₂Cl₂

1. NMP
2. THF
3. CH₂Cl₂

1. NMP
2. THF
3. CH₂Cl₂
Copy of TLC for recording data and incorporation into Laboratory notebook

Silica gel TLC slide

Spot samples here, approx 1.5 cm from bottom

Std    A1

Std = 4-cyanobenzamide derivative of phenylalanine (product standard)
A1 = synthesized 4-cyanobenzamide derivative of phenylalanine in vessel A1