Enzymatic Resolution of 1-Phenylethanol and Formation of a Diastereomer: An Undergraduate ^{1}H NMR Experiment To Introduce Chiral Chemistry

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Stereochemistry is usually introduced in the first semester of undergraduate organic chemistry and is a crucial concept for organic chemistry (racemization, S_N2 reactions), biochemistry (peptides, proteins, helicity of B- and Z-DNA), and inorganic chemistry (chiral catalysis). Unfortunately, students often struggle with the abstract concepts of stereochemistry. There are many experiments described in this *Journal* and others that attempt to enhance student understanding of stereochemistry *(1−20)*. Herein, an organic laboratory experiment is presented that focuses on stereochemistry by introducing students to stereoselective enzyme reactions, resolution of enantiomers, and NMR analysis of diastereomers.

This experiment uses an enzyme for the preparation of an enantiopure product from a racemic precursor. Acylase I from *Aspergillus melleus (21)*, which catalyzes the transformation of a range of aromatic alcohols to esters *(22)* by transferring an acyl group, is used. A racemic mixture of 1-phenylethanol is reacted with vinyl acetate. The reaction is shown in Scheme 1 and reflects the stereoselectivity of the enzyme. The enzyme reaction provides an opportunity to discuss green chemistry. It is also carried out in hexane, which is more suitable for many organic molecules than the aqueous environment most enzymes require *(23)*. Previously described enzymatic methods include the stereospecific, catalyzed reactions of penicillin acylase derivatives *(24)* and their use in the chiral resolution of isomers *(25)*. Students benefit from the hands-on experience and observe firsthand how enzymes can resolve racemic compounds. To determine which enantiomer the enzyme preferred, the students derivatize the unconverted alcohol(s) to a set of diastereomeric esters using a chiral carboxylic acid and a coupling reagent. The chiral acid used in this experiment is more reactive, gives a better NMR result, and is ~85% less expensive than the Mosher acid, MTPA *(26, 27)*.

The experiment is divided into two parts and conducted over three, two-hour laboratory periods 1 week apart. Part 1 is the stereoselective formation of an ester from a racemic alcohol by acylase I, followed by the separation of the product ester and the unreacted alcohol. Part 2 is the formation of a diastereomeric compound from the unreacted alcohol and the use of ^{1}H NMR spectroscopy to determine the chirality of the unreacted alcohol.

Experimental Details

Synthesis

In part 1, racemic 1-phenylethanol (α-methylbenzyl alcohol) was reacted with vinyl acetate, catalyzed by acylase I, in hexane for 1 week at room temperature. No stirring was required and the reaction occurred by simply storing the mixture for 1 week at the student's lab stations. After the reaction was complete, the alcohol and ester products were separated by silica gel column chromatography (monitored by TLC).

In part 2, students carried out three reactions to investigate the stereochemistry of the reaction in part 1. The unreacted 1-phenylethanol was derivatized with (*R*)-(−)-acetoxyphenylacetic acid (*O*-acetylmandelic acid) in the presence of a coupling reagent, ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and a catalyst, 4-dimethylaminopyridine (DMAP), to prepare the ester (Scheme 2). Students then reacted the racemic starting alcohol with (*R*)-(−)-acetoxyphenylacetic acid to make the pair of diastereomeric esters. Finally, they reacted the enantiopure (*R*)- or (*S*)-alcohol with (*R*)-(−)-acetoxyphenylacetic acid to make the diastereomeric ester. Proton NMR analysis was used to assess the stereochemistry of the products.

The details of the experimental setup are provided in the supporting information.

NMR Analysis

The ^{1}H NMR (300 MHz) spectrum from the reaction of the racemic starting alcohol with (*R*)-(−)-acetoxyphenylacetic acid is shown in Figure 1. The two diastereomers can be distinguished by the NMR signals of the methyl doublets near

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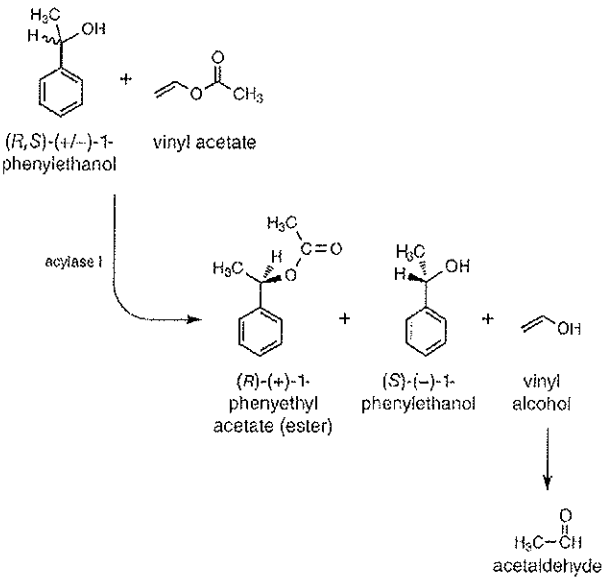
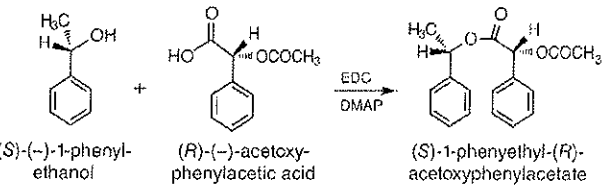
Scheme 1. The Enzymatic Acylation of (*R,S*)-(±)-1-Phenylethanol



Scheme 2. The Formation of (*S*)-1-Phenylethyl (*R*)-Acetoxyphenylacetate from the Unreacted Starting Alcohol



1.5 ppm. An expansion of the 1−3 ppm region of the ^{1}H NMR spectrum of a 50:50 mixture of diastereomers (Figure 2A) shows the benzylic methyl group from the two diastereomers have different chemical shifts. The same region from a spectrum of the diastereomer derived from commercially available (*R*)-phenylethanol (Figure 2B) shows the doublet at 1.54 ppm is attributable to the (*R,R*) diastereomer, whereas the doublet at 1.41 ppm comes from the (*S,R*) diastereomer.

The ^{1}H NMR (90 MHz) spectrum of the ester (Figure 3) from part 1 (Scheme 1) indicates that the majority (approximately 3:1) of the unreacted alcohol was the (*S*)-enantiomer leading to the (*S,R*)-diastereomer, indicating the enzyme preferred (reacted faster with) the (*R*)-enantiomer. The student data showed 76% (*S,R*) isomer and 24% (*R,R*) isomer leading to an enantiomeric excess of 52% by ^{1}H NMR.

Optical Purity

Students determined the optical purity of their unreacted alcohol by measuring its specific rotation and comparing to a purchased standard, (*R*)-1-phenylethanol (99.9% from Aldrich). The specific rotation of the standard was +43.03 and the resolved alcohol −25.02 (both in methanol), giving an optical purity (enantiomeric excess) of 58% (*S*)-enantiomer by polarimetry.

Hazards

1-Phenylethanol, vinyl acetate, dichloromethane, and deuterochloroform are cancer suspect agents. 4-Dimethylaminopyridine

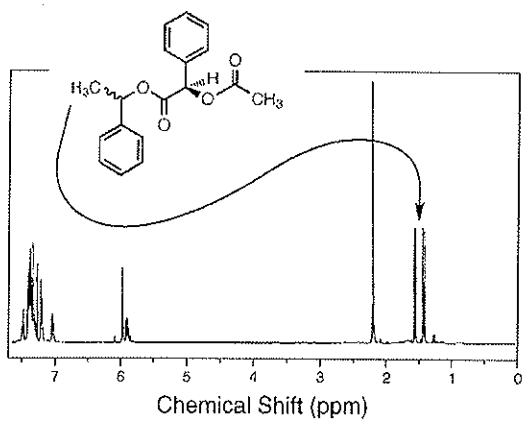


Figure 1. ^{1}H NMR (300 MHz) spectrum of (*S*)-1-phenylethyl (*R*)-acetoxyphenylacetate and (*R*)-1-phenylethyl (*R*)-acetoxyphenylacetate.

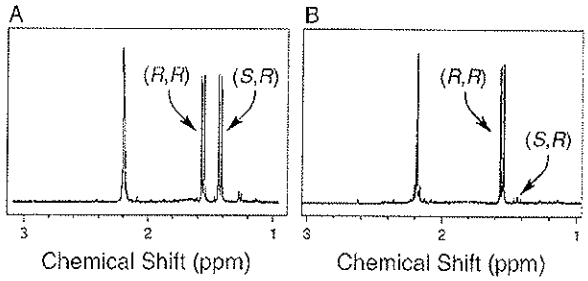


Figure 2. Expanded ^{1}H NMR spectra of Figure 1: (A) 50:50 mixture of the two diastereomers (*S*)- and (*R*)-1-phenylethyl (*R*)-acetoxyphenylacetate and (B) the (*R,R*)-diastereomer derived from commercially available (*R*)-phenylethanol. The trace amount of the (*S,R*)-diastereomer indicates that the commercial alcohol was not 100% pure.

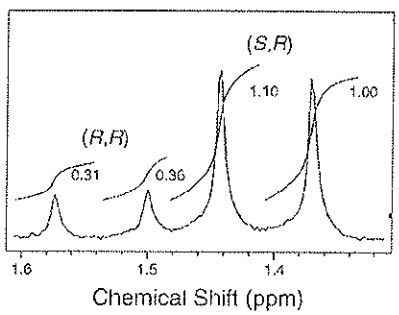


Figure 3. ^{1}H NMR (90 MHz) of 1-phenylethyl acetoxyphenylacetate, after the unreacted 1-phenylethanol was reacted with (*R*)-(−)-acetoxyphenylacetic acid leading to the diastereomers (*R,R*) and (*S,R*). The 3:1 ratio of (*S,R*) to (*R,R*) indicates a preference of acylase I for the (*R*) enantiomer of 1-phenylethanol.

(DMAP) is highly toxic. EDC, 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride is an irritant. These materials must be used in small quantities in fume hoods. Student should use chemically resistant gloves and protective eye wear.

Organization of the Group Work

The students each perform the enzyme-catalyzed transesterification with the racemic alcohol (part 1). Each student

in a group of 3−4 then either reacts the (*R*)-enantiomer of the alcohol, the experimentally unreacted (*S*)-enantiomer (collected from all group members), or the racemic mixture with the acetoxyphenylacetic acid so that each group had a complete set of complexes to analyze (part 2). This allows students to gain enough information to determine which enantiomer reacted preferentially with the enzyme in part 1. The weekly experimental schedule for the laboratory exercise is

Week 1: Each student reacts racemic 1-phenylethanol with vinyl acetate using acylase I from *A. mellеus* as a catalyst. This reaction takes 10−15 min to prepare and can be performed at the end of another lab experiment session. If possible, students should come in during the course of the week to test the reaction mixture via TLC and observe the disappearance of the starting alcohol peak and the increase in the product ester peak.

Week 2: Each student performs TLC analysis of their reaction mixture and then separates the alcohol and ester by microscale column chromatography. Each fraction is analyzed by TLC, and pure fractions are combined. The students in each group are then assigned an alcohol:

Student 1: Reacts the racemic alcohol with (*R*)-(−)-acetoxyphenylacetic acid to make the pair of diastereomeric esters.

Student 2: Reacts the unreacted alcohol with (*R*)-(−)-acetoxyphenylacetic acid to make the diastereomeric ester.

Student 3: Reacts the enantiopure (*R*)- or (*S*)-alcohol with (*R*)-(−)-acetoxyphenylacetic acid to make the diastereomeric ester.

For larger groups: Additional students can replicate another student.

Week 3: Students collect and share ^{1}H NMR spectra and analyze their results to determine which enantiomer of 1-phenylethanol was the preferred substrate in the enzyme reaction. Student 1 obtains a ^{1}H NMR spectrum showing the doublets from both diastereomeric esters (Figure 1). The ^{1}H NMR spectrum from student 2 shows the doublet from the ester of the unreacted alcohol (Figure 3). Student 3 has an ^{1}H NMR of the ester from the pure, known (*R*)- or (*S*)-alcohol (Figure 2B). From this data, the group can deduce which enantiomer of the starting alcohol was consumed by the enzyme and calculate by how much it was preferred.

Costs

An attractive aspect of this enzyme-facilitated enantioresolution is its low cost. On the basis of catalog prices from commercial suppliers, the average cost per student is about $3.50, exclusive of solvents, TLC plates, and other common reagents. The majority of the cost is (*R*)-(−)-acetoxyphenylacetic acid, EDC, and DMAP at about $2.75 per student. In comparison, on a similar scale using an acid chloride, the experiment costs approximately $43 per student (Sigma-Aldrich Handbook of Fine Chemicals 2007−2008).

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Note Added after ASAP Publication

This paper was published on the Web on July 14, 2010. Significant changes were made throughout, including changes in authorship and supporting information; new graphics for Schemes 1 and 2; several new references; renumbered references; and several text changes. The corrected version was published on the Web on January 24, 2011.

Literature Cited

1. Pavia, D. L.; Lampman, G. M.; Kriz, G. S.; Engel, R. G. *Introduction to Organic Laboratory Technique*; Saunders: Philadelphia, PA, 1990; p 228.
2. Gilbert, J. C.; Martin, S. F. *Experimental Organic Chemistry*, 3rd ed.; Harcourt College Publishers: Philadelphia, PA, 2002; p 213.
3. Bettelheim, F. A.; Landesberg, J. A. *Experiments for Introduction to Organic Chemistry*; Saunders: Ft. Worth, TX, 1997; p 113.
4. Williamson, K. L.; Minard, R.; Masters, K. M. *Macroscale and Microscale Organic Experiments*, 5th ed.; Houghton Mifflin Co: New York, 2007; p 786.
5. Wade, L. G. *Organic Chemistry*, 6th ed.; Prentice Hall: Upper Saddle River, NJ, 2006; p 1167.
6. Loudon, G. M. *Organic Chemistry*, 4th ed.; Oxford: New York, 2002; p 438.
7. Bruice, P. Y. *Organic Chemistry*, 4th ed.; Prentice Hall: Upper Saddler River, NJ, 2004; p 213.
8. Cesare, V.; Stephani, R. *J. Chem. Educ.* **1997**, *74*, 1226.
9. Baar, M. R.; Cerrone-Szakal, A. L. *J. Chem. Educ.* **2005**, *82*, 1040–1042.
10. North, M. *J. Chem. Educ.* **1998**, *77*, 630.
11. Patterson, J.; Sigurdsson, S. T. *J. Chem. Educ.* **2005**, *82*, 1049–1050.
12. Lee, M. *J. Chem. Educ.* **1993**, *70*, A115–A158.
13. Lee, M. *J. Chem. Educ.* **1998**, *75*, 217–219.
14. Lee, M.; Huntington, M. *J. Chem. Educ.* **1994**, *71*, A62.
15. Rebolledo, F.; Liz, R. *J. Chem. Educ.* **2005**, *82*, 930–933.
16. Stetea, D.; Arends, I. W. C. E.; Hanefeld, U. *J. Chem. Educ.* **2002**, *79*, 1351.
17. Poiré, C.; Rabiller, C.; Chon, C.; Hudhomme, P. *J. Chem. Educ.* **1996**, *73*, 93.
18. Smith, D. H.; Giner, J.-L.; Faraldos, J. A. The enzymatic resolution of enantiomers and the formation of diastereomers: An undergraduate NMR experiment. In *Abstracts of Papers, 235th ACS National Meeting, New Orleans, LA, April 6−10, 2008* CHED−1525.
19. Lee, S. *Mosher's Technique of Measuring Enantiomeric Excess: A Microscale and Nmr Experiment for Organic Chemistry. The 18th Biennial Conference on Chemical Education*, Iowa State University, Ames, IA, July 22, 2004.
20. Priest, O. *Collaborative Organic Chemistry Laboratory Exercise*: Mosher Amides. *The 19th Biennial Conference on Chemical Education*, West Lafayette, IN, August 2, 2006.
21. Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 6354–6364.
22. Faraldos, J.; Arroyo, E.; Herradón, B. *Synlett* **1997**, 367–370.
23. Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114–120.
24. Rosell, C. M.; Fernandez-Lafuente, R.; Guisan, J. M. *Ann. N.Y. Acad. Sci.* **1995**, *750*, 425–428.
25. Topgi, R. S.; Ng, J. S.; Landis, B.; Wang, P.; Behling, J. R. *Bioorg. Med. Chem.* **1999**, *7*, 2221–2229.
26. Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512–519.
27. Chataigner, I.; Lebreton, J.; Durand, D.; Guingant, A.; Villieras, J. *Tetrahedron Lett.* **1998**, *39*, 1759–1762.

Supporting Information Available

Student handout; notes for the instructor. This material is available via the Internet at http://pubs.acs.org.

Student Handout

This experiment allows you to explore the properties of chiral molecules. You have learned that some compounds exist as enantiomers – non-identical mirror images, such as your left and right hands. Enantiomers have the same physical and chemical properties and react identically with non-chiral reagents, but react differently with other chiral agents. Enantiomers will rotate (or polarize) light by the same amount, but in different directions, which is one way they can be identified.

Diastereomers (or diastereoisomers) are stereoisomers that are not enantiomers. The most common example is a pair of isomers that have more than one chiral center, with opposite configurations at one or more (but not all) of the chiral centers. For example, a molecule with 2 chiral centers can exist in 4 forms:

S,S	R,S
S,R	R,R

The S,S and R,R forms are enantiomers of each other, since they have opposite chirality at both stereocenters. Similarly, the R,S and S,R forms are enantiomers of each other. However, the S,S and S,R forms are diastereomers of each other– they have opposite chirality at some, but not all, chiral centers.

In this experiment, you will be given a chiral alcohol with one stereocenter. You will react the alcohol with an acetate, using the acylase I enzyme as a catalyst. Since the enzyme is chiral, it will preferentially react with one enantiomer. The product mixture will include one enantiomer (R or S) as an acetate ester and the other enantiomer (S or R) as the unreacted alcohol. Working in groups, you will take the unreacted alcohol and combine it with another

agent of known chirality, to create a diastereomer. By comparing the ^{1}H NMR spectra of the product diastereomers, you will be able to determine with which enantiomer the enzyme reacted. This experiment is divided into two parts, conducted over three laboratory periods.

Part 1: The stereoselective formation of an ester from a racemic alcohol by acylase I, followed by the separation of the product ester and unreacted alcohol.

Part 2: Formation of a diastereomer from the unreacted alcohol enantiomer, then ^{1}H NMR analysis of the product diastereomer to determine which alcohol the enzyme preferentially uses as a substrate.

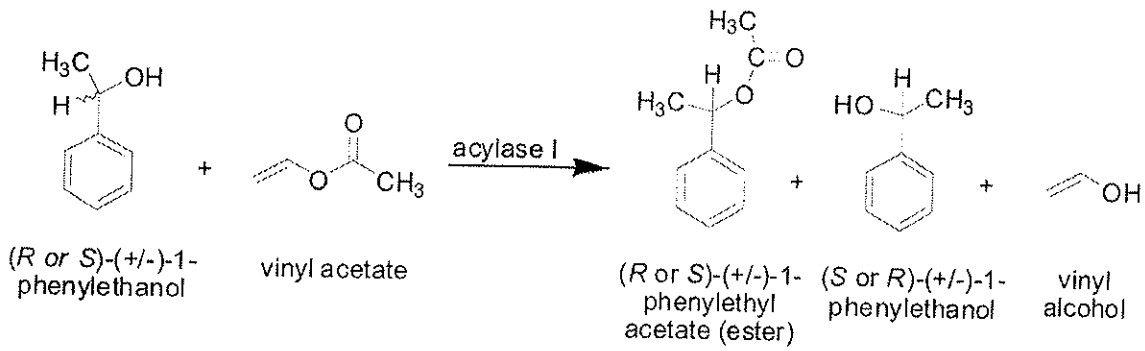
Part 1 (Weeks 1 & 2): The Stereoselective Esterification of an Alcohol by an Enzyme

This week you will combine a racemic alcohol with vinyl acetate to create an ester, using a chiral enzyme as a catalyst. During the second week, you will separate the unreacted alcohol from the product ester.

Enzymes have been used in the laboratory to prepare chiral products from non-chiral precursors for many years (Rosell, C. M., et al. *Ann. N.Y. Acad. Sci.* **1995,** *750,* 425-428 and Topgi, R. S., et al. *Bioorg. Med. Chem.* **1999,** *7,* 2221-2229). Some enzymes can function in diverse or harsh environments, including those required for organic reactions. The acylase I enzyme catalyzes the formation of esters from alcohols. The reaction is called a transesterification reaction because it involves the transfer of an acyl group from an ester to an alcohol to produce a new ester. You will use this enzyme to convert 1-phenylethanol to its acetate ester, 1-phenylethyl acetate. Because the enzyme is much more reactive with one of the two enantiomers of 1-phenylethanol than the other, we can use it to resolve, or separate, the two

enantiomers. One enantiomer of 1-phenylethanol will be esterified and the other enantiomer will remain unreacted.

Reaction 1



(*R or S*)-(+/-)-1-phenylethanol | vinyl acetate | (*R* or *S*)-(+/-)-1-phenylethyl acetate (ester) | (*S* or *R*)-(+/-)-1-phenylethanol | vinyl alcohol

Pre-Lab Preparation:

You will need to read about enzymes in the lab manual and in the textbook. It is strongly suggested you review the concepts of enantiomeric and diastereomeric compounds as well.

Procedure: Enzyme Reaction

You will be working in "reliable data groups" of three or four people. Each member of the group will conduct the enzymatic reaction ("Week 1") and purify the products. Later, each group member will perform different tasks (see Part 2: Diastereomeric Esters of 1-Phenylethanol).

Week 1:

Clean a 10 mL Erlenmeyer flask. Mix - in order - 5 mL of hexane, 200 mg (1.6 mmol) of racemic 1-phenylethanol, and 300 mg (3.5 mmol) of vinyl acetate (CAUTION! Cancer suspect agents!). Mix well. Add 80 mg of acylase I from *A. melleus*. The enzyme will not dissolve.

Try to be within 5% of these weights and record the actual amounts used of each reagent. Tightly cork the flask and leave it in your drawer where it will not spill. The reaction is normally complete in seven days.

During the next week, every 2 or 3 days, one member of the group should sample one of the flasks to follow the course of the reaction. Over time, the alcohol peak or spot should decrease and the product peak(s) should increase. The reaction progress can be followed by TLC by using 4:1 hexane:ethyl acetate to develop the plate.

After 7 days, or when chromatography shows the reaction to be complete, it may be stopped by filtering (vacuum filtration) the mixture to remove the undissolved enzyme. Store the remaining mixture at room temperature until the second lab period.

Week 2:

Fill a 10 mL chromatography column (Kontes glass micro-scale kit) with 8ml of dry silica gel (140 mesh). Set up a rack of clean test tubes for collecting the column fractions. Apply the reaction mixture to the top of the dry silica column. Develop the column with the following poured onto the top of the column, in order: 4 x 2 mL portions of 29:1 hexane:ethyl acetate, then 4 x 2 mL portions of 9:1 hexane:ethyl acetate, then 5 x 2 mL portions of diethyl ether. Analyze the fractions by TLC, using a 3x5 cm silica gel strip on its "side" (spot the samples along the long direction and develop in the short direction), developed with 4:1 hexane:ethyl acetate. Remember to include a sample of the starting alcohol as a control.

Pool your own pure fractions of alcohol and of ester into separate tared test tubes or 10 mL Erlenmeyer flasks. Evaporate the solvents and weigh. Record the amount of unreacted alcohol, and the amount of ester product produced.

Within your group, combine all the pure alcohol fractions. Do the same with the 4 pure ester fractions.

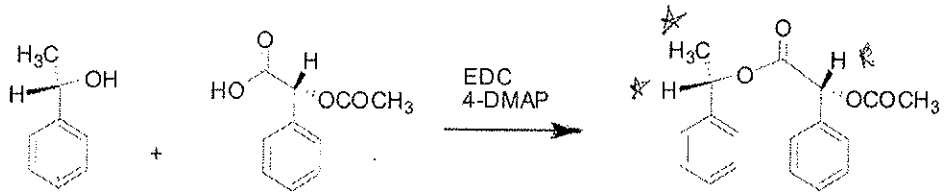
Student Handout

Part 2 (Weeks 2 & 3): Diastereomeric Esters of 1-Phenylethanol

^{1}H NMR is a very sensitive technique, but like most other analytical methods cannot distinguish enantiomers. Diastereomers, on the other hand, have different chemical and physical properties, including ^{1}H NMR spectra. Making a diastereomeric derivative of the chiral compound that is being analyzed using a chirally pure derivatizing agent is a useful technique. The relative proportion of the two enantiomers can be calculated from the resulting ^{1}H NMR spectrum of the diastereomeric mixture.

In this session, you will produce an ester of the unreacted alcohol from the previous reaction and analyze it by ^{1}H NMR and TLC. The ester is formed from the remaining (R or S) alcohol and a chirally pure carboxylic acid so that the products will be diastereomers of each other. An example appears below.

Reaction 2



S-(-)-phenylethanol *R*-(-)-acetoxyphenylacetic acid (*S*)-1-phenylethyl (*R*)-acetoxyphenylacetate

S-(-)-1-Phenylethanol reacts with *R*-(-)-acetoxyphenylacetic acid with the aid of a coupling agent (EDC) and a catalyst (4-DMAP) to make the (*S*,*R*)-diastereomeric ester. Starting with an *R*-alcohol would result in the formation of the (*R*,*R*)-diastereomeric ester.

R-(-)-Acetoxyphenylacetic acid is also known as acetoxymandelic acid. It will be abbreviated as "*R*-acetoxy acid". EDC is a "coupling agent", meaning it is a chemical that reacts easily with one substance (here, the acid) and then is displaced by another substance (the alcohol). 4-DMAP is a non-nucleophilic base and highly toxic. Look up the structures of EDC and 4-DMAP. Optional Reading: "A related NMR technique" (p 693) and the definition of enantiomeric excess (p 689) in Williamson, K. L.; Minard, R.; Masters, K. M. *Macroscale and Microscale Organic Experiments,* 5th ed.; Houghton Mifflin Co: New York, NY, 2007.

The benzyl hydrogen on the alcohol portion of the product, as well as the methyl group, are in a different ^{1}H NMR environment in the (*S*,*R*)-diastereomer compared to the (*R*,*R*)-diastereomer, so they are distinguishable by NMR.

Procedure: Diastereomer Formation

FOR THIS PART YOUR GLASSWARE MUST BE THOROUGHLY DRY!

Weigh 25 mg (2.0 mmol) of your assigned 1-phenylethanol sample (from Table 1) into a 6" test tube. On separate pieces of weighing paper, weigh 2 molar equivalents (2 moles per mole of 1-phenylethanol) of EDC hydrochloride (EDC), 2 molar equivalents of (*R*)-acetoxy acid, and 0.2 molar equivalents of 4-DMAP, within 10% of the calculated weights. Molecular weights: EDC 192 g mol^{-1}, DMAP 122 g mol^{-1}, (*R*)-acetoxy acid 194 g mol^{-1}.

Table 1: Assigned 1-phenylethanol samples for students in groups.

Student	1-Phenylethanol sample description
1	Racemic 1-phenylethanol
2	Unreacted 1-phenylethanol from enzymatic resolution
3	Enantiomerically pure (*R*)-1-phenylethanol
4	Enantiomerically pure (*S*)-1-phenylethanol (or replicate one of the above students)

Mix the solution using a stir bar and then add, in order: 1-phenylethanol, 2 mL of dichloromethane, EDC, (*R*)-acetoxy acid, and finally 4-DMAP. After 15 minutes of stirring at room temperature, remove the stir bar and add an additional 2 mL of dichloromethane. Extract the dichloromethane solution sequentially with one 1-1.5 mL portion of 10% HCl, one 1-1.5 mL portion of brine (saturated NaCl), one 1-1.5 mL portion of 5% NaOH, and finally with one 1-1.5 mL portion of brine, removing the aqueous layer (and only the aqueous layer) after each extraction. Dry the dichloromethane layer with anhydrous sodium sulfate, and filter the solution through a small plug of cotton in a pipette.

Check the purity of the product by TLC, using 4:1 hexane:ethyl acetate as the developing solvent, with a reference spot of the starting alcohol as a control.

Completely evaporate the dichloromethane using air and gentle heating in a water bath (this must be done in the HOOD!). Dissolve the product in approximately 0.8 mL of deuterochloroform and acquire the ^{1}H NMR spectrum.

Student Handout

Laboratory Report Guide for "The Stereoselective Esterification of an Alcohol by Means of an Enzyme" and "Diastereomeric Esters of 1-Phenylethanol" Experiment.

Each student will submit a single laboratory report for the combined "The Stereoselective Esterification of an Alcohol by Means of an Enzyme" and "Diastereomeric Esters of 1-Phenylethanol" experiments.

Make a table containing each group member's ^{1}H NMR data:

Table 1. ^{1}H NMR Data

Sample	Integration at ca. 1.5 ppm	Integration at ca. 1.4 ppm	% at 1.5 ppm*	% at 1.4 ppm	Enantiomeric excess
Diastereomer from unknown chirality alcohol					
Diastereomer from R-alcohol					
Diastereomer from S-alcohol					

* % at 1.5 ppm = (the integration of the doublet at 1.5)/(integration at 1.5 + integration at 1.4)

Optical Purity of the Resolved Enantiomer

Calculate the optical purity of each group member's alcohol using ^{1}H NMR data and include this data in your table.

Enantiomeric excess = % major isomer - % minor isomer

Compare each group member's ^{1}H NMR data to determine if acylase I preferred to react with the *R*- or the *S*-enantiomer of the alcohol. Write a paragraph using the table describing the logic that led you to this conclusion.

Discussion Questions

Pre-lab discussion questions:

1. Explain how Reaction 1 is a "transesterification" reaction.
2. How is the active site of an enzyme chiral? Why is the complex formed by the interaction of each enantiomer of the 1-phenylethanol with the active site of the enzyme a diastereomer?
3. What do you expect to see by TLC during the initial reaction? How will you know when the reaction is "done" (has used all of one enantiomer, if it is going to do that)? What will you see when the reaction is past "done"?
4. Why is a mixture of hexane and ethyl acetate used to develop the column? Why is the mixture changed from more hexane/less ethyl acetate to relatively less hexane and more ethyl acetate?
5. Identify the benzylic proton in (*S*)-1-phenylethyl (*R*)-acetoxyphenylacetate. Show how it is diastereomerically different from the benzylic proton in (*R*)-1-phenylethyl (*R*)-

acetoxyphenylacetate. What is the spin-spin splitting of the benzylic hydrogen? What is the splitting on the methyl group?

6. Show how to calculate "2 molar equivalents". How many grams of EDC are "2 molar equivalents" in this experiment?

Post-lab discussion questions (include your responses in your laboratory report):

1. Did all of the alcohol react? How can you tell from the ^{1}H NMR?
2. Which has a larger R_f, the alcohol or the ester?
3. Which enatiomer of the initial alcohol was preferentially used by the enzyme?

Instructor Notes

1. Other acylase enzymes (porcine, for instance) do not work for this experiment.
2. Setting up the enzyme reaction in Part 1 ("week 1") can be started at the end of the previous lab period as it only takes 15-20 minutes to prepare the sample reaction. The rest of the experiment would be completed over the next two weeks.
3. Recommendations for preparing the column for fractionation: 8 ml of dry silica gel is more than a Pasteur pipette will hold. An appropriate sized column may be available in your microscale kit. If not, one can be easily made from a10 mL disposable pipette.
4. EDC forms a "derivative" of the carboxylic acid similar to an acid chloride. The EDC group is displaced by an alcohol:

   R-COOH + EDC → R-COOEDC (reacts readily with 4-DMAP catalyst)

   R-COOEDC + R'OH → R-COOR'

   *Both reactions occur more readily, under gentler conditions, and in higher yield than the direct reaction of R-COOH + R'OH → R-COOR'
5. A streamlined procedure may be used instead of having each student weigh out tiny amounts of reagent. This saves a substantial amount of reagent and lots of student time. You may prepare solutions of each reagent in dichloromethane for your whole lab section such that 1 mL of solution contains 2 molar equivalents relative to the alcohol. The amount of EDC hydrochloride is 0.77 g mL^{-1}, DMAP 0.05 g mL^{-1}, and (*R*)-acetoxy acid 0.66 g mL^{-1}. These amounts can be weighed out in advance and the solution made the day of the lab. Solutions are good for at least 8 hours. The students add 1 mL of each solution to their 1-phenylethanol in place of the 2 mL of dichloromethane. They must be added in the order of EDC, DMAP, and then acetoxy acid.

6. The ^{1}H NMR Spectra.

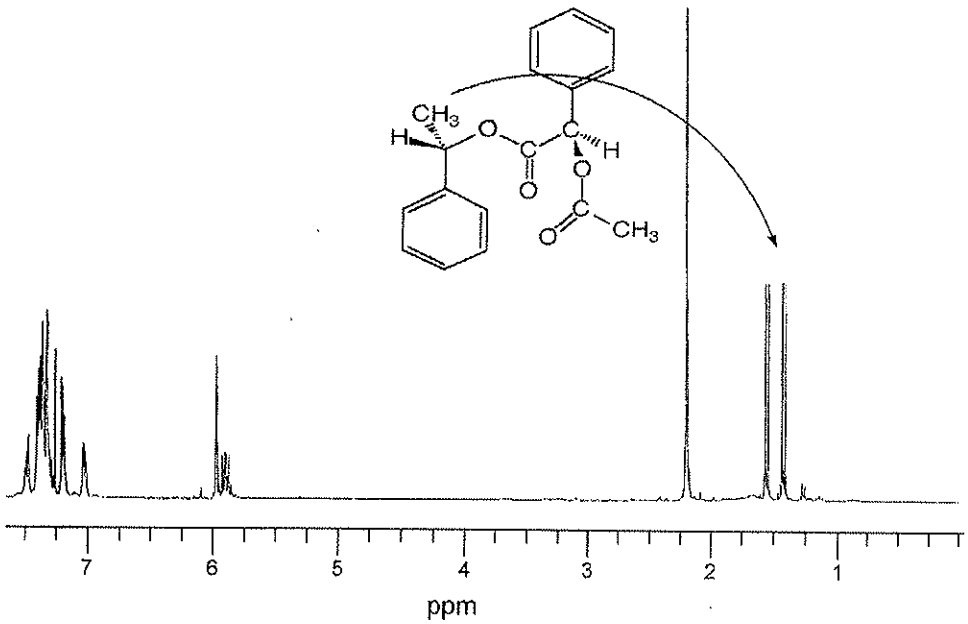


Figure 1. 300 MHz spectrum of 1 *(S)*-1-phenylethyl *(R)*-acetoxyphenylacetate made from the racemic alcohol. The doublet from both the R-alcohol ester and the *S*-alcohol ester are present.

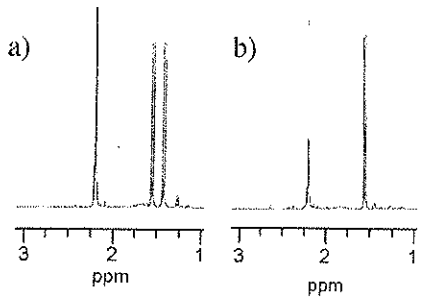


Figure 2. The 1-3 ppm region is expanded to show a) a mixture of the two diastereomers and b) the pure *R,R*-diastereomer, illustrating how clearly the different diastereomers can be seen. A little of the *R,S*-diastereomer is also present.

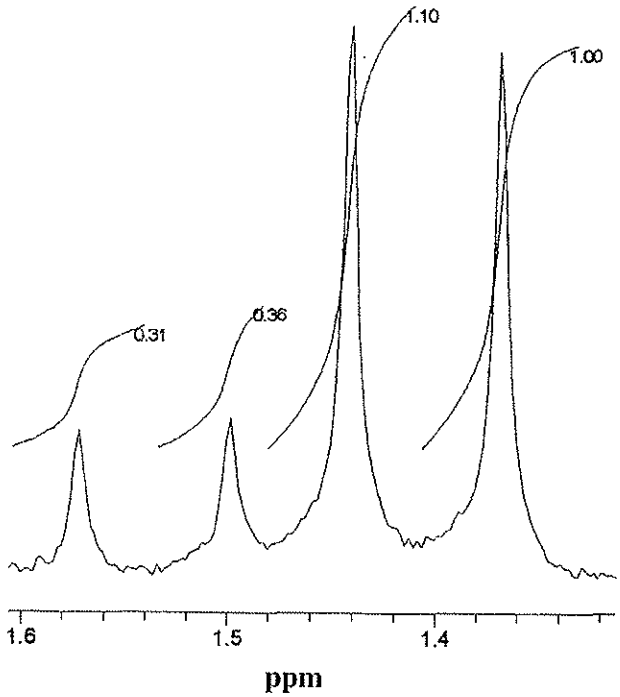


Figure 3. Examples of student data acquired on a 90MHz NMR of ester made from enzyme treated alcohol.

The enantiomeric excess in the treated sample shown in Figure 3 is:

Integration of the *R,R* doublet	0.67 (0.31 + 0.35)
Integration of the *S,R* doublet	2.10 (1.10 + 1.00)
Percent of *R,R* isomer	24% (67/(67+210))
Percent of *S,R* isomer	76% (210/(67+210))

Enantiomeric excess = % major isomer - % minor isomer = 76-24 = **52**

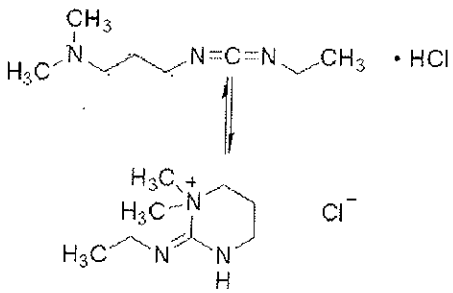
Note: If less enzyme is used, the reaction may not be complete in seven days, resulting in excess *R*-enantiomer remaining. In our laboratory in 2008, using 20 mg of acylase I, the enantiomeric excess of the *S*-alcohol was about 52%.

7. Variations: The extent of reaction of the alcohol depends on the amount of enzyme, the temperature, and the amount of time the alcohol is allowed to react. Any of these can be varied to change the enantiomeric excess. The experimental conditions presented here are for a large amount of enzyme, room temperature, and one week.

   80 mg of acylase should cause all of the *R*-enantiomer of 1-phenylethanol to react, but using only 20 mg may leave some unreacted *R*-enantiomer. Using ^{1}H NMR and/or polarimetry can allow students to calculate an enantiomeric excess for amounts of reaction less than 100%.

   If other alcohols are used, the class may explore which alcohol structures the enzyme "prefers," (i.e., which react faster). (See by Faraldos J.; Arroyo, E.; Herradón, B.; *Synlett* **1997**, *4*, 367-370.)

8. Structure of EDC:



9. Structure of 4-DMAP:

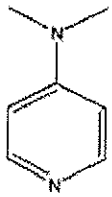


Table of Reagents

Reagent	CAS Number	Purchased from	Catalog Number	Comments
R-(+)-1-phenylethanol	[1517-69-7]	Sigma-Aldrich	77848	
S-(-)-1-phenylethanol	[1445-91-6]	Sigma-Aldrich	77849	
Sec-phenylethyl alcohol	[98-85-1]	Sigma-Aldrich	P13800	cancer suspect agent, toxic
vinyl acetate	[108-05-4]	Sigma-Aldrich	V-150-3	cancer suspect agent, flammable
acylase I from *Aspergillus* sp.	E.C.3.5.1.14 N-acylamino acid amide hydrolase	TCI America	0688	porcine acylase does not work
4-DMAP, 4-dimethyl-aminopyridine	[1122-58-3]	Sigma-Aldrich	522813	highly toxic, corrosive
EDC, 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride	[29952-53-8]	Sigma-Aldrich	161462	irritant
R-(-)-2-acetoxy-phenylacetic acid, (-)-O-acetyl-D-mandelic acid	[51019-43-3]	Sigma-Aldrich	253030	NMR derivitizing agent, *J. Chem. Soc., Perkin Trans.* 2, **1983**, *1*, 83-88.
S-(+)-2-methoxy-phenylacetic, O-methyl-L-mandelic acid	[26165-26-1]	Sigma-Aldrich	248983	
R-(-)-2-methoxy-phenylacetic acid, O-methyl-D-mandelic acid	[3966-32-3]	Sigma-Aldrich	248967	NMR derivitizing agent, *Tetrahedron: Asymmetry* **2006**, *17*, 1979-1984.