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Laboratory Experiment

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Nature's Chiral Catalyst and Anti-Malarial Agent: Isolation and Structure Elucidation of Cinchonine and Quinine from *Cinchona calisaya*

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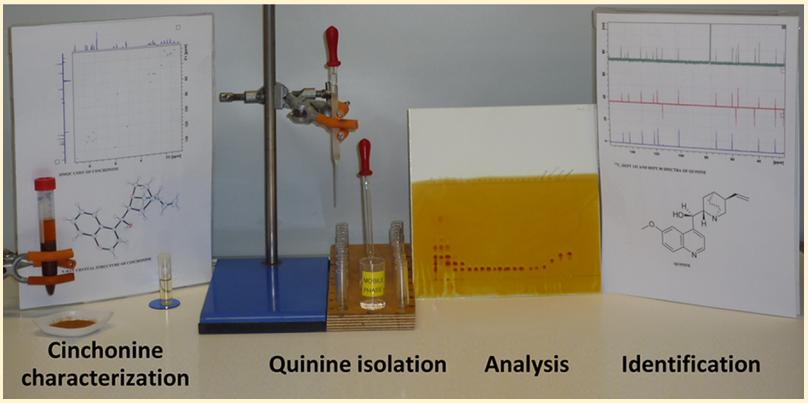
Supporting Information



ABSTRACT: Nature is a well-recognized source of compounds of interest, but access is often an issue. One pertinent example is the cinchona alkaloids from the bark of *Cinchona calisaya*. In this experiment, students at the third-year undergraduate level undertake the selective isolation and characterization of two of the four main alkaloids present in the bark. Beginning with powdered bark, acid–base extraction, followed by selective crystallization, serves to yield cinchonine, nature's chiral catalyst, from the complex mixture. Slow crystallization provides suitable quality crystals for X-ray analysis. Students readily appreciate the three-dimensional nature of this chiral catalyst, which aids subsequent NMR spectroscopic analysis. Manipulation of the mother liquor by thin-layer and flash column chromatographic techniques proves a simple but elegant method to furnish quinine, nature's anti-malarial agent. Both alkaloids are treated to an intensive structure elucidation workshop comprising 1- and 2-dimensional NMR, infrared, and mass spectrometry. The method is economical, polished, and robust, bringing the student on a journey from crude plant material to medicinally important natural products in three, 2-h laboratory sessions. Moreover, questions in the student handout and model answers in instructor's notes, respectively, require that students engage further in topics associated with the context of this practical.

KEYWORDS: *Upper-Division Undergraduate, Laboratory Instruction, Organic Chemistry, Hands-On Learning/Manipulatives, Biosynthesis, Chromatography, Drugs/Pharmaceuticals, Natural Products, NMR Spectroscopy, X-ray Crystallography*

The purpose of this experiment is first to provide the students with a historical overview of cinchona bark, its alkaloids (Figure 1), and their application. Second, the students learn the essential skills required to isolate and purify cinchonine and quinine from the bark of *Cinchona calisaya*. The method is low-cost, refined, and reproducible. It relies on an understanding of the principles behind the selective isolation of basic compounds from neutral and acidic natural products, which includes knowing the correct choice of extracting solvent for the selective isolation of cinchonine from its closely related alkaloids, cinchonidine, quinine, and quinidine. An understanding of the principles behind crystallization as a means of purification of natural drug substances is also highlighted. As an added bonus, flash column chromatography of the mother liquor provides a simple but elegant method of quinine isolation from the complex mixture. The use of 1- and 2-D NMR, IR, MS, and X-ray spectroscopy as analytical tools to perform a complete structure elucidation study on the isolated compounds is then conducted. The experiment complements

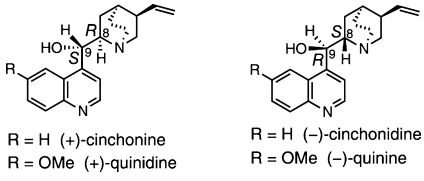


Figure 1. Cinchona alkaloids.

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the many other laboratory-based experiments on the isolation of nature's medicines including valtrate from *Centranthus ruber*,[1] galantamine from *Leucojum aestivum*,[2] lovastatin from red yeast rice,[3] parthenolide from *Tanacetum parthenium*,[4] betulin from birch bark,[5] curcumin from turmeric,[6] and thiarubrine A from *Ambrosia artemisiifoia*.[7]

BACKGROUND

Cinchona bark is the dried bark from the stem and branches of species of *Cinchona* (Rubiaceae),[8] large trees indigenous to South America. The various species, including *C. calisaya*, *Cinchona legeriana*, and *Cinchona succinubra*, vary in their relative alkaloid content.[8] This may range from 4 to 17%, the larger percentages coming from hybrid species. The four main alkaloids in cinchona bark are quinine and cinchonine and their respective diastereoisomers, quinidine and cinchonidine, which together account for approximately 30−60% of the total alkaloid content.[8] These alkaloids have a rich history. Already in use to cure fever before 1600 by Peruvian native Indians, their scientific name originates from the myth that in the early 1600s, Lady Chinchón, wife of the Spanish viceroy of Peru, was cured from malaria using an ancient herbal remedy, the "quinquina" bark. Around 1630, Jesuits exported cinchona bark (thereafter also known as "Jesuit's bark") to Europe where it soon became the remedy of choice for malaria, widespread at the time.[9] With a growing demand for cinchona bark and the threat of extinction by overharvesting, European governments set about establishing cinchona plantations in their tropical colonies. By the 1930s, the Dutch plantations of *C. ledgeriana* in the Indonesian island of Java provided the bulk of the world's production. The advent of World War II saw the allied forces cut off from cinchona trees in Java, their sole source of quinine. This led to an increased impetus, especially in the United States, for developing a synthetic route to quinine:[9] the claim by Woodward in 1944 of having successfully accomplished this was hailed by *The New York Times* as "one of the greatest scientific achievements in a century".[10] Interest in the cinchona alkaloids continues to this day. They are a versatile class of natural products, serving both as medicinally important compounds (quinine is used in the treatment of malaria and leg cramps, quinidine as an anti-arrhythmic agent) and as privileged catalysts and ligands (the major use of cinchonine and cinchonidine) for asymmetric synthesis.[11] These uses are further explored in the accompanying Supporting Information.

EXPERIMENTAL OVERVIEW

The experiment, conducted over three, 2-h laboratory sessions, is particularly suited to upper-level undergraduate students who have a basic understanding of the chromatographic and spectroscopic techniques used in the identification of natural or synthetic compounds. Throughout the experiment, students gain first-hand experience of various techniques involved in the extraction, isolation, and purification of medicinally important natural products from the crude plant material. These include acid−base extraction on the powdered bark to attain an alkaloid-rich fraction from a complex matrix and subsequent extraction of cinchonine and related alkaloids into a suitable solvent, ethyl acetate. Taking advantage of the low solubility of cinchonine in ethyl acetate,[12] crystals of cinchonine slowly form from the mixture over several days and are of suitable quality for X-ray crystallography (week 1). The students then isolate the crystals and determine their percentage yield and purity by thin-layer chromatography (TLC). The mother liquor is not discarded! It is reduced in vacuo and the residue subjected to thin-layer and flash column chromatographic techniques to furnish quinine (week 2). In the third week of the experiment, an extensive structure elucidation study of cinchonine and quinine is carried out in the form of a workshop, using ^{1}H, H−H, ^{13}C, HMQC, and HMBC spectra. This gives students a firm understanding of the applications of 1- and 2-dimensional NMR spectroscopy. Moreover, assignment of the exact resonance positions of the cinchonine peaks is greatly facilitated by having its X-ray crystal structure (Figure 2). Students can

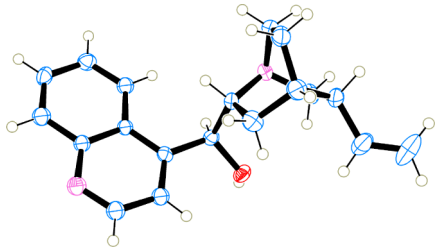

Figure 2. X-ray crystal structure of cinchonine.

quickly understand from the 3-dimensional nature of the molecule why the methylene protons on the quinuclidine ring are chemically inequivalent. The students can appreciate this chiral catalyst in 3 dimensions, from the steric bulk of the quinuclidine moiety to the chiral pocket of the β-hydroxyamine functionality central to enantioselective catalysis. Furthermore, students learn how to interpret infrared and mass spectral data to complement the information gathered from NMR spectroscopy. In particular, the similarities and differences of cinchonine and quinine are emphasized.

From a class of 58 students, the average yield of cinchonine isolated from 3.0 g of *C. calisaya* was 3.5 mg, whereas the average yield of quinine isolated was 5.0 mg. Considering the minimal time, effort, and resources involved (student-friendly in every sense), the end result compares favorably with catalytic asymmetric total synthetic approaches to quinine (19 steps with overall yields of 0.96% by Stork et al.;[10] 16 steps with overall yields of ca. 5% by Jacobsen et al.[11]). The complete experiment required three, 2-h sessions with a maximum of 15 students conducting the experiment in a given session.

EXPERIMENTAL SECTION

Isolation of Cinchonine by Acid−Base Extraction Followed by Selective Crystallization

In week one of the experiment, powdered *C. calisaya* (3.0 g) from the Herbal Apothecary[13] was accurately weighed out and placed directly into a 15 mL centrifuge tube. A 1.0% aqueous solution of trifluoroacetic acid (8.0 mL) was added to the powdered material. The screw cap lid was placed onto the tube and tightened gently. The contents of the tube were shaken vigorously by hand for 15 min, centrifuged (3000 rpm, room temperature, 10 min), and 3.5 mL of the supernatant was transferred to another 15 mL centrifuge tube containing ethyl acetate (4.0 mL) and 1.0 M aqueous NaOH (5.0 mL). The screw cap lid was attached on the tube and tightened gently. Again, the contents of the tube were shaken vigorously by hand for 5 min. The sample was centrifuged (3000 rpm) for 10 min at room temperature. Approximately 3.5 mL of the upper organic layer was removed using a Pasteur pipet and placed into

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a preweighed, labeled sample vial. Particular care was taken to avoid removal of the lower aqueous layer. A lid was attached onto the sample vial and the crystals of cinchonine were allowed to form slowly over a period of one week. In week 2 of the laboratory experiment, the mother liquor was carefully removed using a Pasteur pipet. The crystals that formed were washed thoroughly with ethyl acetate (~1 mL) by slowly rotating the vial for 2 min. Using a Pasteur pipet, the washings were then carefully removed, taking care to avoid removal of the crystals. The washing procedure was repeated. The crystals were dried under high vacuum for 10 min. The sample vial containing the purified crystals was reweighed. The mass of cinchonine obtained from 3.0 g of cinchona bark was recorded.

Isolation of Quinine by Flash Chromatography

In week two of the experiment, the mother liquor and washings from isolation of cinchonine were combined and reduced in vacuo. The residue was taken up in the minimum volume of dichloromethane/methanol (10:1) to allow for easy transfer onto the flash column. The flash column was prepared as described in the Supporting Information. The mobile phase of choice was an isocratic system consisting of dichloromethane/ethyl acetate/propan-2-ol/diethylamine (20:70:4:6). The volume of eluent collected in each fraction was approximately 0.5 mL. The fractions containing pure quinine were identified using silica gel TLC employing the above mobile phase, for which quinine has an R_f of 0.16. These fractions were combined, reduced in vacuo in a preweighed round-bottomed flask, the contents dried under high vacuum and the yield of pure quinine was then calculated. Overall, this technique was reproducible but the student must use the correct quantity of silica and the minimum volume of solvent to allow for easy transfer of the residue onto the column.

Structure Elucidation Studies

In week three of the experiment, IR, mass spectrometry, and detailed NMR spectroscopic analyses confirmed that the substances isolated were cinchonine and quinine. Both molecules have broadly similar IR spectra with the expected characteristic absorption peaks for O−H, C−H, C═C, and alcoholic C−O stretching. An additional peak of interest in the IR spectrum of quinine—a strong absorption in the fingerprint region (1228 cm^{-1}) for the C−O stretch of the ether functionality—distinguishes it from its demethoxy analogue, cinchonine.

The high-resolution mass spectrum (HRMS) reveals the MH^+ at m/z 295.1818 and 325.1917 for cinchonine and quinine, respectively, as the most abundant ion, with the expected mass difference attributed to the methoxy functionality of quinine. In the ^1H and ^{13}C NMR spectra of quinine, the methoxy protons resonate downfield as a singlet at 3.89 ppm. Although the DEPT-135 spectrum still shows eleven peaks, one of these (at 55.6 ppm) is found to be a $-CH_3$ carbon by its absence from the DEPT-90 spectrum. Although the aliphatic region is comparable, there is a notable difference in the aromatic region: the six protons of cinchonine (four doublets and two triplets) give way to the five of quinine (four doublets and a double double doublet) and an extra quaternary carbon (to accommodate the methoxy). Complete assignment of all signals is provided in the Supporting Information, as is complementary information presented as questions and model answers. Following completion of the experiment, each student submitted a detailed report. Student feedback on the experiment was positive. They liked the relatively straightforward isolation procedures to isolate two important natural products and in the case of cinchonine, viewing the structure in 3-dimensional form following X-ray analysis of the crystals isolated from a representative student sample. Students were pleased with the workshop session as they particularly liked the small group, interactive format. Moreover, the manner in which the experiment blends into biosynthetic, pharmacological, pharmacokinetic, and clinical material delivered in lectures was particularly valued. Thus, the series of questions covered in the Supporting Information was welcomed by the students to give them a more "bench to bedside" appreciation of their subject.

HAZARDS

Some potentially hazardous reagents and flammable solvents are used.[14] Ethyl acetate, propan-2-ol, diethylamine (all highly flammable), trifluoroacetic acid, and deuterated chloroform are toxic by inhalation, in contact with the skin and if swallowed. Dichloromethane and deuterated chloroform have limited evidence of a carcinogenic effect. Sodium hydroxide and trifluoroacetic acid are corrosive, causing severe burns. Care must be taken when handling these chemicals, which must be used in the fume hood, as must silica gel. Contact with skin and eyes should be avoided and suitable personal protective equipment worn. Protective gloves should be worn when handling the powdered *C. calisaya* sample. Ultraviolet (UV) radiation can cause severe damage to the eyes. Do not look directly into the light source.

SUMMARY

The experiment has been performed with consistent reproducibility by 58 third-year pharmacy students. The method is economical, polished, and robust, bringing the student on a journey from crude plant material to isolation and characterization of medicinally important natural products in three, 2-h laboratory sessions.

ASSOCIATED CONTENT

Supporting Information

Student handout; and instructor's notes; NMR spectra. 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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