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Laboratory Experiment

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Searching for Synthetic Antimicrobial Peptides: An Experiment for Organic Chemistry Students

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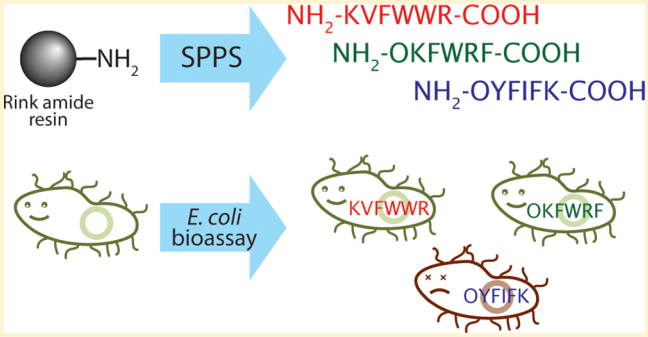
Supporting Information

ABSTRACT: This laboratory experiment provides undergraduate students enrolled in organic chemistry the opportunity to design and synthesize their own peptide, which is then tested for antimicrobial activity. After reading a primary scientific paper on antimicrobial peptides, students design and synthesize their own hexapeptide that they hypothesize will have antimicrobial activity. The students characterize their products by analyzing liquid chromatography−mass spectrometry and antimicrobial bioassay data for their synthesized peptide. The students are able to complete the synthesis and prepare their samples for analysis in three 3−4 h lab periods; instructors perform LC−MS and a bioassay with the peptides and provide data to students for analysis. This exercise is flexible and can be altered to include students performing the bioassay, or to meet different time constraints or target student populations. This experiment allows students to increase their knowledge of solid phase peptide chemistry and gain experience with developing and testing hypotheses through experimental design.



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The value of learning science by doing science should not be underestimated. Many important learning skills—including how to ask questions, how to critically analyze data, and how to construct arguments—can be developed in the teaching laboratory.[1−3] The teaching laboratory also allows for students to participate firsthand in scientific inquiry: they can observe, plan an experiment, formulate appropriate questions, develop rational hypotheses, design experiments, and analyze the results of these experiments.[1,4] Moreover, the laboratory provides an ideal venue to implement a *transformative teaching* model that emphasizes comfort with ambiguity, the search for uncertainty, and learning from failure.[5] Indeed, interest in including authentic research experiences in the teaching laboratory has grown over the years.[6−9] Engaging with experimental design, in particular, has been an area of focus for chemistry laboratory activities.[10−14]

At Pomona College, the upper level laboratory courses (e.g., Advanced Analytical Chemistry and Biochemistry) place an emphasis on engaging students in scientific inquiry. At the same time, much of the introductory curriculum (General Chemistry and First-Semester Organic Chemistry) focuses on expository instruction,[15] with an emphasis on developing laboratory manipulative skills, algorithmic problem solving and retention of content knowledge. Although it is recognized that pedagogical approaches can change throughout the students' time in the chemistry department, it is also acknowledged that the transition from expository toward open-inquiry laboratories might be uncomfortable, or even jarring.[16] Thus, an experience was sought out that would provide a scaffold for developing hypotheses and designing experiments, thereby easing the transition of students toward more open-inquiry laboratories and scientific research experiences.

A multiweek experiment was developed in which students synthesize a hexapeptide and subsequently analyze the results of a bioassay performed by the instructor or TA to measure the antimicrobial activity against a common strain of the Gram-negative bacterium *Escherichia coli*. The learning objectives (LOs) of the laboratory are

1. Communicate the general mechanisms involved in solid phase peptide synthesis
2. Use liquid chromatography−mass spectroscopy (LC−MS) data analysis to evaluate product formation in organic synthesis
3. Formulate a testable hypothesis based on presented data/information
4. Design and outline an experimental study to test a hypothesis, demonstrating mastery of technical issues such as controls and sample size

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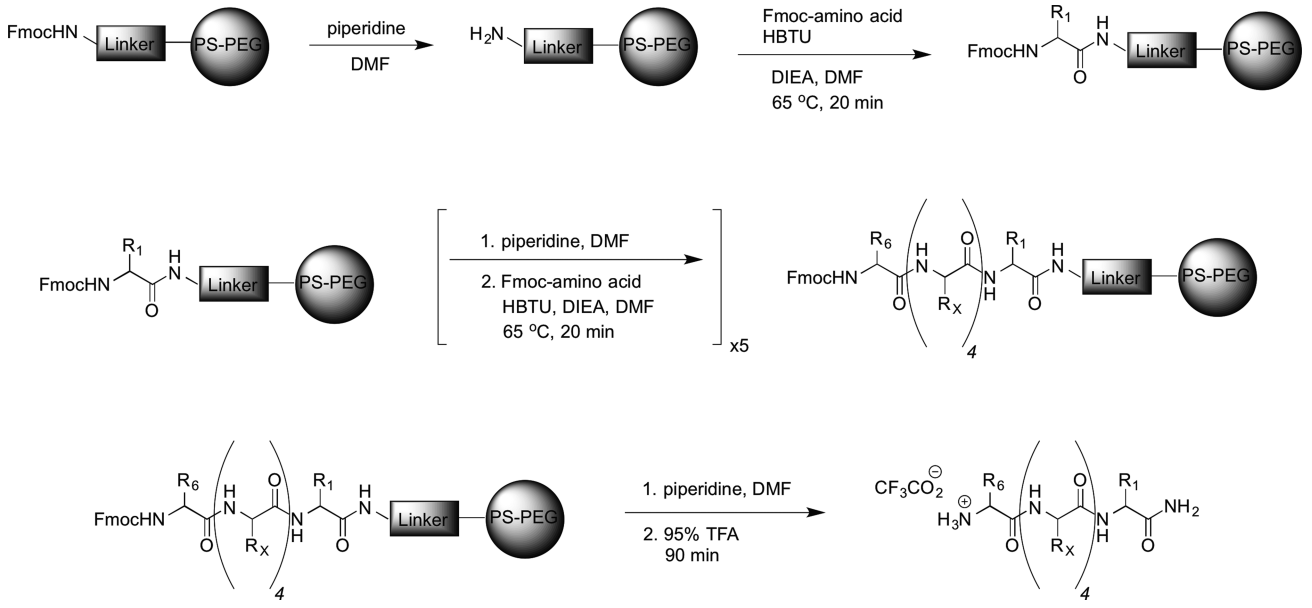
Scheme 1. Solid-Phase Synthesis of Hexapeptides



This laboratory was implemented in Second-Semester Organic Chemistry. On average, 79% of students who take this course go on to take Biochemistry, Advanced Analytical Chemistry, or both at some point following completion of organic chemistry. This laboratory could also be implemented in a medicinal chemistry course, or as part of an integrated course in which organic chemistry students synthesize the peptides and then provide purified products to microbiology, biochemistry or biophysics students for follow-up analysis of the effects of the peptides on bacterial growth or membrane dynamics.

This activity differs from other peptide-based laboratory exercises previously published in this *Journal* in that the students have a large degree of ownership over the entire process.[17−22] Depending on the rubric used, this exercise is a narrow project-based or a level 1−2 type inquiry-based laboratory activity.[4,15,23] The coupling of a bioassay with the synthesis also makes this experiment unique. To begin, the students are provided with a problem—antimicrobial resistance—and are introduced to the idea of antimicrobial peptides through a prelab video and a primary research paper.[24−26] The students are then guided through the process of developing a hypothesis and an experimental plan via a worksheet. Over three laboratory periods, they synthesize their peptide, which is used for both LC−MS analysis and a bioassay. Instructors and teaching assistants perform the LC−MS runs and the bioassays. The students are expected to analyze all of the data collected with their product peptide to evaluate whether they made the correct peptide and whether their initial hypothesis was valid.

It was postulated that the synthesis of biomedically relevant peptides would appeal to the premedical students who are typically enrolled in Second-Semester Organic Chemistry. Thus, a teaching lab involving the synthesis of an antimicrobial peptide could meet the instructors' learning objectives and dovetail with the professional interests of the students. The experiment would also introduce the students to solid-phase synthesis, a technique that is central to many industrial and academic synthesis programs, particularly those involving the development of bioactive peptides. Indeed, throughout the experiment, endeavors were made to mimic the scale and methodology used by practicing chemists. It was reasoned that one of the advantages of making longer peptides over multiple weeks is that students have time to repeat processes if mistakes are made, and overall, students have the opportunity to iterate a protocol numerous times, allowing for an increase in both competence and confidence on the part of the student.

EXPERIMENT SUMMARY

Overview

Students work in pairs to synthesize a hexapeptide of their own design following a modified version of a standard solid-phase peptide synthesis (SPPS) protocol.[18,27] This experiment is performed during three lab periods, over three consecutive weeks. Amino acids are coupled in the first 2 weeks. The last week is dedicated to isolation of the deprotected peptide and solution preparation for submission of peptide samples for LC−MS and bioassay analysis. Instructors run the LC−MS samples using an autosampler, as is common in an academic research or industry setting. The bioassays, through which the minimum inhibitory concentration (MIC) needed to inhibit *E. coli* growth are measured, are performed by the instructors and teaching assistants. Each student group receives the LC−MS chromatogram and mass spectrum for their product and spreadsheets containing bioassay results are deposited on the course management site. The students are responsible for analyzing this data to determine the success of their synthesis and the potential of their peptide as an antimicrobial agent.

The instructors perform the LC−MS analysis and bioassays outside of laboratory hours in order to save class time; however, both procedures are straightforward and could easily be performed by the students themselves if time allows. As both the LC−MS and bioassays are performed in parallel, all student products, whether the student synthesized the correct product or not, are tested for antimicrobial activity. Details of the experiment and the laboratory preparation, which includes a modular laboratory setup to expedite student work, may be found in the Supporting Information (e.g., Instructor's Notes, which includes bioassay information, and LC−MS Data).

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Student Preparation

In preparation for the laboratory, students were first asked to watch an online video that introduced background information on antimicrobial peptides, the antimicrobial bioassay, and experimental design considerations including the development of a testable hypothesis and the use of appropriate controls (see Supporting Information, PreLab Video Slides). The face-to-face prelab that took place in the first week of the laboratory then focused on the mechanisms involved in SPPS (see Supporting Information, PreLab Video Slides).

After viewing the prelab video, students were asked to read an assigned paper[26] on antimicrobial hexapeptides and complete a prelab worksheet (see Supporting Information, PreLab Worksheet). The worksheet guided the students through the process of developing a testable hypothesis involving a hexapeptide as an antimicrobial agent against *E. coli*. The students were expected to use the data presented in the assigned paper to develop a reasonable hypothesis. The worksheet also instructed the students with which amino acids they would have access to and guided the students through the process of developing an experimental plan that detailed how they would synthesize their hexapeptide and test its efficacy as an antimicrobial.

Procedure Overview

A sealable plastic disposable chromatography column (1.5 mL) containing a frit is used for the reaction. Students load their column with 125 mg of Tentagel Rink amide resin. The peptides are built onto a copolymer of polystyrene (PS) and polyethethylene glycol (PEG) joined to an amide linker. Peptides are elongated via an iterative cycle of 9-fluorenylmethoxycarbonyl (Fmoc) deprotection (the amino acids used are Fmoc-protected at their α-amino group) using piperidine, and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU)-mediated amino acid coupling at elevated temperature (Scheme 1, details in Supporting Information, Lab Manual). The amino acids used in this synthesis have their side chains orthogonally protected where necessary.

Initially, the resin is swelled through washes of dichloromethane (DCM) followed by *N*,*N*-dimethylformamide (DMF). Fmoc deprotection is accomplished with exposure of the resin to 20% piperidine in DMF followed by washes of DMF. The coupling steps are run in 30% *N*,*N*-diisopropylethylamine (DIEA) in DMF, and use 4 mol equiv (with respect to the resin bound peptide) of the Fmoc-protected amino acid and 4 mol equiv of HBTU. Coupling reactions are incubated at 65 °C for 20 min, after which the solution of amino acid coupling reagents is filtered off and the resin is washed with DMF. The couplings are done at elevated temperatures to benefit potentially difficult syntheses.[28] After each amino acid coupling step, a Kaiser test is performed on several resin beads in order to test for the presence of free amines. A positive Kaiser test is indicative of incomplete coupling and the incomplete reaction step is repeated.

After coupling the final amino acid, one last wash with 20% piperidine in DMF is performed to remove the last Fmoc protecting group, followed by washes of DMF, methanol and DCM. The hexapeptides are cleaved off the resin through exposure to 95% aqueous trifluoroacetic acid (TFA) for 90 min at room temperature (the C-terminus is capped as an amide postcleavage). The cleaved peptide in solution is isolated via vacuum filtration followed by rotary evaporation. The students then prepare a solution of their peptide in 0.1% TFA in acetonitrile for LC−MS analysis and activity testing against *E. coli*.

IRB Approval

The study was reviewed and approved (exemption granted) by the Institutional Review Board of Pomona College.

HAZARDS

Students are required to wear personal protective equipment at all times. All solvents and solutions used were dispensed in fume hoods. The use of a mixture of solvents, including DCM, DMF, and methanol, is considered best practice for SPPS and was used here to familiarize the students to common practices in academic and industry settings.[27,29] The small scale inherent in SPPS further minimizes risks when dealing with these hazardous chemicals. If there are concerns with the use of methanol and halogenated solvents, those washes could be replaced with extra DMF washes, although the authors have not tested such a procedure. TFA is highly toxic and corrosive, as are the Kaiser test solutions, which contain KCN and phenol, along with pyridine; extra care should be observed for these chemicals with respect to handling and ventilation. Diethyl ether and piperidine are flammable and should be kept away from sources of heat. All other information pertaining to the chemicals used in this laboratory experiment can be found in the respective Safety Data Sheets. The hazards regarding the hexapeptides synthesized by the students are unknown so they should be handled with care.

RESULTS AND DISCUSSION

This experiment has been performed in the second semester of the organic chemistry laboratory sequence with groups of two to three students (88 students divided into 43 groups for Year 1 and 77 students divided into 37 groups for Year 2). Throughout the synthesis, Kaiser tests consistently indicated that the 20 min couplings at 65 °C sufficiently removed free amines from the reaction mixtures. In the most recent iteration, synthesis yields for hexapeptides ranged from 8 to ∼ 100% (yields greater than 100% were likely due to incomplete drying of the peptides), with an average of 44% (after excluding undried peptides or peptides whose final mass did not match the desired product). Even with the syntheses that had very low yields (e.g., 8%), there was more than enough material for both LC−MS analysis and the bioassay. With regard to the success of making the desired peptide, both positive and negative results were observed, as would be expected for an authentic research experience. LC−MS analysis indicated that most syntheses (74%) produced correct and pure product (see Supporting Information, LC−MS Data). While some of the unexpected products may have been due to errors on the students' parts (e.g., adding the wrong amino acid during a synthesis), it is likely that undesired side reactivity, incomplete deprotections and/or failed couplings may have negatively impacted these syntheses.

Regardless of whether the students synthesized their desired peptide, all student products were tested in the antimicrobial activity assay that involved treating *E. coli* with different concentrations of each peptide to determine its MIC. Although only one out of the 67 unique peptides (0 of 35 in Year 1; 1 of 32 in Year 2) produced so far demonstrated notable antimicrobial activity (O−Y−*F*−I−*F*−*K*−NH_2),[30] in their written reports, all students were able to accurately analyze

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their bioassay data and suggest next steps to create a better antimicrobial peptide (Figure 1). This experiment could also be

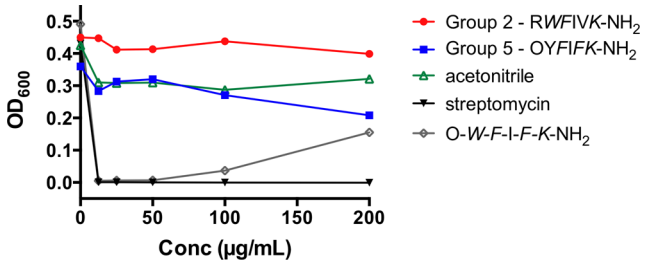

Figure 1. Antimicrobial bioassay evaluating the MIC of peptides against *E. coli*. Group 2's peptide and acetonitrile (negative control) show no inhibitory effect on *E. coli* growth at any of the concentrations tested. Streptomycin and peptide O−*W*−*F*−I−*F*−*K*−NH_2[26] (positive controls) have MICs of ≤12.5 μg/mL, the lowest concentration tested. The increase in OD_{600} at high concentrations of peptide O−*W*−*F*−I−*F*−*K*−NH_2 is likely due to aggregation of the peptide. Group 5's peptide shows some inhibitory activity at the highest concentration tested, 200 μg/mL. Data points represent the mean of two technical replicates.

easily adapted to make shorter or longer peptides that can be tested for biological activity. Some peptides as short as three amino acids, for example, have shown antimicrobial or neurological activity.[31,32]

Overall, this experiment largely achieved its stated learning goals. On the written laboratory final exam, students scored an average of 79% (range of 17−100%) and 88% (range of 22−100%) on questions related to peptide synthesis in Years 1 and 2, respectively (see Supporting Information, Exam and Key). With regard to nonpeptide questions on these exams, the students scored an average of 79% and 82%. These results suggest that the students learned peptide synthesis as well as, if not better than, other material covered in the course (LO 1).

All students who completed this laboratory were asked to write a report, introducing the project, explaining their approach and presenting an analysis of their results. Over the two years, 95% of the students demonstrated that they were able to properly analyze their LC−MS data and draw correct conclusions about their peptide product (LO 2). In future iterations, we may have students share their data such that they can draw larger conclusions about structure−activity relationships based on pooled analyses.

With regard to LO 3 and 4, all students in both years were able to successfully complete the prelab worksheet and came to the first laboratory session with an appropriate hypothesis based on the provided data.[26] All students also prepared an experimental design for the project; however, 18% of students mistakenly planned to synthesize their peptides from the N-to-C terminus, rather than from C-to-N. Their error was corrected prior to the start of synthesis. In Year 2, using a pre- and post-test, the students' ability to develop a hypothesis based on provided data and design an experiment to test their hypothesis was assessed; these questions were not related to peptide synthesis and were meant to assess the students' ability to apply LO 3 and 4 to new situations. The assessment results suggest that prior to this laboratory, the students already had a high level of competency with regard to these learning objectives (see Supporting Information, Assessment). That is, there was no measurable difference between the pre- and post-test populations. This might not be true, however, for all student groups that take on this laboratory exercise. At the same time, we were intrigued by these results and are currently pursuing this line of study with students earlier in their college career.

In Spring 2016, we invited all students who had enrolled in the course during the previous two years to participate in a short survey about their experiences in Second-Semester Organic Chemistry laboratory. Twenty-five students completed the survey (see Supporting Information, Assessment). Compared to the other laboratories they completed during the course, a higher percentage of students: clearly remembered the peptide lab, strongly agreed that it helped them understand experimental organic chemistry, strongly agreed they enjoyed the lab, and strongly agreed that the lab provided useful preparation for subsequent lab courses. The survey included a single item quiz question to assess student's long-term retention of the key point from the peptide lab; 32% of the students correctly answered the quiz item.

SUMMARY

Students completing this laboratory exercise gain knowledge on solid-phase chemistry while also developing hypotheses and executing experiments of their own design. The coupling of SPPS and the use of a bioassay that tests for antimicrobial activity of the synthesized peptides also exposed students to a biomedically relevant research topic while improving their knowledge of peptide chemistry and characterization of synthetic products by LC−MS analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00912.

Lab manual. (DOCX)
Instructor's notes. (DOCX)
List of chemicals and equipment. (DOCX)
Prelab worksheet. (DOCX)
Slides used in the prelabs. (PDF)
Examples of the characterization data collected from student samples. (PDF)
Laboratory exam questions. (PDF)
Laboratory Exam Key. (PDF)
Assessment data. (PDF)
Slides used in the video prelabs. (PDF)

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Notes

The authors declare no competing financial interest.

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