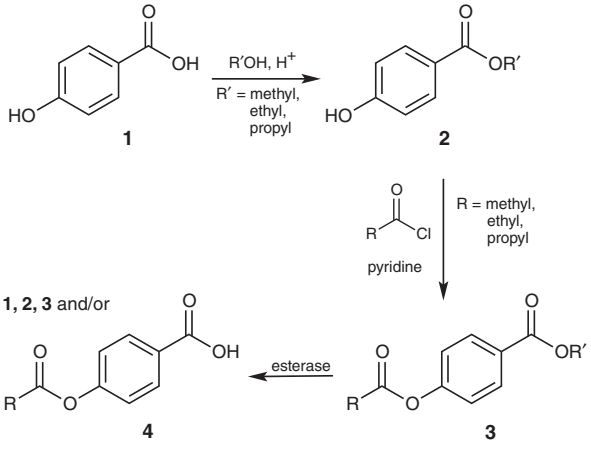
The Preparation and Enzymatic Hydrolysis of a Library of Esters

Elizabeth M. Sanford* and Traci L. Smith
Department of Chemistry, Hope College, Holland, MI 49422-9000; *sanford@hope.edu

Second-year level organic chemistry lab strives to equip students with a repertoire of techniques for the synthesis and characterization of organic compounds. Often left out of standard organic lab are newer tools that organic chemists have adapted from biochemistry and molecular biology such as the use of enzymes. Laboratory training that demonstrates the use of biological tools to solve chemical problems will help produce more interdisciplinary-minded and skillful students, currently a mandate across the sciences *(1)*.

With interdisciplinary training in mind, we sought an existing experiment that used multistep organic synthesis of reactions typically covered in second-semester introductory organic chemistry and involved an affordable enzymatic synthesis on a preparative scale with extensive characterization of each step including in-depth ^{1}H NMR analysis. The experiment "Regiospecific Ester Hydrolysis by Orange Peel Esterase" designed by Bugg et al. in this *Journal (2),* gave us a starting point in providing a multistep synthesis based on carboxylic acid chemistry and using an affordable enzymatic synthesis. It, however, was not designed to compare chemical to enzymatic synthesis, was not run on preparative scale, and did not include extensive characterization. We were able to adapt it to do both chemical and enzymatic steps on large scale and developed a spectroscopic analysis of the products including the product of the enzymatic hydrolysis (Scheme I). The original publication used thin-layer chromatography (TLC) as characterization for the enzymatic hydrolysis.

We also couched the experiment in a case study that made students work cooperatively to prepare a library of esters and then to try to selectively hydrolyze the compounds with two different enzymes *(3)*. Students then had to share their data and each had to come to a conclusion about the effectiveness of enzymatic hydrolysis for the stated goal of the case study. Including group work and having each student make a defendable recommendation as an "expert" were also goals of this experiment *(4)*.



Scheme I. Sequence of reactions with possible outcomes for enzymatic hydrolysis.

The case study told students that a start-up company had natural sources for precursors to a valuable antifungal compound. The naturally found precursors were diesters of type **3** and two contained chiral R′ groups. The antifungal compounds were hydroxy esters of type **2**. The value of having an inexpensive natural source of an enantiomerically pure chiral synthon was presented to the students as a potential gold mine for this start-up company. The only step needed to convert the naturally abundant precursors to the desired antifungal agents was a selective hydrolysis to convert the naturally occurring diesters of type **3** to **2.** This selective hydrolysis, however, was not chemically possible. It was then the students' job to synthesize a library of model compounds based on **3** to see whether the desired hydrolysis was possible enzymatically and whether they could further refine their choice of substrate to make the enzymatic hydrolysis as effective as possible.

Students had to collectively synthesize and characterize the methyl, ethyl and propyl esters of *para*-hydroxybenzoic acid, **1,** via a Fischer esterification and then prepare all combinations of the diester of these compounds using acetyl-, propionyl-, and butyryl chlorides via alcoholysis. Each of these diesters was then subjected to enzymatic hydrolysis with orange peel esterase (as a crude extract) or commercially available pig liver esterase. Each product was characterized by melting point, IR, and ^{1}H NMR. In theory the enzymatic reaction could give 4-hydroxybenzoic acid **1**, **2**, **3** (no reaction), or **4**. According to the case study, the desired hydrolysis product was **2**. Using ^{1}H NMR, students had to figure out what compounds were produced in the enzymatic reaction. Each student independently completed three sequential reactions. Students then pooled data for the enzymatic reaction and analyzed and defended their results and conclusions in writing. This experiment was completed with ninety-eight students in three, five-hour laboratory periods. In addition three fifty-minute laboratory lectures were devoted to explaining the case, the mechanism of Fischer esterification, alcoholysis of an acid chloride, and chemical and enzymatic hydrolysis of an ester.

Experiment

Each student played the role of a technician in an industrial lab who was responsible for preparing one substrate and hydrolyzing it with an assigned enzyme. In part 1 there were three different reactions; in part 2 there were nine different reactions; and in part 3 there were eighteen different reactions run.

Parts 1 and 2

Students were given a general protocol for a Fischer esterification *(5)* and for the alcoholysis of an acid chloride *(6)* that they could each use for their particular substrate assignment. The products were characterized with melting point, IR, and ^{1}H NMR. After each step, students had to provide the instructor with a description of the full characterization and a conclusion whether the material was adequate to proceed to the next step. Products were obtained in high yield and purity for both steps.

Part 3

Students performed the enzymatic hydrolysis on at least 0.1 g of diester. They followed the reaction by TLC and worked up the product when hydrolysis was complete or at the end of three hours. The enzymatic hydrolysis led to some pure products and some product mixtures. Identification of the products was done using ^{1}H NMR. In the case of mixtures, using the aromatic protons to calculate the ratio of products proved to be most straightforward. Students characterized their products using melting point, TLC, IR, and ^{1}H NMR. They shared yield data and product identification and composition with their classmates.

Each student wrote a report as a technician that described his or her individual three-step synthesis with characterization of each step. Each student then used the compiled class data to decide which, if any, substrate and enzymatic hydrolysis could be used to produce the target compounds described in the case.

Hazards

Methanol, ethanol, propanol, diethyl ether, pyridine, acetyl chloride, propionyl chloride, butyryl chloride, and acetone are flammable. Methanol is toxic. The acid chlorides are all corrosive and lachrymators. The acids and bases used are all corrosive. All of the compounds used are harmful or irritants and should be handled in a manner consistent with the appropriate material safety data sheet. Acid chlorides were dispensed to students in their fume hood. Students using butyryl chloride were instructed how to wash glassware and dispose of waste to eliminate stench.

Results

Overall the esterifications were straightforward. The enzymatic hydrolysis data show that both pig liver esterase and orange peel esterase can selectively hydrolyze diester **3** to the compound targeted in the case study, **2**. Pig liver esterase was most efficient at the hydrolysis for all substrates and selectively cleaved **3** to **2** regardless of the identity of R and R′. Orange peel esterase also cleaved **3** to **2** but was more selective in that the identity of the R′groups mattered leading to near complete hydrolysis when R′was methyl and partial or no hydrolysis when R′was ethyl and propyl. This experiment can be expanded to be inquiry based by having students decide which compounds to prepare and using a larger number of esterases.

Conclusion

An investigative case study was developed that involves a sequential three-step synthesis. Students are challenged to use enzymatic synthesis to solve a chemical problem. ^{1}H NMR was used extensively to analyze the product of each step including product mixtures in some cases. Students had to approach the project cooperatively and share data. At the end of the experiment each student had to act as an expert and make a recommendation concerning the route to the desired compounds. Students were surveyed about their learning by rating their understanding gained from the experiment from 1 to 5 where 1 is not at all and 5 is a great deal. The average score on five questions relating to the enzymatic reactions was 3.9. The score on analyzing real-world data was 3.8. The score on how to come to and defend a real-world decision was 3.5. The score on completing a three-step synthesis was 4.4. All students who responded to the question of whether to do this lab again said yes and rated it very favorably. A majority of the students felt the case study added to their interest. A majority of students also indicated that they were able to make connections to their coursework in biology. When asked what they gained from bringing the chemistry and biology perspectives together, the majority responded with positive statements such as "an understanding of the connection between the two and hands-on experience with both", "how one discipline can complement another, and how if a goal can't be achieved using traditional methods, approach it using another discipline", "It made me have a better appreciation for how we need chemistry as biologists", and "It gave an insight into the biology world for chemists and showed just how closely connected the two branches were." Many students also indicated that they found successfully completing three sequential reactions rewarding.

Acknowledgments

Development of this lab was supported by a Howard Hughes Medical Institute Undergraduate Science Education Program Award to Hope College. We acknowledge Gregory Borst, Joanne Stewart, Leah Chase, and Greg Murray for their input in the development of this lab and Jason Gillmore, William Mungal, and all of our organic chemistry lab students for its successful implementation.

Literature Cited

1. The National Academies Press. *http://www.nap.edu/catalog/10633.html* (accessed Mar 2008) and *http://www.nap.edu/books/0309085357/html/* (accessed Mar 2008). Burroughs Wellcome Fund News and Publications. *http://www.bwfund.org/news/special_reports/special_reports_the_role_of_the_private.html#top* (accessed Mar 2008).
2. Bugg, T. D. H.; Lewin, A. M.; Catlin, E. R. *J. Chem. Educ.* **1997,** *74,* 105–107.
3. Millis, B. J.; Cottel, P. G., Jr. *Cooperative Learning for Higher Education Faculty;* Oryx Press: Phoenix, 1998.
4. King, P. M.; Kitchener, K. S. *Developing Reflective Judgment;* Jossey-Bass Publishers: San Francisco, 1994.
5. Williamson, K. L. *Macroscale and Microscale Organic Experiment,* 2nd ed.; Houghton Mifflin: Boston, 1994; pp 385–388.
6. Doyle, M. P.; Mungall, W. S. *Experimental Organic Chemistry;* John Wiley and Sons: New York, 1980; pp 263–266.

Supporting *JCE Online* Material

http://www.jce.divched.org/Journal/Issues/2008/Jul/abs944.html

Abstract and keywords

Full text (PDF)
Links to cited URLs and *JCE* articles

Supplement
Detailed student instructions and materials, instructor notes, experimental and survey results, and lists of chemicals and their hazards