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Laboratory Experiment

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Fighting Tuberculosis in an Undergraduate Laboratory: Synthesizing, Evaluating and Analyzing Inhibitors

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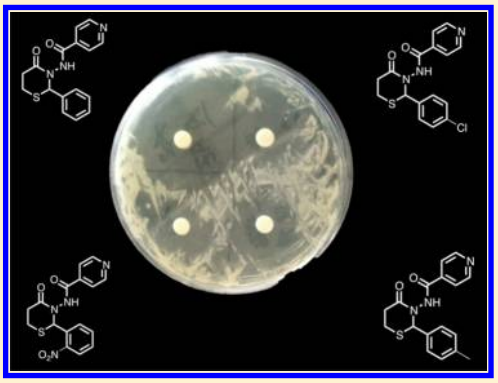
Supporting Information

ABSTRACT: A drug discovery project has been successfully implemented in a first-year general, organic, and biochemistry (GOB) health science course and second-year organic undergraduate chemistry course. This project allows students to apply the fundamental principles of chemistry and biology to a problem of medical significance, practice basic laboratory skills, and understand the interdisciplinary aspect of the drug discovery platform. Students collectively synthesize a small library of antituberculosis compounds and subsequently screen them using a Kirby−Bauer disc diffusion assay for inhibitory activity against *Mycobacterium smegmatis*, a known safe surrogate of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The last unit of the project involves students using PyMOL, a free computer program, to examine a cocrystal structure of the activated inhibitor−enzyme complex, allowing students to identify the molecular interactions responsible for the binding affinity.



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INTRODUCTION

Drug discovery has evolved to become a multidisciplinary platform involving many core sciences: chemistry, biology, biochemistry, and computational science.[1] Scientists working in the biotechnology and pharmaceutical industries must be comfortable working in interdisciplinary teams in order to design and develop drugs successfully. Therefore, it is important that college students gain experience working on problems with an interdisciplinary component. Several recent articles have reported using drug discovery experiments that consist of both the organic synthesis of inhibitors and the subsequent biological analysis of the synthesized compounds. Utilizing this approach as a means for conveying organic chemistry concepts has been shown to increase student engagement by presenting the concepts and techniques in a medicinal application context.[2,3] The laboratory project described here is a medicinal chemistry project reflecting the multifacet nature of modern day drug-discovery projects. The laboratory project was constructed to promote student engagement, and the practice of basic chemistry and biological techniques.

Overview

The project focuses on identifying inhibitors against one of the most deadly diseases, tuberculosis (TB). TB, caused by the microorganism *Mycobacterium tuberculosis*, affects approximately one-third of the world's population. TB is a readily recognized disease, which allows for the facile engagement of students with minimal knowledge of microbiology and infectious diseases.[4]

The laboratory experiment utilizes a combinatorial approach to construct a small library of biologically active derivatives of isoniazid, the most commonly prescribed anti-TB drug.[5] Isoniazid is an inhibitor of the enzyme 2-*trans*-enoyl-acyl carrier protein reductase (InhA), which catalyzes a key step in the synthesis of mycobacterial fatty acids (Figure 1).[6,7] During the experiment, students synthesize isoniazid derivatives using a one-pot, three-component condensation reaction consisting of isoniazid, a benzaldehyde derivative, and mercaptopropionoic acid in the presence of *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide (EDC) in tetrahydrofuran (THF) (Scheme 1). Upon isolation of the desired compound, students evaluate the compound's activity using a Kirby−Bauer disc diffusion assay. The compounds are assayed against *Mycobacterium smegmatis*, a safe surrogate of *Mycobacterium tuberculosis*.[8] Finally, students examine the molecular interactions between the active metabolite and the enzyme using PyMOL, thus allowing students to identify some of the fundamental chemistry interactions responsible for the observed biological activity.[9] Because isoniazid and the derivatives described here

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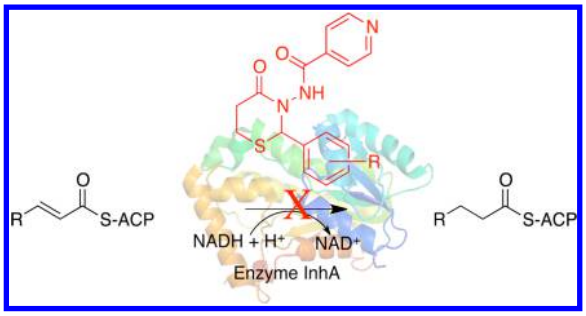
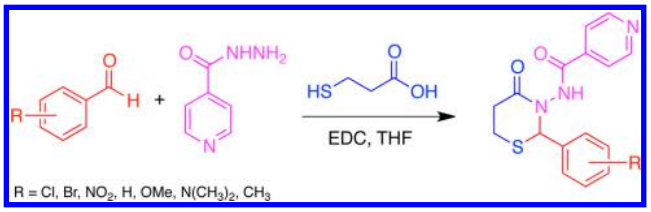


Figure 1. Depiction of the enzymatic reaction that is being inhibited by isoniazid derivatives. The inhibitors target InhA, which is shown in the background of the scheme.

Scheme 1. General EDC-Catalyzed Condensation Reaction for Producing a Derivative of a First-Line TB Drug, Isoniazid[a]



[a]In pink.

utilize a prodrug methodology, they are all metabolized to a common active metabolite, allowing for the use of a common cocrystal structure to be examined.[10]

THE EXPERIMENT

Synthesis

Students work individually or in groups (2 or 3) to synthesize one of eight derivatives of isoniazid in a single, 3 h laboratory period using a modified procedure of Ramani et al.[10] The reaction is a one-pot, three-component condensation reaction with isoniazid, EDC coupling, and a nucleophilic reaction. Students monitor the progression of the reaction by thin layer chromatography (TLC). After approximately 75 min, the reaction is quenched with water, causing the desired product to precipitate. The compound is collected via vacuum filtration and identified using melting point and IR spectroscopy. The compound is stored in a vial until the ensuing laboratory period.

Kirby–Bauer Diffusion Assay

In the second laboratory session, students analyze the inhibitory effects of their derivative against *Mycobacterium smegmatis*. Students prepare the appropriate inhibitory solution (see Supporting Information). A suspension of *M. smegmatis* cells are pipetted onto an agar plate and spread throughout the plate using a sterile spreader. Inhibitory solutions are pipetted onto individual plating discs and placed on the agar plate. Students should avoid placing discs in close proximity to other discs and the edge of the plate (see Supporting Information). Inoculated plates are incubated at 37 °C for approximately 24 h or until significant bacterial growth is observed (Figure 2). Following the appropriate incubation period, students analyze the potency of the compounds by measuring the zones of inhibition surrounding each disc.

Visualizing the Inhibition

The cocrystal structure of InhA-inhibitor (active metabolite) complex has previously been solved. Because isoniazid is a prodrug, the cocrystal structure contains the active metabolite. The derivatives synthesized here are also metabolized to this common active metabolite.[10] A PyMOL session file highlighting the inhibitor-enzyme interactions in the cocrystal is included in the Supporting Information (Figure 3).[11] The use of PyMOL allows for an exploratory activity that facilitates identification of intermolecular interaction between the active metabolite and enzyme by students.[12] Students are given a

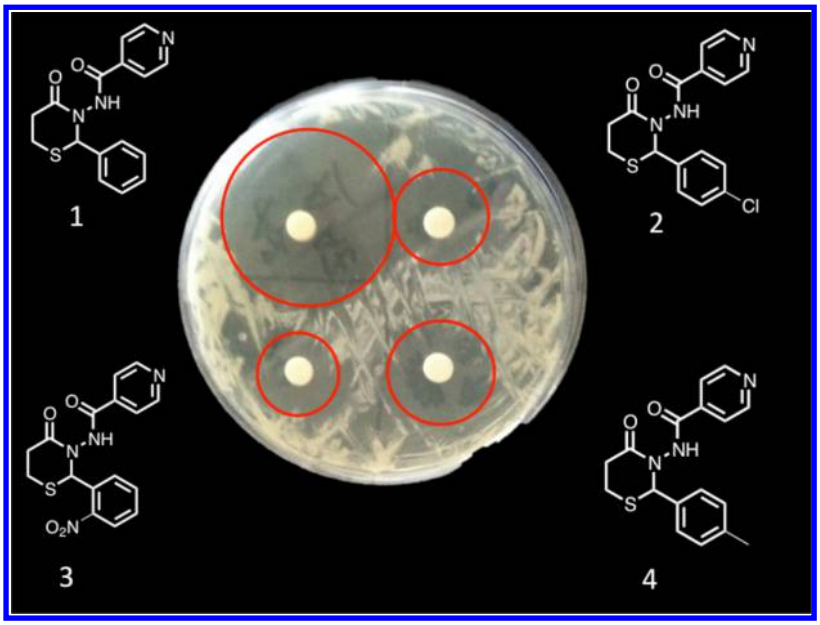


Figure 2. Student data; a Kirby–Bauer assay from one of the organic lab sections after 24 h of incubation at 37 °C. The structures of the inhibitors are shown by each of the respective discs. The red circles highlight the zones of inhibition, with large zones of inhibition corresponding to greater potency.

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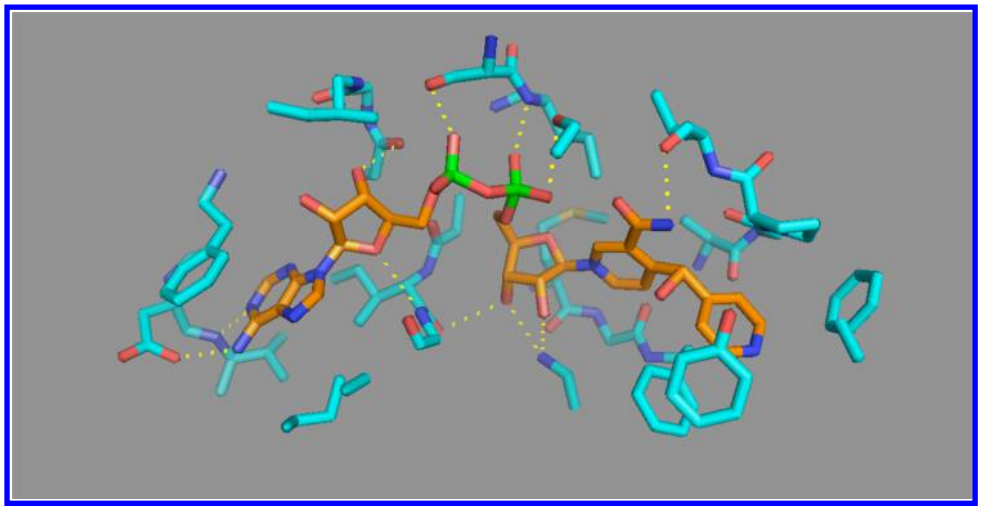

Figure 3. Screen shot of the active site with an inhibitor bound. Students use PyMOL to examine the interactions that contribute to the binding affinity.

handout with this snapshot and are asked to identify key intermolecular interactions between InhA and the inhibitor.

HAZARDS

Synthesis of the isoniazid derivative employs organic synthetic techniques and thus standard laboratory precautionary measures should be taken. Tetrahydrofuran is a flammable liquid that could also cause skin irritation. Dimethyl sulfoxide (DMSO) can penetrate the skin, as well as carry other compounds through the skin. DMSO and ethanol are both combustible liquids. Isoniazid can cause skin and eye irritation with direct contact. Although the compound is used in the treatment of TB, it should not be digested, as it can be hazardous. All of the substances should be handled with care and used in the fume hood. Appropriate laboratory attire should be worn, such as laboratory coats and suitable protective eye wear. *Mycobacterium smegmatis* is a safe surrogate for *Mycobacterium tuberculosis*; however, *Mycobacterium smegmatis* (biosafety 1) remains a bacteria and first-line precautionary measures, such as wearing gloves and lab coat, should be applied when handling the microorganism. The exact hazards of the synthesized products are unknown, but students should handle the compounds with caution. The inhibitors should not be ingested and kept away from the eyes.

RESULTS

The synthesis and biological analysis of *Mycobacterium tuberculosis* inhibitors were successfully completed by students in two chemistry courses at Merrimack College: (1) "Chemistry for the Health Professions II" (a first year course heavily populated by students majoring in sports medicine and athletic training (65 students)) and (2) "Organic Chemistry II," a second year course (45 students). Students capably synthesized and isolated a sufficient amount of compound for inhibitory analysis via the ensuing Kirby–Bauer disc diffusion assay. Students in the second year course were expected to preform the laboratory experiment at a higher level. Thus, greater accuracy in pipetting and meticulousness was expected from the students. First year students were not expected to analyze the product using IR, but secondary students were. Students were not asked to analyze the products by NMR due to the number of students and the practicality of doing at our institution, but it could be a modification other educators could incorporate. Lastly, second year students were expected to display a great understanding of how the chemical reactions proceed. Syntheses of the inhibitors were successful in both the first and second year laboratories. Percent yield for the reactions ranged from 40–90%, but even students obtaining particularly low yields had a sufficient amount of product to conduct the subsequent assay. The Kirby–Bauer Disc Diffusion Assay offers a quick and effective method for evaluating a compound's potency. Assay results can be readily interpreted: the size of the inhibitory zone is directly proportional to the potency of the compound. By comparing several derivatives on a single growth medium, students were able to observe the biological results of moderate chemical modifications to a molecular scaffold. Further, the use of PyMOL allowed students to visualize the molecular basis of the observed inhibitory activity from a molecular three-dimensional vantage point. PyMOL allows students to manipulate (rotate, zoom in/out) the structure for facile examination of the structure.[12] The program also allows students to identify the residues involved in a particular interaction, such as hydrogen bonding, van der Waals forces, hydrophobic/hydrophilic interactions, and π stacking. A snapshot of the PyMOL session file is shown in Figure 3, which highlights the active metabolite in the active site. Thus, allowing students to gain a greater understanding of small molecules can inhibit enzymes, using fundamental chemical interactions. The general consensus from student-written evaluations acknowledged a greater understanding of fundamental chemical interactions in a biological context.

A key component of the project is the open-ended possibilities it offers. The experiment can be tailored to the appropriate level of a course. The one-step synthesis and direct application of the Kirby–Bauer assay can be readily executed by students of nearly all skill levels in the biological sciences. However, this lab can also be modified to be appropriate for an advanced chemistry or biochemistry course. In addition, this project could be designed with an iterative process built into the construction of the chemical library. Students could be given the opportunity to synthesize their own library of compounds based on the activity of the compounds; students would synthesize a subsequent library based on their preliminary results. The project also offers a unique

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opportunity for collaboration of students in multiple courses and over a time-span of more than one semester. For example, students may synthesize and test compounds in a chemistry course, conduct susceptibility testing across multiple microbe species in a microbiology course, and isolate and characterize resistant mutants in a genetics course.

The pedagogical results were measure using a post laboratory questioner, direct student feed back and the evaluation of laboratory reports from this project as compared to previously submitted laboratory reports from the students. One of the primary goals of the project was to create an engaging laboratory experiment that would garner the interest of students from a wide variety of majors, which was successful based on student feed back. The second objective was to facilitate students' understanding of how chemistry applies to biological applications. This objective was also successful based on the student data that was collected.

CONCLUSION

Providing a pragmatic approach to combat an infamous disease, such as tuberculosis, helped captivate students' interest in a multiweek experiment. Students practiced basic lab techniques, such as pipetting, filtration, monitoring reactions by TLC, bacterial plate culture, and solution preparation. The project was well received by chemistry, biology, biochemistry, and health science majors. Feedback from students was very positive through written evaluations.

ASSOCIATED CONTENT

Supporting Information

A complete laboratory handout, instructor guide and PyMOL session file are available. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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