Benzodiazepine Synthesis and Rapid Toxicity Assay

James T. Fletcher* and Grit Boriraj
Department of Chemistry, Creighton University, Omaha, Nebraska 68178
**jamesfletcher@creighton.edu*

This laboratory experiment introduces students to some basic principles of medicinal chemistry by guiding them through the synthesis of a druglike molecule and testing the molecule's biological activity by performing a rapid toxicity assay. To be practical for a large enrollment college second-year organic chemistry laboratory course, the experiment had to adhere to the following restrictions: (i) able to be completed within three hours; (ii) amenable to small-scale setup and purification to minimize costs and waste produced; and (iii) afford a meaningful educational experience to large class sections (>25 students per section, >250 overall students). This article describes an experiment meeting such criteria that involves the synthesis of a benzodiazepine derivative and the evaluation of its biological activity using a brine shrimp toxicity assay.

Background

Benzodiazepines are a class of heterocycles that consist of a benzene ring fused with a seven-atom ring, in which two of the atoms are nitrogen. Many common depressants such as Valium and Xanax are composed of benzodiazepine rings and are often prescribed as sleep aids or antianxiety drugs. The benzodiazepine ring to be synthesized in this experiment is a regioisomer variant of the benzodiazepine ring system found in Valium and Xanax (Figure 1). As this experiment demonstrates, such a complicated ring structure can be created from simple building blocks via relatively elaborate condensation reactions between *ortho*-phenylenediamine and two equivalents of a ketone such as acetone (Scheme 1).

When any new drug candidate is synthesized, it must undergo a series of trials to measure its overall toxicity as well as its therapeutic efficacy. One common laboratory technique used to first approximate toxicity is a serial-dilution assay against a relevant cell or organism. In such assays, the drug molecule candidate is diluted over a wide range of concentrations and each tested in parallel so that the minimum concentration of the drug's bioactivity can be identified. Examples of such assays include the lysing of red blood cells, reported as the minimum concentration upon which hemolysis occurs (typically reported as a HD(50) value, or 50% hemolytic dose), or the killing of a whole organism such as bacteria, reported as the minimum lethal dose (typically reported as a LD(50) value, or 50% lethal dose). This testing allows highly toxic molecules to be identified before proceeding on to more advanced (and expensive) studies to measure therapeutic efficacy.

In this experiment, the principle of assaying for minimum lethal dose is demonstrated by performing a dilution−toxicity assay with a benzodiazepine product against the common aquatic organism *Artemia salina*, also known as brine shrimp or "sea monkeys". Utilizing this organism has several advantages in the instructional laboratory environment *(1)* including its rapid observable response time (minutes instead of hours or days), the lack of special incubation required to culture the organism and run the assay, the nonhazardous nature of the organism (relative to blood products or bacteria), the inexpensive nature of the organism (sold as fish food in pet stores), and the lack of bureaucratic oversight required for its use (as it is an invertebrate and not classified as an animal by the National Institutes of Health).

Experiment

This reaction involves two successive condensation reactions of acetone with *ortho*-phenylenediamine to form a diimine intermediate, followed by a cyclization step to form the final benzodiazepine product. This reaction is performed using commercially available and inexpensive *ortho*-phenlenediamine as a reactant, sulfamic acid as the catalyst, and acetone as both the solvent and coreactant (Scheme 1) *(2)*. After stirring 30 min at room temperature in a 20 mL screw-cap vial, the crude product is isolated from the sulfamic acid catalyst via small-scale extraction between dichloromethane and water in the reaction vessel. The organic layer is removed via pipet transfer and dried over Na_2SO_4, and volatiles are removed via rotary evaporation. The resulting crude reaction product is purified via trituration with a

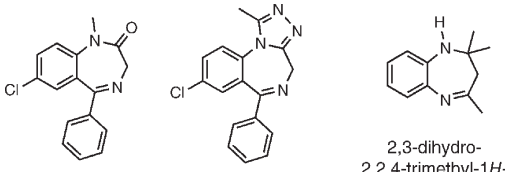
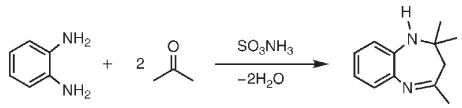


Figure 1. Structures of 1,4-benzodiazepine compounds Valium and Xanax and the 1,5-benzodiazepine product of the experiment.

Scheme 1. Preparation of 2,3-Dihydro-2,2,4-trimethyl-1*H*-1,5-benzodiazepine[a]



[a] Note that sulfamic acid is a catalyst.

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hexane/2-propanol mixture. Following vacuum filtration, the purified product (a varying white to tan crystalline solid) can be analyzed via melting point, GC−MS, ^{1}H NMR, or ^{13}C NMR as class size and time frame of experimental implementation permits.

Typical student yields for this reaction ranged from 16 to 80% crude product and from 5 to 55% purified product, with the scale of the reaction (280 mg of *ortho*-phenylenediamine) providing most students a more than sufficient quantity of material to complete the assay portion of the experiment (a minimum of 15 mg of product is needed). Most students performing this lab had no trouble reaching the stage of isolated purified product after 60−90 min of lab time.

Using their purified products, students performed a serial-dilution assay to measure the toxicity of their benzodiazepine product toward brine shrimp *(1)*. Students prepared concentrated stock solutions of their product in ethanol, diluted these ethanol solutions into the aqueous suspensions of brine shrimp, and were asked to calculate the resulting assay concentrations of their benzodiazepine as 5% ethanol-in-water solutions. Two different benzodiazepine concentrations were assayed, 2.5 mg/mL and 5 mg/mL, along with a 5% aqueous ethanol control. Students were asked to note observations of their three assay samples every 5 min and approximate the percentage of shrimp with decreased motility relative to normal motility in each of the three assay tubes. Most students noted obvious differences between the drug and control samples within 15 min of assay time, and most students had sufficient time within a three-hour lab period to make between 25 and 45 min worth of observations.

Hazards

ortho-Phenylenediamine is toxic. Acetone, hexanes, and 2-propanol are toxic and flammable. Sulfamic acid is a corrosive solid. Dichloromethane is reasonably expected to be carcinogenic. The benzodiazepine product should be considered toxic. All compounds should be handled in a manner consistent with their material safety data sheets, including proper hand and eye protection. All procedures should be performed using proper ventilation.

Discussion

The range of student yields of crude and purified product was significantly lower than that reported in the literature *(2−8)* and can partially be attributed to the shorter reaction times used in this experiment (30 min versus a minimum of 3 h). The reaction can be monitored by GC−MS analysis, where the diimine intermediate is detected within minutes and slowly cyclizes into benzodiazepine product. When this experiment was being developed, it was observed that the diimine:benzodiazepine product ratio at the 15 min time point is 62:38, at 20 min is 34:66, and at 30 min is 13:87. Hence, students who wait the prescribed reaction time when performing their experiment are rewarded with increased yields. One major impact on purified product yield was the state of the crude reaction product, which ideally appeared as a somewhat oily solid. If dried insufficiently with Na_2SO_4 or if volatiles were not sufficiently evaporated before trituration, the crude product appeared as a tan oil (with no visible solid) and little or no purified product was obtained from its trituration. The instructors observed that this experiment was effective in distinguishing those students who performed their work diligently from those who worked quickly and carelessly.

Student feedback regarding the brine shrimp assay portion of the experiment was overwhelmingly positive. One fairly common error was students not properly calculating the concentration of their initial ethanol stock solutions relative to the desired final 5% aqueous ethanol assay concentrations. The concentrations described here were selected to ensure observable results within a 30 min assay time frame. Lower concentration assay samples could be easily added to this series; however, for higher concentration assays (e.g., for 20 mg/mL assay sample) the quantity of product to be dissolved in ethanol approaches the saturation limit. Not only did students seem to appreciate the ability to quickly observe differences in biological activity by eye, but they also were interested in the observation that the 5% ethanol control tube also showed an observable decrease in shrimp motility over time (although less extreme than the benzodiazepine samples).

This experiment was performed in eight lab sections of approximately 30 students each. It was completed within a single three-hour laboratory period, which included approximately 30 min of prelab instruction. Student interest in this experiment was notable, primarily owing to the brine shrimp assay portion of the experiment. Any student anxiety about sacrificing a living animal was significantly reduced upon seeing exactly what a brine shrimp organism looked like and being told that such organisms were purchased from a pet store as fish food.

When performed relatively late in the organic chemistry sequence, this experiment allows the instructor to emphasize both NMR interpretation and the carbonyl condensation reaction mechanism. Alternatively (and as it was implemented by the authors), it could also be performed relatively early in the semester with instructor emphasis on the hands-on skills of product isolation and purification, as well as the preparation of stock solutions and calculation of resulting assay concentrations. In smaller class sizes with the available resources, GC−MS analysis could be used to monitor reaction progress, owing to the ability to observe reactant, intermediate, and product molecules using this analytical method.

Recent reports in the primary literature have demonstrated a variety of methods for synthesizing 1,5-benzodiazepine regioisomers via acid-catalyzed condensation−cyclization reactions between 1,2-diaminobenzene and ketone reactants *(2−8)*. Although not explored in this study, variations on this experiment could include the execution of this reaction via ultrasound *(3)* or microwave heating *(4)*, the evaluation of different acid catalysts *(2, 5, 6)*, or the implementation of a green-chemistry approach by performing such reactions in water *(7, 8)*.

Acknowledgment

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Supporting Information Available

Student handout; postlab questions; GC−MS reaction monitoring; ^{1}H and ^{13}C NMR characterization; instructor notes; list of chemicals; safety hazards. This material is available via the Internet at http://pubs.acs.org.

