Synthesis of Plant Auxin Derivatives and Their Effects on *Ceratopteris richardii*

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A Collaborative Experiment between Undergraduate Organic and Biochemistry Laboratories

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In *BIO2010: Undergraduate Education to Prepare Biomedical Research Scientists (1),* the National Research Council emphasizes the importance of developing strong connections between scientific disciplines and the necessity of giving students practice with experimental design, quantitative analysis of biological problems, and communication skills. This report also encourages faculty to develop independent or group research projects in which students attack unanswered questions to practice these skills. In an editorial in this *Journal (2),* John Moore emphasized the role chemists can play in carrying out the recommendations of the BIO2010 report by stating, "Cross-disciplinary teaching is something that neither chemists nor biologists can do alone, and it is perhaps the most difficult aspect of improving undergraduate education in the sciences."

This lab is a collaborative project between second-semester organic chemistry students and biochemistry–cell biology students that incorporates inquiry-based learning, experimental design, generation of a data set of substantial size that can be analyzed quantitatively, and the presentation of final results in a joint poster session.[1] The organic chemistry students synthesize derivatives of auxin-type compounds, which are plant hormones, and the biochemistry–cell biology lab students use *Ceratopteris richardii* gametophytes in a bioassay to test these compounds for auxin-like activity. We have been team-teaching a biochemistry–cell biology laboratory for several years at this college. Developing this laboratory exercise has been an enjoyable experience and has enriched both our teaching of other courses in biology (RF) and chemistry (CS) and our own research. We have found that it is truly rewarding to work across disciplines with other faculty and with students.

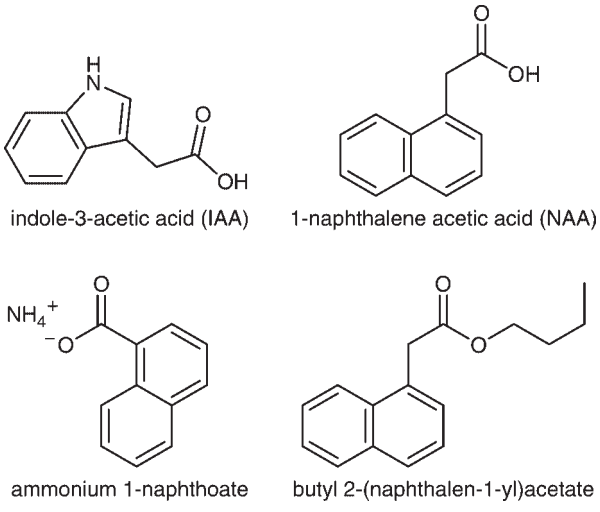

Figure 1. Structure of control auxins and examples of student synthesized derivatives.

Bioassays are commonly used to test the biological activity of chemicals *(3),* and other exercises have been presented in which students synthesize plant hormones *(4).* To the best of our knowledge, there are no other published accounts of a collaborative experiment in which organic chemistry students synthesize chemicals that are then tested in a bioassay by other students. The first time we taught this lab exercise, we used commercially available auxins and auxin-regulating compounds. In our opinion, the lab is much more effective in its present form because the organic students feel that they are synthesizing compounds for a specific purpose, and the biochemistry students enjoy testing a compound that was made by their fellow students and that has never been tested for auxin activity in a bioassay.

Experimental Overview

The second-semester organic students designed and synthesized their derivatives during the first five weeks of the semester. The biochemistry groups then spent three weeks evaluating the effects of the auxins on the gametophyte. After the experiments were completed, the students were given two weeks to create their posters for the combined poster session. During the lab periods in which the students are not working on the project, they perform the more traditional experiments in their respective labs. The organic class was divided into groups of two or three students. They made derivatives of the auxins indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) (Figure 1).[2] This section of the project was planned to help the students learn how to develop their own syntheses as well as how to approach organic syntheses on a preparatory scale. Some of the syntheses that the students chose to do were based on previous reactions they had seen in their first-semester organic chemistry laboratory course. It should be noted that the class size for both the biochemistry class and the organic class was approximately sixteen students. This experiment works well with a group of this size. Since the synthetic portion of the experiment is inquiry-based (students are just given basic instructions and requirements), this lab may not be suitable for large lab classes. The inquiry-based approach leads to many office hour visits by the students with questions concerning their synthetic designs.

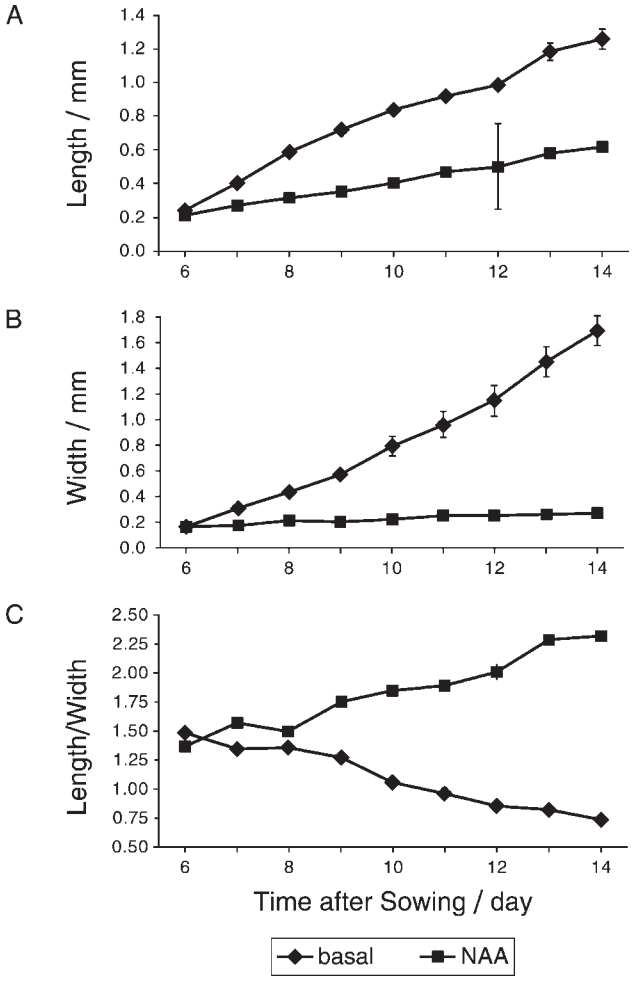

Figure 2. Growth measurements for gametophytes cultured on basal (no hormones) or NAA media for 14 days: (A) length of gametophytes, (B) width of gametophytes, and (C) length/width ratio of gametophytes. Error bars indicate standard error. In data points without visible error bars, the size of the symbol exceeds the size of the error bars.

The auxin derivatives (or other known auxins) were then evaluated for their biological activity by the biochemistry–cell biology students using gametophytes of the fern *C. richardii*. Each student group monitored the development of gametophytes on three types of media: (i) no hormones, (ii) medium containing NAA, a known auxin, and (iii) medium containing one auxin derivative from the organic class. Over a two-week period, students measured the length and width of the growing gametophytes daily and made other morphological observations using a research dissecting microscope. The students then used Excel to calculate length-to-width ratio, averages, standard deviation, and standard error for their measurements. At the end of the semester, a public poster session took place in which both the organic chemistry and the biochemistry–cell biology students presented their results.

Experimental Methods

Synthesis of Auxin Derivatives

The organic chemistry students spent the first two weeks of the semester designing their synthesis. This portion of the organic laboratory is inquiry-based because we gave the students the basic requirements of their auxin derivatives and allowed them to choose a derivative and design the synthesis of that derivative. There were three requirements for their derivatives: (i) the derivative must be based on IAA or NAA, (ii) the compound must be soluble enough to make a 10^{-4} M solution, and (iii) the procedure they use must be safe and approved by their instructor. It was the responsibility of the student to find a suitable synthesis and determine the reagents and their availability. Designing a synthesis on their own proved to be the most problematic part of the lab for most students, since in the first-semester organic chemistry lab they were provided with a "cookbook" approach to organic chemistry where they were given a set of instructions and reagents and performed a dictated experiment. This lab not only provides the students with experience in synthesis design and scale-up techniques, they had to learn how to research experiments by using the library and online resources as well. They also learned how to modify a known reaction to suit their specific needs.

Both of the auxins we chose to modify (IAA and NAA) have a carboxylic acid functional group. This acidic moiety was the group that most students chose to enhance in their syntheses. Each group was allowed to devise their own synthetic strategies toward their desired product. We supplied them with a few texts to use as guides for synthetic methods and procedures *(5)*. Prior to any lab work, the students first consulted with us, providing a detailed description of the synthesis and an MSDS sheet for every reagent and their product, if available. The groups were limited to using the reagents present in the science division's inventory. This reduced cost and allowed them to start immediately, as opposed to waiting for reagents to be shipped. All of the reagents used in their experiments are available through the Aldrich Chemical Company.

The last three weeks of the organic portion of the experiment were dedicated to synthesis. All groups had to successfully perform their synthesis on a microscale prior to scaling them up. These three weeks proved to be a trial and error period for most groups. Groups that chose simpler one-step reactions, such as esterifications, usually were able to complete the project in two weeks. Those that chose multistep syntheses took three weeks. Along with their compound, students were required to obtain proof of the structure and its purity.

Auxin Bioassay

We chose *Ceratopteris richardii* for this experiment because it is widely used for biological research and biology teaching at both the undergraduate and K–12 levels *(6)*. Like all ferns, *C. richardii* exhibits alternation of generations and thus has both a haploid gametophyte generation and a diploid sporophyte generation. Researchers and teachers use the gametophyte generation for experiments because of its short generation time (14 days), its simple body plan (one cell layer

thick, 2D growth), small size (1–2-mm diameter at maturity), and the ease of sowing spores and conducting observations. *C. richardii* has been used to study cell biology, developmental biology, and sex determination mechanisms *(7–9).* Hickok and his colleagues at the University of Tennessee at Knoxville have developed *C. richardii* as a model system for teaching biology, and they maintain a Web site *(10)* that includes background information, photographs, and inquiry-based experiments using this organism. *C. richardii* spores, media, and teaching kits are available from Carolina Biological Supply Company.

This bioassay is based on the results of RF's research on the effects of auxins on *Ceratopteris richardii* gametophyte development, which show clear differences between gametophytes grown on medium without hormones and gametophtyes grown on medium containing 10^{-5} M NAA, a synthetic auxin *(11).* Gametophyte characteristics compared included length, width, length-to-width ratio, sexual differentiation, and reproductive characteristics. Length-to-width ratio calculations were included because developmental biologists use the length-to-width ratio as a quantitative measurement of shape. Three gametophytes were chosen for observation on each of four Petri plates for a total of twelve gametophytes for each media treatment. The quantitative data were analyzed using analysis of variance with SPSS, with hormone as the main effect and the plate nested within hormone treatment. Individual treatment means were tested for differences using Bonferroni-corrected least significant differences. At 14 days, gametophytes grown on medium without hormones differed significantly in length ($p < 0.001$), width ($p < 0.001$), and length-to-width ratio ($p < 0.001$) from gametophytes cultured on medium containing NAA (Figure 2).

Gametophytes grown on these two types of media also differ dramatically in their appearance. The gametophytes grown on NAA have much larger cells than gametophytes cultured without hormones and produce rhizoids along their body length, while gametophytes grown on medium without hormones produce rhizoids at the base of their bodies (Figure 3).

RF has examined concentration series of several auxin and auxin-regulating compounds, and all compounds tested except NAA were biologically active without causing lethal effects at a concentration of 10^{-4} M. It is possible that students would find that their auxin derivatives are active at a concentration other than 10^{-4} M, but we restrict students to using their auxin derivative at 10^{-4} M because of space and time considerations. Each student group compared the development of *C. richardii* gametophytes on three types of sterile medium: (i) medium without hormones, (ii) medium with 10^{-5} M NAA, and (iii) medium containing 10^{-4} M of an auxin derivative synthesized in the organic chemistry lab. Workstudy students who set up our other laboratory exercises prepared all media.[3] Students sterilized spores, sowed them onto Petri plates, and then examined the development of gametophytes for two weeks using a research dissecting microscope. Observations included measurements of lengths and widths using an ocular micrometer, visual observations (cell shapes and sizes, gametophyte color, gametophyte sperm production, production of a lateral lobe by the gametophyte), and digital photographs of gametophytes. Three gametophytes were chosen for observation on each of four Petri plates for a total of twelve gametophytes for each media treatment. Thus, students measured 36 gametophytes for eight days. Students entered lengths and widths into an Excel spreadsheet, and Excel was used to convert ocular units to mm, to calculate averages, standard deviation, and standard error for each treatment, and to produce a graph from the data. More complex statistics such as analysis of variance could be done by collaborating with a statistics class to do data analysis, or by importing the students' Excel data into a statistical data analysis program such as SASS or SPSS.

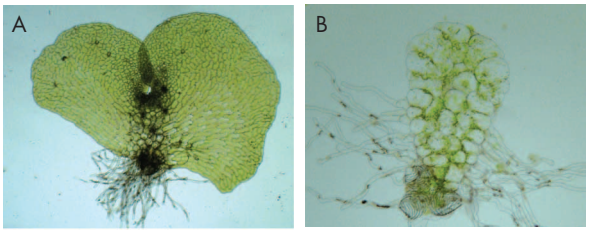

Figure 3. 14-Day-old gametophtyes cultured on (A) medium without hormones and (B) medium with 10^{-5} NAA (scale bar = 100 μm). Color versions of these images are featured on the cover of this issue.

Hazards

Organic Chemistry Lab

Since every lab group had their own specific syntheses, they each met individually with instructor to discuss the hazards associated with their reactions. All students followed general lab safety procedures (safety glasses, gloves, etc.) and were under supervision by their instructor during the lab.

Biochemistry–Cell Biology Lab

Since the tissue culture plates are sealed, students are not exposed to any chemical hazards during the experiment. Preparatory staff should wear goggles and gloves while weighing out NAA and other chemicals for making media, and glass Pasteur pipets used for sterilizing spores should be disposed of in the glass trash container.

Discussion

Synthesis

In the three years we have used this experiment, we have mixed results with the syntheses. Some students chose to modify experiments from the first-semester organic chemistry lab, such as ester synthesis. Others chose to perform completely new reactions in which they had no previous experience or familiarity. We had synthetic yields as high as 90% and as low as 10%. Some students also designed compounds that were not soluble enough in water to be useful. In some cases, students had enough time to go back and get better yields or try to make a more soluble derivative. We were most interested in having the students learn the process of independent research and how to do it safely, rather than getting the best yield or even having a soluble derivative. In most cases (over 90%), students were able to pro-

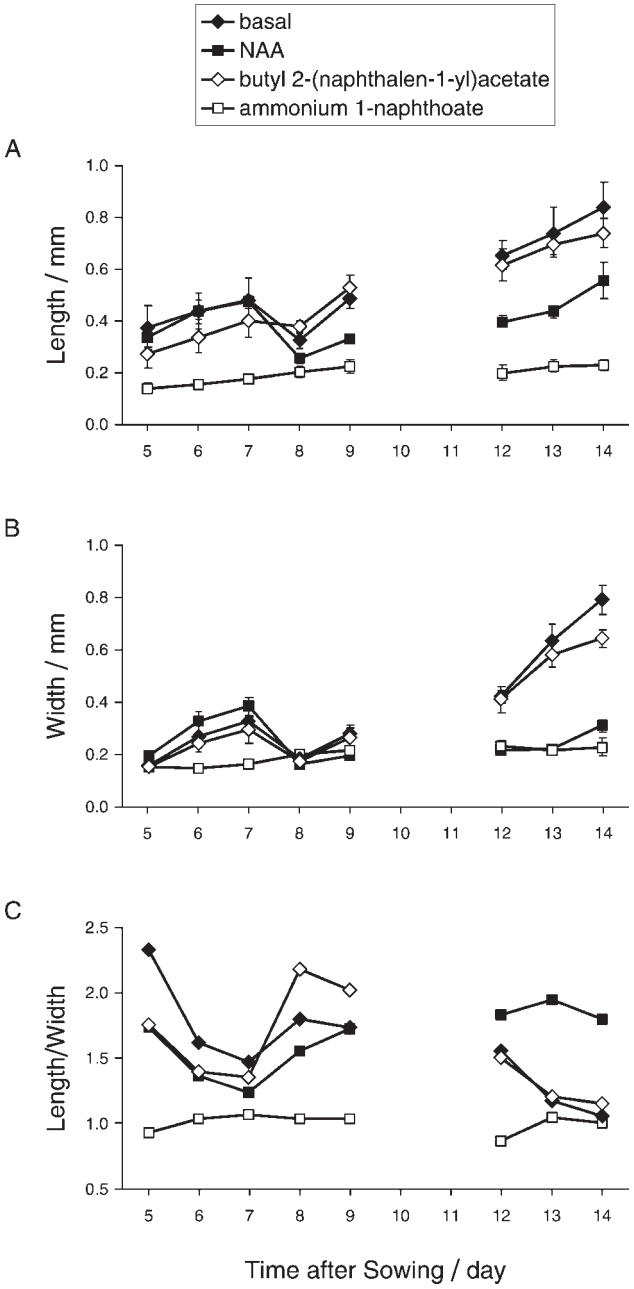

Figure 4. Example student graphs of growth measurements for gametophytes cultured on basal, NAA, butyl 2-(naphthalen-1-yl)acetate, or ammonium 1-naphthoate media for 14 days. Students did not have access to the lab on the weekend, and so data points were not recorded for days 10 and 11: (A) length of gametophytes, (B) width of gametophytes, and (C) length/width ratio of gametophytes. Error bars indicate standard error. In data points without visible error bars, the size of the symbol exceeds the size of the error bars.

vide us with water-soluble derivative at the end of the experiment. In those cases where solubility was an issue or they never got their product, we gave the biochemistry–cell biology students a commercially available auxin from our chemical inventory.

Bioassay

Student experiments typically fall into two groups. Either the auxin derivative causes no change in the growth of gametophytes or the auxin derivative clearly causes an auxin-like growth response in the gametophyte. Occasionally, the auxin derivative is toxic and prevents spore germination or the auxin derivative causes novel effects. In student data, differences at 14 days between gametophytes grown on medium with no hormone, medium containing NAA, or medium containing an auxin derivative can usually be most easily and clearly seen by looking at width measurements (Figure 4B), while length measurements (Figure 4A) and length-to-width ratios (Figure 4C) do not always differ as clearly. In our example student data, gametophytes cultured on medium without hormones differed significantly at 14 days from gametophytes cultured on NAA medium in length ($p = 0.005$), width ($p < 0.001$), and length-to-width ratio ($p < 0.001$). Gametophytes grown on butyl-2-(naphthalene-1-yl)acetate mimicked the appearance of gametophytes grown on medium without hormones, and at 14 days differed significantly in width from gametophytes grown on NAA ($p < 0.005$), but did not differ significantly in length ($p = 0.183$) or length-to-width ratio ($p = 0.021$) from gametophytes grown on NAA. Gametophytes grown on ammonium 1-naphthoate mimicked the appearance of gametophytes grown on NAA medium, and 14-day-old gametophytes of these two groups differed significantly in length ($p < 0.001$) and length-to-width ratio ($p = 0.006$), but not in width ($p = 1.000$).

Gametophytes grown on ammonium 1-naphthoate differed significantly at 14 days in length and width (both $p < 0.001$) from gametophytes grown on medium without hormones, but did not differ significantly in length-to-width ratios ($p = 1.00$) from gametophytes grown on medium without hormones. Since students are just learning to use the ocular micrometer small differences in gametophyte size can be lost in the measurement process. Most of our student groups take turns measuring the gametophytes, a practice that also leads to variations in measurements. Discussing the numerical data with students can increase their understanding of quantitative analysis and of the importance of consistent, careful measurements. The biochemistry–cell biology students thus gained experience in sterile technique, use of the research dissecting microscope, ocular micrometer, and digital camera, and the management of a data set of substantial size (36 gametophytes measured daily for 8 days).

Poster

At the end of the semester, the organic students and the biochemistry–cell biology students presented a joint poster session. Each poster included abstract, introduction, materials, methods, results, and discussion sections. The organic students presented their synthesis first followed by the corresponding presentation of biochemistry–cell biology students who used their auxin derivative. Students were allowed access to the corresponding groups' data prior to the presentation. They could then discuss how the change in auxin structure affected the growth of the gametophytes, and integrate the synthesis and bioassay results in their poster presentations.

Acknowledgments

We would like to thank our CHM216 and BIO\CHM340 students for testing this experiment and providing us with valuable feedback.

[W]Supplemental Material

A detailed handout to students, background material, preparation details, grading sheets as well as further information for instructors are available in this issue of *JCE Online.*

Notes

1. A part of this work was presented on March 13, 2005 at the 229th American Chemical Society Meeting in San Diego, CA.

2. If the instructor needs to shorten the duration of the project, this part of the project could be shortened to one week by giving students a predesigned synthesis.

3. In a small class, students could prepare their own media, but we have found it more efficient to start the lab with the sowing of spores. This allows the students some practice with sterile technique without taking an entire lab period for media preparation.

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