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Laboratory Experiment

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Drug Synthesis and Analysis on a Dime: A Capstone Medicinal Chemistry Experience for the Undergraduate Biochemistry Laboratory

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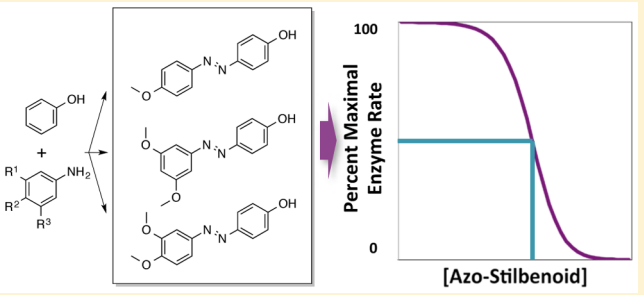
Supporting Information

ABSTRACT: Integrative, research-based experiences have shown tremendous potential as effective pedagogical approaches. Pharmaceutical development is an exciting field that draws heavily on organic chemistry and biochemistry techniques. A capstone drug synthesis/analysis laboratory is described where biochemistry students synthesize azo-stilbenoid compounds and test the biological activity of those compounds as well as a known inhibitor on mushroom tyrosinase using UV/vis-based kinetic assays. In this paper, three such successful azo-stilbenoid inhibitors of tyrosinase, representative student generated data, technical aspects of the experiments, and an interpretation of student feedback on the project as a whole are presented.



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Essential skills that biochemistry students should master in an undergraduate curriculum have been identified by the biochemistry and molecular biology community in a series of national workshops and regional meetings sponsored by ASBMB and funded by a Research Coordination Network-Undergraduate Biology Education Track (RCN-UBE) grant from the National Science Foundation (NSF) for The Biochemistry and Molecular Biology Concept Inventory Project. These foundational skills include experimental design, analysis and interpretation of data, communication and comprehension of science, and working safely in teams.[1] In addition to these broader skills, there are a number of specific technical skills critical for success in the biochemistry laboratory such as accurate pipetting, solution preparation, and proper use of instrumentation. It is also desirable for students to be able to integrate prior material and apply it in a new application with unknown outcomes.

In order to address all of these desired goals, a capstone multiweek research-based kinetics experiment was designed for an undergraduate biochemistry laboratory course that includes the organic synthesis of an azo-stilbene compound and subsequent testing of its ability to inhibit tyrosinase. This medicinal chemistry experience gives students exposure to drug synthesis and testing in the biochemistry laboratory and serves to integrate skills with concepts from our biochemistry curriculum. Such integrative capstone experiences have been shown to be high impact educational exercises that carry many of the same benefits as undergraduate research, such as improved oral and written expression and critical thinking.[2−5] More importantly, these gains have been found to be independent of academic divisions, GPA levels, and gender.[2−5]

Originally synthesized a century ago as dyes, azo-stilbene compounds have demonstrated potential as pharmaceuticals, tools for chemical biology, and food additives.[6] Polyphenolic versions of these compounds, known as azo-stilbenoids, have been previously identified as inhibitors of tyrosinase.[7] There are a variety of known synthetic methods for obtaining these compounds. In the synthesis chosen for this laboratory, electron rich anilines are reacted with sodium nitrite under acidic conditions to generate a diazo intermediate, which subsequently undergoes electrophilic aromatic substitution with phenol under mildly basic conditions (Scheme 1). The desired highly colored azo-stilbenoids precipitate in remarkable purity from acidic aqueous solution. The described synthesis can easily be performed in one lab period of 3 h and 10 min and has been used successfully by undergraduate research students at St. Mary's College of Maryland to make a potent and photoisomerizable tubulin polymerization inhibitor.[8] Each

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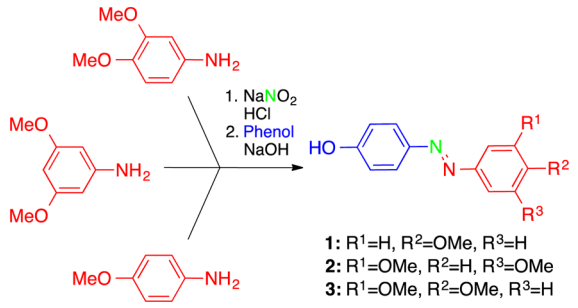
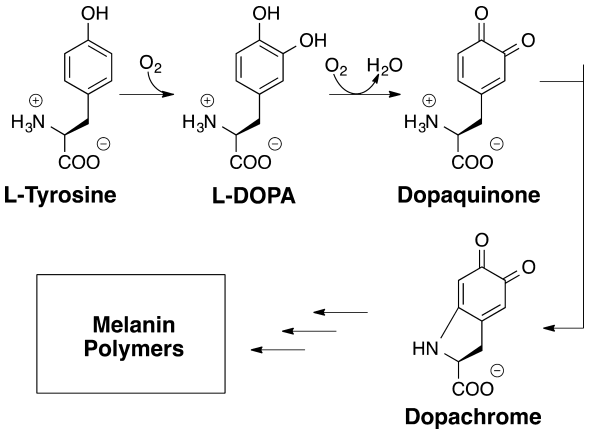
Scheme 1. Synthesis of Azo-Stilbenoids



student group synthesizes one proposed compound. After synthesizing their azo-stilbenoid, each group of students analyzes the identity and purity of their chosen compound by ^{1}H NMR spectroscopy, calculates the percent yield, makes a stock solution of their compound, and tests its activity as a tyrosinase inhibitor.

Tyrosinase is a well-studied enzyme, which is responsible for melanin production from tyrosine (Scheme 2). Overproduction

Scheme 2. Sequential Oxidation of L-Tyrosine by Tyrosinase To Produce Colored Product Dopachrome



of melanin is implicated in a variety of skin pigmentation disorders, making tyrosinase a therapeutic target of significant potential.[9,10] Tyrosinase is also commonly used to study enzyme kinetics in an undergraduate biochemistry laboratory as it displays classic Michaelis–Menten kinetics, and there are several well-established and commercially available compounds that inhibit it by diverse mechanisms.[11−15] Mushroom tyrosinase is also a commercially available and stable enzyme that holds activity in solution for at least a week if kept at 4 °C. In addition, an intermediate product of tyrosine oxidation by tyrosinase is dopachrome, which can be measured using a UV/vis spectrometer, making kinetic assays easy to implement in an undergraduate laboratory.

EXPERIMENTAL SECTION

The materials provided to the students for each week of this lab experiment, along with the pedagogy document outlining the learning outcomes, learning opportunities, and assessment, can be found in the Supporting Information of this paper. In the first week of the experiment, students work in teams of 2 or 3 to perform the azo-stilbenoid synthesis (Table 1). In addition, they complete preliminary kinetic assays and use their data to

Table 1. Schedule of Laboratory Activities

Week	Laboratory Activity
1	Azo-stilbenoid synthesis
	Assays to determine tyrosinase volume needed
	Assays to determine concentrations of known inhibitor to use
2	Calculate yield of azo-stilbenoid inhibitor
	Evaluate inhibitor purity/identity by ^{1}H NMR
	Generate inhibitor stock solution in DMSO
	Known inhibitor kinetic assays
3	Azo-stilbenoid inhibitor kinetic assays
4	Finish any remaining experiments, if needed
	Work on kinetics lab report

design their experimental methods, which include determining the ideal volume of tyrosinase stock solution and the concentrations of a known inhibitor (cyanide, azide, cinnamic acid, sodium benzoate) to use in subsequent experiments. In week 2, students evaluate the identity and purity of their compound and use Lineweaver–Burk analysis to determine the type of inhibition and calculate the K_I and/or K_I' of their known inhibitor. They also calculate the yields of their azo-stilbenoid compounds and prepare 50 mM solutions in dimethyl sulfoxide (DMSO). In the final week of the experiment, students determine the IC_{50} of their synthesized azo-stilbenoids. At the conclusion of the project, each student group submits a detailed written report in the form of a research paper outlining their results.

HAZARDS

Phenol and anilines are potential health hazards and should be handled with care. In addition, sodium nitrite is a known poison and carcinogen, so appropriate protective equipment should be worn. Sodium azide and sodium cyanide are highly toxic in solution and extremely toxic as gases, so much care should be used in their handling and to prevent generation of gas by acidification of their waste streams. The NMR solvent, $CDCl_3$, is a potential carcinogen and is a known health hazard. It should be worked with in a fume hood, and appropriate protective equipment should be worn. DMSO is known to rapidly penetrate the skin and carry compounds dissolved in it through the skin. Disposable gloves only provide very brief protection against DMSO. If a glove is exposed to DMSO, it should be removed immediately and replaced. Finally, all azo-stilbenoid products should be handled using gloves to avoid exposure as they are designed to be biologically active.

RESULTS AND DISCUSSION

Chemical Synthesis

Students were generally able to produce their desired azo-stilbenoids in high purities although yields varied widely. Yields for successful product synthesis typically ranged from 50% to 80%. Some product was lost during filtration, which decreased the percent yield. Occasionally, a group was unable to produce an azo-stilbene precipitate, often because they did not closely follow the synthesis directions. For example, temperature plays an important role in a successful synthesis as the reaction must be cold enough to prevent evolution of the N_2 gas and decomposition of the electrophilic intermediate. If a student group was not initially successful with the synthesis, they either re-performed the synthesis or used product from another group. As very little product is needed to perform the inhibition

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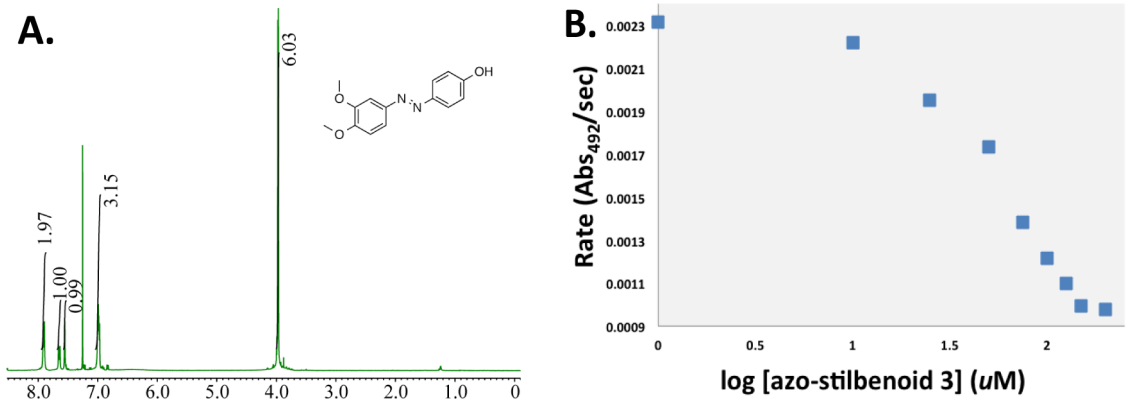

Figure 1. Representative experimental student data for compound **3**: (A) ^{1}H NMR spectrum; (B) IC_{50} data.

testing, the synthesis was designed to produce some excess product to share among unsuccessful groups. A representative ^{1}H NMR spectrum of one of the student synthesized products, compound **3**, taken with a JEOL 400 MHz NMR, is shown in Figure 1A. Representative spectra for the other two student synthesized products, compounds **1** and **2**, as well as a full integration analysis for all three compounds, are presented in the Supporting Information.

There were several important considerations when carrying out the synthesis of these azo-stilbenoids. First, the use of electron rich anilines was critical as the rate of electrophilic intermediate formation is related to the nucleophilicity of the aniline. As such, the use of electron rich anilines was required to keep reaction times short enough for completion during a single laboratory period. Conveniently, electron rich substituents are common to the reported stilbenoid and azo-stilbenoid tyrosinase inhibitors.[7] Three anilines were identified that produced exceptional results in the synthesis and kinetic assays, though there are large numbers of commercially available anilines that could be utilized for this laboratory. Others have been tried by students in different semesters; at least one new aniline was tried each semester as this added to the research-based experience of the experiment, but only the three most successful were reported here. At least one group, and ideally at least two groups, based on lab section size, tested each of the available anilines.

For the synthesis, it was critical to keep the reaction temperature below 0 °C until the phenol had reacted to prevent decomposition of the aryl diazo intermediate into nitrogen gas. As this reaction was not particularly sensitive to aerobic conditions, it was run in a beaker or Erlenmeyer flask. Likewise, the solid products of this reaction were stable at room temperature and under aerobic conditions for months. Additionally, this reaction was particularly attractive for production of small molecule inhibitors in an undergraduate laboratory setting because the reaction was run in ethanol and had excellent atom economy, and the product simply precipitated in relatively high purity from acidic aqueous solution, removing the necessity for expensive, wasteful, challenging, or time-consuming purification procedures.

Inhibition Assays

Using Lineweaver–Burk plots to determine inhibition type helped students understand aspects of enzyme kinetics, including how different types of inhibitors impact kinetic parameters. As use of Lineweaver–Burk plots has been previously reported for undergraduate biochemistry laboratories using tyrosinase,[13,15] this discussion will focus on the IC_{50} analysis of azo-stilbenoids. In general, students were able to obtain high quality dose–response plots (enzyme activity vs inhibitor concentration) for their azo-stilbenoid compounds (Figure 1B and Supporting Information). For the three compounds reported here, IC_{50} values ranged from 50 to 150 μM. Using IC_{50} values for azo-stilbenoid analysis was more amenable since it required fewer measurements and was therefore less time intensive. Additionally, the determination of both K_I and IC_{50} was a useful exercise as IC_{50} data are ubiquitous in medicinal chemistry literature, yet are not prominently discussed in most biochemistry textbooks. Specifically, it was useful to outline the appropriate use of each value and introduce the advantages and limitations that IC_{50} measurements and Lineweaver–Burk analyses have with respect to measuring enzyme inhibition.

Research-Based Laboratories

These experiments were designed as research-based laboratories where students selected an azo-stilbenoid to synthesize and were not given full sets of instructions but instead were asked to develop optimal procedures on their own. Such experiences, where the outcomes of the experiment are unknown, are uncomfortable but valuable exercises for undergraduate students. Each iteration of the experiment has grappled with a new set of variables. For example, L-tyrosine (K_m = 0.5 mM)[16] and L-DOPA (K_m = 1.5 mM)[16] are both substrates of tyrosinase and, while early versions of the laboratory used L-DOPA as a substrate, its fast reaction kinetics and spontaneous oxidation in air necessitated its replacement with L-tyrosine. Students then worked to determine the optimal biochemical assay conditions, such as tyrosinase and inhibitor concentrations. Another variable the students had to address was absorbance interference between the highly colored azo-stilbenoid compounds and dopachrome. As a result, it was necessary to determine an ideal wavelength for measuring dopachrome generation as well as the maximum inhibitor concentration that could be used to minimize interference from the absorbance of the inhibitor (λ = 492 nm).

Capstone Nature of the Experiment

This experiment is considered a capstone experience or signature work as described by the Association of American Colleges and Universities (AAC&U) where students demonstrated their ability to integrate learning from multiple sources through working on a question or problem that was important to the student and important to society.[17] This experiment required students to synthesize and integrate skills from

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biochemistry as well as their organic chemistry and general chemistry laboratory courses. The reaction itself facilitated the integration of a variety of critical chemical concepts. In prelaboratory meetings, pK_a values and the importance of pH at various stages of the reaction were discussed. In addition, regiochemistry of electrophilic aromatic substitutions and resonance structures were featured prominently when discussing the expected products of the reaction, since the para-directing nature of the phenolic −OH can be justified using resonance and steric arguments. These discussions, in addition to discussions of rational design and tyrosinase's natural substrates, assisted students in selecting their aniline. The students indicated special interest in this drug design element of the project.

Once the product was isolated, students demonstrated even more general and organic chemistry skills such as percent yield calculations and solution preparation. ^{1}H NMR spectra of the products in $CDCl_3$ were obtained, which allowed each group to analyze the identity and purity of their products. Given the differences in symmetry, number of aromatic protons, and methyl groups in the target compounds, the NMR spectrum for each compound was quite distinct, making identity analysis achievable. At institutions where NMR analysis is not available, the purity of the product can easily be qualitatively assessed by silica gel thin layer chromatography because the product of these reactions is easily identified as a bright yellow or orange spot (See Supporting Information).

While students were asked to utilize their organic chemistry knowledge, the project also integrated numerous biochemical concepts. For example, students prepared a phosphate buffer solution that was used for all of the kinetics assays. For this laboratory, they were given only the desired concentration, volume, and target pH. Many biochemistry students struggle with buffer chemistry even though it is a topic covered extensively in both the biochemistry lab and the lecture course after its first introduction in general chemistry. Thus, further practice with buffer preparation and use helped reinforce pH concepts and allowed students to master this skill. Additionally, students routinely performed dilution calculations, used spectrometers, and prepared graphs using Excel throughout the multiweek lab. In particular, performing dilution calculations was a skill that some students found challenging so additional practice was beneficial, particularly in the application of a real world enzyme assay where total volume of each assay was kept constant. Accurate pipetting and mixing, which were themes throughout the course, were key to obtaining quality data. Students needed to safely handle chemicals and properly dispose of both aqueous and organic waste. The AAC&U has stated that students who engage in these kinds of "high impact" capstone experiences are more likely to complete college, are more engaged in their work, and show higher levels of deep and integrative learning.[17]

Written Assignment

The lab report consisted of an abstract, introduction, methods, results, and discussion. This format brought together the writing skills that were emphasized throughout the semester including preparation of figures, figure legends, and proper citation of the literature. While the students were provided with a list of the various sections that were required in the lab report with brief descriptions, this was not an exhaustive list of what was to be included in those sections. The students determined what should and should not be included. Students were required to write a thorough introduction, which gave background on different types of enzyme inhibition and specifically described tyrosinase inhibitors with appropriate citations. Students presented their results in figures with figure legends, showed relevant calculations, and compared their results to published studies. For example, it has previously been shown that cyanide acts as a mixed noncompetitive inhibitor for tyrosinase with catechol compounds,[18] while inhibition of tyrosinase using azo-stilbenoid compounds has also been described.[7] Instructors observed that the students did well at writing a detailed introduction, using appropriate citations from the literature. Students were successful at creating figures with appropriate figure legends. They were less successful in correctly performing all of the calculations to determine kinetic parameters, particularly K_I values. The discussion section showed the biggest range in scores among student groups.

Student Evaluation

To investigate students' perceptions of their experiences in this lab, a Likert scale survey was created and disseminated at two small liberal arts institutions, St. Mary's College of Maryland and University of Mary Washington. The questions were developed by first identifying the learning outcomes of the lab experience and then reformatting these in Likert format (the Likert questions and learning outcomes used can be found in the Supporting Information for this paper). For example, one learning outcome was "Students will be able to have increased confidence in working on research questions without a known outcome." This outcome was evaluated by the Likert question "This experiment increased my confidence in tackling research questions without a known outcome". Once created, the Likert scale was evaluated by the instructors involved in the study and a chemical educator to determine whether they sufficiently evaluated the desired learning outcomes and were appropriate for use with the students. After IRB approval, the seven question Likert scale was administered in a paper/pencil format to students on or soon after the final day of the lab experience. In the third semester of running the experiment at St. Mary's College of Maryland, data were collected from 36 students, and in the second semester of the experiment at the University of Mary Washington, data from 11 students were collected.

Overall, the experiment was well received by the students (See Table S1). A majority of students reported gains in several areas including understanding of the concepts of Michaelis−Menten kinetics, understanding of the experiments and calculations required to determine the kinetic parameters of an enzyme, and confidence in performing experiments with an unknown outcome. Faculty in these courses have also observed an improved ability of students to integrate diverse concepts from their previous courses, as well as gains in specific technical skills such as accurate pipetting and solution preparation. These outcomes fit nicely with the priorities of the Biochemistry and Molecular Biology Concept Inventory Project as outlined above.

The data reflect an overall positive experience by the students. Some of the comments made by the students on the survey were particularly interesting. Several comments indicated that the project was indeed increasing student understanding of the concepts in the lab but not in the ways one would expect. For example, several students commented that the work they invested in the final report outside of the laboratory contributed to their understanding of the concepts from the lab, while reporting that performing the experiments

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contributed only moderately to their understanding. These comments may indicate that students did not recognize writing the final lab report as a part of the overall lab experience. These students were nonetheless reporting gains in understanding of kinetics and inhibition calculations as a result of the project.

At St. Mary's College of Maryland, some students commented that they disliked the uncertainty associated with the length of the laboratory. While it was certainly possible to finish all of the experiments within the designated lab period, not all groups were able to complete the necessary lab work in 3 h and 10 min. For shorter lab periods, the laboratory could be divided over more periods or data from different groups could be shared. This format was validated at the University of Mary Washington where the laboratory is 2 h and 45 min, and the schedule of laboratory activities used is shown in Table S2. An extra week was added to the schedule, and no students expressed this concern in their feedback. Nonetheless, we have found that completion of the lab work on time is strongly correlated with adequate prelaboratory preparation, since correctly calculating the volume of each reaction component in each reaction can occupy an inordinate portion of lab time.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.6b00048.

Laboratory handout, instructor notes, student survey results, pedagogy document, laboratory schedule, instructions for written lab report, representative student results, instructions for alternative purity analysis by TLC, sample nonlinear regression analysis, and ^{1}H NMR and IC_{50} data (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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