MASSACHUSETTS INSTITUTE OF TECHNOLOGY

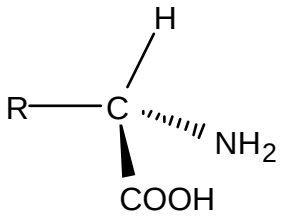
Department of Chemistry
5.310 Laboratory Chemistry

EXPERIMENT #1

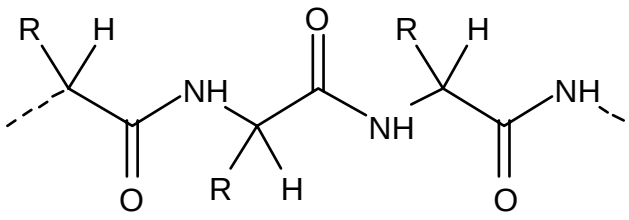
CHARACTERIZATION OF AN UNKNOWN α-AMINO ACID[1]

I. INTRODUCTION

L-α-amino acids are water-soluble organic molecules having the general structure:



The 20 L- α-amino acids (differing in the nature of the side-chain R) known as the essential amino acids are the building blocks of proteins. Amino acids are usually linked together by amide bonds (with loss of water) as given below.



Amino acids and proteins play many important roles in biochemistry.[2]

In this experiment you will receive an "unknown" amino acid to identify using several procedures. This will introduce you to some of the basic manipulative techniques of preparative chemistry and quantitative volumetric analysis. You will:

(1) Prepare a derivative with a melting point characteristic of the parent amino acid. In this instance, the p-toluenesulfonate[3] of the unknown amino acid will be prepared and isolated.

---

[1] Modified by M D. Gheorghiu to use water as a reaction medium for tosylation and to accommodate microscale quantities of the chemicals. The experiment includes contributions from past instructors, course textbooks, and others affiliated with course 5.310.

[2] For a general background discussion, see any undergraduate biochemistry textbook, for example Lehninger, A.L.; Nelson, O.L; Cox, M.M. *Principles of Biochemistry* 2nd ed. Worth, New York, NY 1993.

[3] tosyl, abbreviated here as Tos, is: [4-methylbenzenesulfonyl]; 4-CH_3-$C_6H_4SO_2^-$

(2) Purify the tosyl derivative by recrystallization and determine its yield and melting point.
(3) Determine the equivalent weight of the purified tosyl derivative by titration using a standardized solution of 0.05M sodium hydroxide.
(4) Identify the structure of the unknown amino acid from the melting point and equivalent weight of the purified amino acid derivative.

Table 1. Melting points of some DL-amino acids and their tosylderivatives.

Name	R	Amino Acid Formula weight Melting point °C	Tosyl derivative Formula weight Melting point °C
Glycine	-H	75.07 240 (dec)	229.25 149-150
DL-Alanine	$-CH_3$	89.09 289 (dec)	243.38 138-139
DL-Serine	$-CH_2OH$	105.09 240 (dec)	259.2 212-3 (dec)
DL-valine	$-CH(CH_3)_2$	117.15 295-7 (sublimes)	271.33 166-7
DL-threonine	$-CH(CH_3)OH$	119.12 244 (dec)	273.3 181-182
DL-leucine	$-CH_2CH(CH_3)_2$	131.18 293-6 (sublimes)	285.36 122
DL-ornitine*****	$-CH_2CH_2CH_2NH_2$	132.17 239 (dec)	286.35 (mono) 440.53 (ditosyl) 212.5-213.5 (dec)
DL-glutamic acid*****	$-CH_2CH_2COOH$	147.13 200-2 (dec)	301.31 172
DL-methionine	$-CH_2CH_2SCH_3$	149.21 280 (dec)	303.39 104-105
DL-phenylalanine	$-CH_2Ph$	165.19 266-267 (dec)	319.37 134-135
DL-tryptophane	$-CH_2$-β-indolyl	204.23 289-90 (dec)	358.41 176

*****ornitine has two NH_2 groups and maybe mono or di tosylated
*****glutamic acid has two COOH groups

II. SAFETY

You will be handling a number of chemicals during this experiment, some of which must be treated with care in order to avoid harm to yourself or to your surroundings. None of these chemicals should be ingested or allowed to come in contact with your skin or eyes. An asterisk denotes the chemicals described in this section when they are first used in the procedure. Additional safety information is provided in Appendix A of this lab manual.

1. 2M **NaOH** solution and 0.05 M standardized solution of NaOH: This strong base is caustic and should not come in contact with hands or clothing. If some of it gets onto your skin, rinse it immediately with plenty of water.

2. **Toluenesulfonyl chloride**: Tosyl chloride is corrosive, vesicant, lachrymator, moisture-sensitive and caustic (mainly due to the liberation of HCl on contact with water). The chemical has a strong, unpleasant odor (weigh it in the hood and keep it covered). F.W.=190.6 g/mol

3. 2M **HCl**: Very acidic liquid. Contact with skin will cause burns. If any gets on you or your clothing, strip it off and rinse exposed parts of your body with cold running water. More dilute acids are correspondingly less hazardous.

4. **Ethanol**: Flammable liquid. The type used in this laboratory is not safe to drink.

5. **Melting Point Standards**: The standard compounds used to calibrate your thermometer are organic chemicals varying in melting point. None are particularly hazardous, but they should not be ingested.

6. **Phenolphthalein**: Toxic organic dye. It should be handled with the usual precautions.

III. PROCEDURE

General Comments

The directions for preparation will give a satisfactory yield and purity for a considerable range of compounds, although they are not necessarily optimal for a given compound. **Careful attention to detail is necessary to get good yields and accurate analysis**.

Some operations are not contingent upon successful completion of others, i.e., the Mel-Temp may be calibrated, even if the derivative is not yet purified satisfactorily. These operations may be performed concurrently with prior approval of your teaching

assistant**. One of the keys to success in this course is to make efficient use of your time by recognizing which operations may be conducted simultaneously.**

Five days are allotted for this experiment. Many students will be able to complete the experiment with acceptable results in four days. The procedures given below are broken down by day and represent the progress of the typical student. **Ambitious and efficient students may be able to accomplish much more in a given afternoon.**

Reading Assignments

Reading assignments pertaining to both theoretical and technical aspects of the experiment are provided for each experiment. These readings provide theoretical background and practical information and are intended to help you prepare for the experiment and may help prevent time consuming mistakes.

MHSM	Jerry R. **M**ohrig, Christina Noring **H**ammond, Paul F. **S**chatz and Terence C. **M**orrill	Techniques in Organic Chemistry, WH Freeman, New York, NY, 2003 (required text, available in the COOP)
JWZ	**J**ames **W. Z**ubrick	"The Organic Chem Lab Survival Manual," 5th ed., Wiley, New York, NY, 2001 (available in Room 4-449 and stockroom)
TM	---	Laboratory **T**echniques **M**anual vol. I and vol. II, printed at MIT, 1979 (available from the Stock Room)
SWH	D. A. **S**koog, D. M. **W**est, and F. G. **H**oller	"Fundamentals of Analytical Chemistry", 7th ed, Saunders, Forth Worth, TX, 1996 (available in Room 4-449)
GM	John C. **G**ilbert and Stephen F. **M**artin	"Experimental Organic Chemistry, a miniscale and microscale approach" 2nd ed. Saunders, Fort Worth, TX, 1998 (available in Room 4-449)

Day 1

- Safety — **MHSM** Chapter 1 pp. 2-16
- Laboratory Notebook — **MHSM** Chapter 3 pp. 23-26
- Handbook and on-line data bases — **MHSM** Chapter 4 pp. 27-31
- Handling chemicals — **SWH** p.779 (p.795 in 6th ed.)
- Laboratory glassware — **MHSM** Chapter 2 pp. 17-23
- Collecting, washing and drying crystals — **MHSM** Chapter 9 pp. 82-83, 85-86
(Buchner funnels, filter flasks, traps) — **JWZ** Chapter 13 pp. 94-98
- Balances — **SWH** pp. 780-791 (797-806 in 6th ed.)
- Measuring mass and volume — **MHSM** Chapter 5 pp. 32-40
- Hot plates — **MHSM** Chapter 6 pp. 42-43
- Water baths, cooling methods — **MHSM** Chapter 6 pp. 46-47

Day 2

- Recrystallization, Drying the crystals — **MHSM** Chapter 9 pp. 78-89
- Melting point determination — **MHSM** Chapter 10 pp. 93-103

- Pasteur filter pipets — **MHSM** Chapter 8.5 pp. 65-66

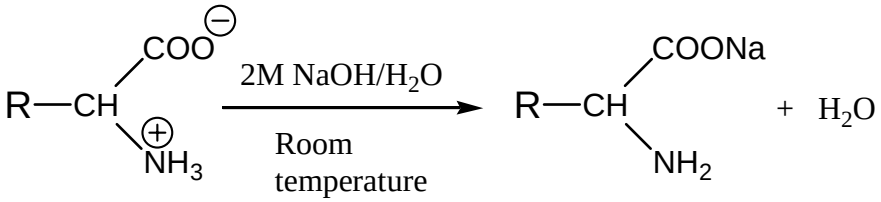
Day 4

- Directions for the use of a buret — **SWH** pp. 798-805 (pp. 820-825 in 6th ed.) omit sections on pipets
- Calculation of Percent yield — **MHSM** Chapter 3.2 pp. 26-27

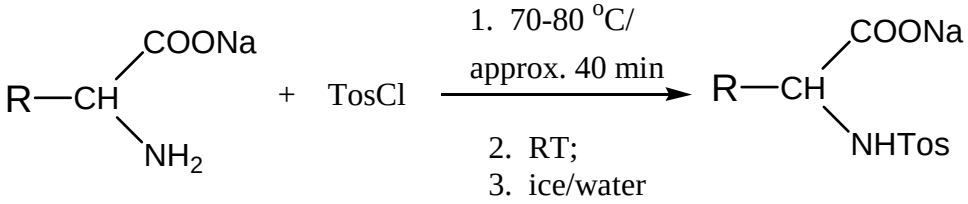
NOTE: GM Chapter 2 is an excellent source of information on a wide variety of microscale techniques

Day# 1: Preparation of the *p*-toluenesulfonate ("tosyl") derivative.[4]

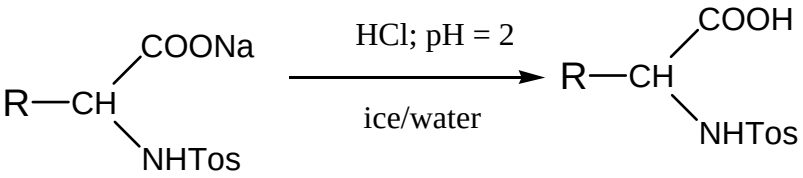
Step #1:

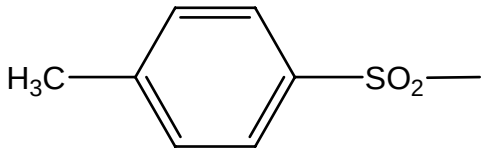


Step #2:



Step #3:



Tos: 

[4] Reactions of acyl or sulfonyl chloride with amines or phenols, carried out with aqueous NaOH are sometimes referred to as **Schotten-Bauman** reactions.

Before you start the experiment, fill a small crystallizing dish half full with water and warm it to ca. 70-80 ºC on the Mini-Hot plate stirrer (heat setting ca. 2). Above the beaker install a clamp and clamp fastener. Place a 25-mL round bottom flask with a 0.5 inch magnetic stir bar in the clamp such that the lower part of the flask is in the water bath. Check to see that the magnetic stirrer will stir. Be sure the magnetic stirrer is centered over the stir plate. Consult your TA if stirring appears to be a problem. Set up an additional clamp to hold the thermometer in place for monitoring the water bath temperature.
Place the desiccant from your desiccators in a labeled (with your name) beaker in the 100 ºC oven on Day 1 or Day 2 as instructed by your TA**.**
Note: desiccators are designed to keep dried things dry NOT to dry out **soaking** wet things like freshly filtered product.

Step #1: Dissolve the unknown amino acid (UAA) in 2M NaOH.

Weigh on the Sartorius BP 110 balance a 0.9 g sample (0.88-0.92 g weighed to ±0.001g) of the unknown DL-α-amino acid (UAA, 99% purity from Aldrich) provided by your Teaching Assistant. **(Be sure to record your sample number-as well as all data-in your laboratory notebook.)** Transfer the sample into the 25-mL round bottom flask with the 0.5-inch magnetic stirbar. Add 15 mL 2M NaOH.* Heat while stirring for at most 1 minute (some samples will require just 10-15 seconds). This converts the amino acid to its sodium salt yielding a clear solution. Remove the flask from the water bath and cool it for 5 minutes at room temperature.

Step #2: Tosylation of the UAA.

Add 2.2 g of p-toluenesulfonyl chloride (TosCl)* (use the balance in the hood) to the alkaline amino acid solution. A funnel made from a sheet of weighing paper may help the transfer. Be sure all the TosCl sits on the bottom of the round bottom flask. Cover the top of the flask with aluminum foil. Clamp the flask into the water bath and start stirring. After all the TosCl has been "dissolved," continue heating an additional 10 minutes at 70-80 ºC. The total reaction time should NOT exceed 40 minutes. Record all start and stop times and temperatures along with some intermediate times and temperatures. Remove the flask from the water bath and cool first at room temperature, then in an ice bath. Filter the cold solution using the Hirsch funnel (cover the bottom with a wet 17 mm filter paper) into a 50 ml filtering flask (don't forget to attach the neoprene filter adapter). Transfer the liquid to a 50-mL beaker.

While waiting calculate the number of moles of TosCl used in your reaction. You will need to compare this to the number of moles of amino acid used (once you have successfully identified it) to determine which species is the limiting reagent (and thus calculate theoretical and percent yields).

Step #3: Precipitation of the UAA tosyl derivative.

Add 2 M HCl* dropwise from the drop bottle. Count and record the number of drops used. Soon a white crystalline compound should start precipitating. If no precipitate is apparent check the pH of the solution every 50 drops. When precipitation is evident check the pH of the solution when there is no visual evidence of additional precipitation. To check the pH with the Universal pH Indicator paper, transfer with a glass rod a drop from the solution to a small piece of the pH paper (**DO NOT DIP THE pH PAPER INTO THE SOLUTION, a pink or red colored UAA derivative might result**!). **Whether or not precipitation has occurred** when the acidity has reached pH~2, immerse the beaker for about 5 minutes (or until precipitation occurs for more stubborn unknowns) in an ice bath (made from a beaker or crystallizing dish, take care to assure that the flask cannot tip over). See your TA for advice if crystallization is a problem.

Step #4: Filtration and removal of the traces of HCl.

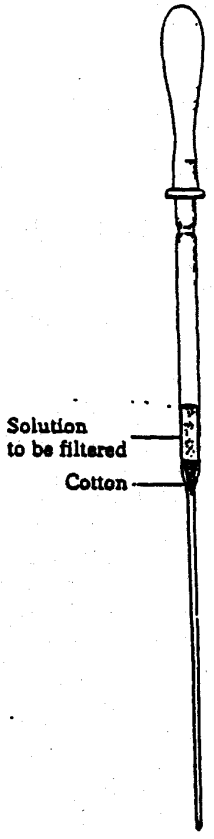
Collect the crystals by vacuum filtration on a Hirsch funnel. Wash (suction by gravity not vacuum) with 3-5 portions of approximately 0.5 mL ice water to remove salts and excess HCl. The wash water as it exits the funnel should be clearly out of the pH=2 range, but close to pH=4 due to the water solubility of the acidic tosyl derivative. After completion of washing with suction by gravity, apply vacuum suction for an additional 10-15 minutes to remove as much water as possible from the derivative. **Air**-dry the UAA derivative until the next lab day. Be sure to record the appearance of your derivative.

<u>Days #2 & 3: Recrystallization. Calibration of the melting point apparatus.</u>

Step #4a: Weigh and record the yield of the air-dried crude product in your notebook. **Set aside a few crystals in a vial** or a covered beaker (placed in a safe location in your desk) for later melting point determination.

Step #5: Recrystallization.

a. Dissolve the remaining crude crystals (but not more than 0.5g, weighed to ±0.001g) in a minimum amount of boiling water (on a hot plate) in a 30-mL beaker. Note how rapidly most of the material dissolves and stop adding solvent when you suspect that almost all the desired material has dissolved. If the tosyl derivative is not dissolving, add **a few drops of ethanol*** until it dissolves, and warm the solution again. Undissolved material noted at this point could be an insoluble impurity that never will dissolve.



Filtration of a solution in a Pasteur pipette.

Allow the solution to boil, and if no further material dissolves, dilute the solution with enough water (approximately 10% excess) so that the solute **will not crystallize** at room temperature. **Record** the volumes of water (and ethanol) used.

b. Prepare a filter pipet by using a length of copper wire to push a ***tiny*** bit of cotton into a Pasteur pipet (If too much cotton is used, it will be difficult to force the solution through the plug). Transfer the **HOT** solution to be filtered into this filter pipet using another Pasteur pipet. Force the liquid through the filter using air pressure from a pipet bulb. If crystals start to form in the pipet **stop** add a little more hot solvent to the solution and a drop or two hot solvent to the filtration pipet. Rinse the pipet and cotton with fresh hot solvent.

c. The filtered solution is concentrated by simply boiling off some of the solvent. A boiling stick may be placed in the solution during this process, but do not forget to remove it before initiating crystallization.

d. Cool the solution slowly without disturbing the container so that large crystals may form. It is best to allow the reaction to cool in a beaker filled with cotton or paper towels which act as insulation, and thus slows cooling.[5,6] Once the undisturbed tube has cooled to room temperature, it can be placed in an ice bath to maximize the amount of product that comes out of solution. Scratch the sides of the flask to induce crystallization if it has not already occurred. Note the appearance of the crystals as they begin to form and record their characteristics in your notebook. After crystallization appears complete, cool in the ice bath for an additional 10-15 minutes.

e. Collect the product by vacuum filtration on a Hirsch funnel; use a microspatula to dislodge crystals which adhere to the beaker. Catch the filtrate in a clean dry filter flask. If crystals remain after the test tube has been emptied, the filtrate can be poured back into the flask to help complete the transfer. A **small** amount of fresh, cold water should be used as a final wash to free the crystals of mother liquor.

A significant fraction of the product may be left in the filtrate and should be recovered. This may be done by evaporating to a smaller volume and cooling until crystallization takes place. **Always save the filtrate from a recrystallization until you are sure it contains nothing worth recovering.** A portion of the material may be recrystallized a second time to establish purity (i.e., melting point does not change).

Transfer the combined lots of recrystallized product to a preweighed, dry watch glass and dry to constant weight in a 50 ºC oven. Record the yield of purified material obtained. (Note: if constant weight is obtained, samples for step #7 maybe prepared,

---

[5] Some compounds, particularly those that melt below 100 ºC, may separate as oil when water is added and the mixture cools. Although the oil may solidify at room temperature, the product is likely to be impure and recrystallization from a hydrocarbon solvent, such as hexane or hexane - ethyl acetate, should be tried. It is essential that the product separate from the outset and not as an oil which then solidifies.

[6] Go ahead with step #6 while waiting for things to cool, crystallize, dry in the oven, etc. A major saving in time and increase in working efficiency is possible through careful preplanning.

weighed and carefully stored for future titration.) When the identity of you unknown is established calculate the percent yield of your crude and recrystallized product based on the limiting reagent.

Step #6: Calibration and Determination of Melting Point.

A Mel-Temp ^{R} apparatus with a digital thermometer and a 90-mm melting point capillary is used. Calibrate the thermometer by taking the melting points of four pure compounds that melt over the range of 50-200°C. Pure melting point standards* are provided in the laboratory. Prepare a graph of reported versus observed melting points for a particular Mel-Temp and digital thermometer. Hand draw lines between points on the curve as the relationship need not be linear over all temperature ranges. For future reference **record the identification number of the Mel-Temp and the digital thermometer**. Consult the Chemical Rubber Company HANDBOOK OF CHEMISTRY AND PHYSICS to verify the melting points of the standards used.

*****Students will be divided into teams. Each team will calibrate one Mel-Temp with each member of the team determining one point (i.e. melting point standard) on the curve for this instrument. You will use the same Mel-Temp apparatus for entire semester.**

To load the melting point capillary push the open end into some of the crystals (which must be carefully dried beforehand), invert and tap the sealed end gently on the desk top until the crystals slide down. The crystals should not occupy more than 2-3 mm of the tube. If you have difficulty getting the crystals to go to the bottom of the tube, take a 65 mm long stem funnel and place it upside down on the bench top. Drop the capillary tube down the stem. The impact will not have enough force to break the tube, but it will force the crystals to the bottom.

If you have no previous knowledge of the melting point, it saves time to do a rough determination by setting the Variac to about 60 and scanning the 50-250 ºC range (5 ºC to 10 ºC per minute). When the approximate melting range has been found, a new tube should be prepared and the run repeated using a much slower (<1 ºC per min) rate of temperature increase. Melting point ranges should not exceed four degrees for the former and two for the latter. Always recrystallize a product to constant melting point.

Determine the uncorrected melting point of the unknown acid and compare to the calibration plot. Record in the notebook these results together with any pertinent observations made during heating i.e., evidence of decomposition, color changes, etc. When submitting your notebook pages, include a table showing yields and corrected melting points of the crude and purified products as well as the thermometer calibration curve.

Day #4: Equivalent weight of Amino Acid Derivative and Identification of Unknown

Step #7: Determination of the Equivalent Weight of the Amino Acid Derivative.

Place about 0.1-0.2 grams of recrystallized unknown acid derivative in a vial and dry for an hour in the 100 ºC oven if the compound has melting point of 130 ºC or over. If the melting point is below 130 ºC, dry in the 50 ºC oven. At the end of the drying period place the vial in a desiccator to cool to room temperature.

Weigh by difference at least three 0.030-0.040 g **dry** samples of the derivative (on the **analytical balance**, in the Balances Room), into numbered 15-mL Erlenmeyer flasks. Dissolve the unknown acid samples in 2.5 mL of water (swirl and warm) and add as necessary 2-3 drops of 95% ethanol and warm further.[7] When fully dissolved allow the solution to cool to room temperature. Add 1 drop of phenolphthalein* indicator and titrate using a 10 mL buret filled with standardized 0.05 M sodium hydroxide solution (available from the automatic buret, record the actual concentration). Attach a plastic tip to the end of buret to reduce the size of the drops and to increase the accuracy of the titration. (Record starting and ending volumes in the notebook.) The titration may be carried out rapidly at first, but the endpoint should be approached carefully. With low-carbonate NaOH, the endpoint should be sharp and easily located to within a fraction of a drop.[8] Try to obtain the same intensity of pink at the endpoint for all your titrations. A barely detectable shade of pale pink is the desired color of the indicator at the endpoint. A bright pink solution indicates that the titration overshot the endpoint.

Make all buret readings by estimating the value to the nearest 0.01 mL, allowing time for drainage. Run a sufficient number of titrations to assure a precise and presumably accurate result.

If three titrations do not result in the desired precision in equivalent weight (i.e., ±5% of the mean), it will be necessary to conduct additional titrations. Include a table with your notebook pages that gives the calculated equivalent weight of the amino acid from each titration, the average, and the 95% confidence limits of the mean. Estimate your uncertainty in each measurement you have made (i.e., in weighing, using the buret, and endpoint determination). Use these values to calculate a propagation of error. Compare your propagated error with the observed precision.

Step #8: Identification of Unknown Acid

From the observed melting point and equivalent weight of the p-toluenesulfonate derivative, attempt to identify your unknown acid. Show the accepted values for these quantities taken from Table I of this experiment.

---

[7] Some acids may need an additional 1 mL of alcohol and heating near boiling to effect the complete solution that is necessary for good results.

[8] The phenolphthalein endpoint is taken as the first distinct pink color that persists for 10 seconds or more after thorough mixing. The color is not permanent but will fade in a matter of minutes or less as a result of absorption of CO_2 from the air.

When you are convinced of the identity of your unknown you may write it in your notebook and sign next to it. Take your notebook to your TA for grading the identity of the unknown. If it is correct you are ready to begin the written report. If it is not correct you may try to determine the source of your error in identification and make a second (or third) determination. There is a penalty for each additional attempt (see grading sheet).

All lab work must be completed during the five days scheduled for the experiment and multiple attempts at identification <u>in no way affects the written report due day</u>.

<u>Day #5: Finish remaining work on this experiment</u>.