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Incorporating Fermentation into Undergraduate Laboratory Courses

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Incorporating Fermentation into Undergraduate Laboratory Courses

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Abstract

Laboratory courses in universities have a responsibility to introduce current research practices and trends in scientific research to adequately prepare students for work in the field. One such research practice gaining popularity in recent years is that of green chemistry. Since the 1960s, increasing concern over the release of toxic chemicals into the environment has led to a push for more environmentally responsible chemistry. A growing faction of chemists has begun to adopt methods to eliminate chemical waste and support green chemistry. Fermentation is an ideal technique to demonstrate environmentally sustainable chemistry in an undergraduate laboratory class. Fermentation of complex natural products, as opposed to traditional organic synthesis, is beneficial as it supports a number of principles of green chemistry; it is conducted at ambient temperature and pressure, uses inexpensive and innocuous materials, makes use of renewable resources, and does not require a fume hood. Skills implemented during fermentation can be easily taught to upper-level Chemistry and Biochemistry undergraduate students, who typically have limited exposure to complex natural products in their coursework. Such a course would be interdisciplinary in nature, incorporating fungal biology and metabolism as well as organic chemistry. Students would learn a variety of skills, including growth media selection and preparation, inoculation of fungal cultures, extraction of natural products, and purification and characterization of metabolites. Experiments of this nature would allow for discussions of several areas of research: green chemistry, natural products and their application to medicine, identification of functional groups in complex molecules by spectroscopy, and introduction to biochemistry and metabolism. Roquefortine C, a prenylated indole alkaloid readily produced by a variety of species of *Penicillia*, is an excellent candidate for demonstrating fermentation in a laboratory classroom setting, owing to its ease of purification from other metabolites and its unique structural features.

Keywords: Fermentation, Green chemistry, Laboratory instruction, Natural products, Roquefortine C

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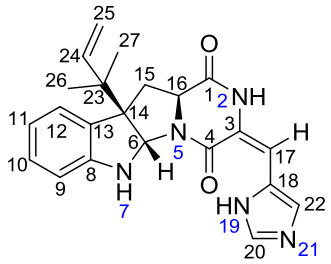
Introduction

Fermentation is an industrial process in which microorganisms such as bacteria or fungi are exploited for production of useful compounds. Though typically associated with the beer and wine industries, fermentation also allows for complex natural products to be produced in a cost-effective manner. Penicillin, arguably the most famous example of an industrially fermented natural product, is still currently produced by fermentation (Ozcengiz and Demain, 2013).

Since the onset of the modern environmental revolution in the 1960s, the practice of sustainable or green chemistry has been widely adopted, due in part to its strong promotion by the Environmental Protection Agency and other international regulatory agencies (United States Environmental Protection Agency, "Green Chemistry"). Green chemistry is a research philosophy aimed at developing chemical products and processes that minimize the use and generation of hazardous substances and maximize incorporation of all materials used in a chemical process. There are a number of principles of green chemistry embodied in fermentation. In particular, fermentation makes use of renewable feedstocks rather than depleting synthetic starting materials. Complex chemical structures are generated during fermentation without the use of chiral auxiliaries or protecting groups, reducing unnecessary derivatization. Additionally, many fermentation processes are carried out at ambient temperature and pressure, requiring minimal energy input.

The goal of the proposed experiment was to identify molecules that are easily obtained by fermentation and design an undergraduate laboratory experiment that incorporates fermentation into the traditional organic synthesis coursework. To offer a proof of concept for this strategy, we have selected a synthetically challenging compound that can be obtained in gram quantities by fermentation. *Penicillium roqueforti*, a fungus found in Roquefort cheese and a number of other blue-veined cheeses, produces a variety of complex and structurally interesting compounds including roquefortine C (Figure 1), a prenylated indole alkaloid produced by a majority of *Penicillium* fungi (Ohmomo et al., 1975). Fungi are particularly useful microorganisms in fermentation as they produce an array of diverse natural products, many of which have potential as therapeutic agents. Roquefortine C has been a compound of interest for a number of years due to its structural complexity and uniqueness (Shangguan et al., 2008) and also for its role as a biosynthetic precursor to a number of biologically active related metabolites (Ali et al., 2013; Garcia-Estrada et al., 2011; Han et al., 2013; Koizumi et al., 2004; Kosalkova et al., 2015; Reshetilova et al., 1995; Ries et al., 2013; Zheng et al., 2013). Using roquefortine C as a model compound, we have developed an experiment to introduce undergraduate students to the fields of fermentation and complex natural products.

Figure 1: *Numbering of Roquefortine C*



Roquefortine C

Experiment

Approximately 3 weeks or five 4-hour lab periods are required for the fermentation and purification of roquefortine C. In the first lab session, freeze-dried samples of *Penicillium crustosum* (a more readily available alternative to *P. roqueforti*) are rehydrated for a minimum of one hour in double distilled (dd) water and inoculated onto malt extract agar plates [1]. The agar plates are prepared using Blakeslee's formula (Taylor and Francis Group, LLC, Atlas, 2010). Fungal samples are then allowed to grow on plates for 3-5 days in darkness at room temperature until colonies of blue-green fungus are observed. In the second lab session, Czapek-Dox yeast extract (CDY) liquid media is then prepared using 35 g/L Czapek-Dox broth (Taylor and Francis Group, LLC, Atlas, 2010) and 5 g/L yeast extract [2]. Borosilicate glass trays (9 by 13 inch) are then filled with autoclave-sterilized media [1]. The CDY broth is inoculated with fungal culture using a sterile loop (or equivalent) once cooled. *P. crustosum* samples are allowed to grow in liquid culture for 12-14 days until a sufficient mycelial mat covers the surface of the liquid media. Mycelial material can be harvested during the third lab session by filtering off liquid media, macerating the mycelial mat using a mortar and pestle, and lyophilizing to remove water. During the fourth lab session, lyophilized samples can then be extracted in 1:1 chloroform/acetone for 3 hours. Organic extracts can then be concentrated by rotary evaporation to give the crude mycelial extract. Roquefortine C is purified from the other metabolites during the fifth lab session by flash chromatography (R_f 0.26, 85:10:5 chloroform/methanol/acetone). Spots are monitored by UV (365 nm) and phosphomolybdic acid (PMA), which stains roquefortine C a rusty red color. IR (KBr): 3199, 1681, 1607, 1435, 1412, 1215, 1102, 752 cm^{-1} (lit.(Ohmomo et al., 1975) IR (KBr) 3180, 1690, 1660, 1604 cm^{-1}). $[\alpha]_D^{20}$ -700.4701 (c 0.77, $CHCl_3$) (lit.(Ohmomo et al., 1975) $[\alpha]_D^{15}$ -764 (c 0.50, pyr.)). ^1H NMR

[1] Facilities that have no/limited access to an autoclave may substitute 30 minutes of boiling media in a water bath for 15 minutes of autoclave sterilization at 121 °C.
[2] Czapek-Dox broth can be purchased from a chemical supplier or made in house.

(500 MHz, CDCl3) δ: 12.93 (s, br, 1H, NH19), 9.28 (s, br, 1H, NH2), 7.68 (s, 1H, H20), 7.26 (s, 1H, H22), 7.17 (d, J = 7 Hz, 1H, H12), 7.09 (t, J = 7 Hz, 1H, H10), 6.76 (t, J = 7 Hz, 1H, H11), 6.58 (d, J = 8 Hz, 1H, H9), 6.28 (s, 1H, H17), 5.97 (dd, J = 11, 17.5 Hz, 1H, H24), 5.63 (s, 1H, H6), 5.14 (s, 1H, H25), 5.11 (d, J = 5 Hz, 1H, H25), 5.08 (s, 1H, NH7), 4.05 (dd, J = 5.5, 11 Hz, 1H, H16), 2.60 (dd, J = 6, 12 Hz, 1H, H15), 2.46 (t, J = 12 Hz, 1H, H15), 1.14 (s, 1H, H26), 1.02 (s, 1H, H27). ^{13}C NMR (500 MHz, CDCl3) δ: 167.33 (C1), 159.68 (C4), 150.15 (C8), 143.56 (C24), 137.06 (C20), 135.68 (C22), 129.33 (C13), 128.77 (C10), 125.71 (C3), 125.40 (C12), 121.89 (C18), 119.38 (C11), 115.01 (C25), 111.60 (C17), 109.38 (C9), 78.63 (C6), 61.76 (C16), 59.05 (C14), 41.15 (C23), 37.00 (C15), 23.10-22.72 (C26-C27). HRMS (EI): m/z calcd for $C_{22}H_{24}N_5O_2$ 390.1930, found 390.1930.

If desired, synthesis of isoroquefortine C, the 3,17 Z-photoisomer of roquefortine C, can be accomplished by irradiating roquefortine C with a broad spectrum UV light bulb (such as Exo-Terra UVB 200 Intense Compact Fluorescent Lamp, 26 W) in ethanol (2.3 mM) under inert atmosphere for 1-2 days. Isoroquefortine C is then purified by flash chromatography (R_f 0.19, 95:5 CH_2Cl_2:MeOH). Spots are monitored by UV (365 nm) and phosphomolybdic acid (PMA), which stains isoroquefortine C a rusty red color. IR (KBr): 3149, 2969, 1685, 1609, 1438, 1216, 1082, 748.31 cm^{-1} (lit.(Schiavi et al., 2002) IR (KBr) 1680, 1630 cm^{-1}). $[\alpha]_D^{22}$ -281.5146 (c 0.96, $CHCl_3$) (lit.(Schiavi et al., 2002) $[\alpha]_D^{20}$ -390 (c 0.05, $CHCl_3$)). ^{1}H NMR (500 MHz, CDCl3) δ: 11.70 (s, 1H, NH21), 10.84 (s, 1H, NH2), 7.60 (s, 1H, H20), 7.11 (m, 2H, H22, H12), 7.04 (t, J = 7.5 Hz, 1H, H10), 6.70 (t, J = 7.5 Hz, 1H, H11), 6.63 (s, 1H, H17), 6.55 (d, J = 7.5 Hz, 1H, H9), 5.94 (dd, J = 11, 17.5 Hz, 1H, H24), 5.61 (s, 1H, H6), 5.07 (m, 2H, H25), (Scott et al., 1979)5.02 (s, 1H, NH7), 4.03 (dd, J = 6, 11.5 Hz, 1H, H16), 2.53 (dd, J = 6, 12.5 Hz, 1H, H15), 2.42 (t, J = 12 Hz, 1H, H15), 1.09 (s, 1H, H26), 0.98 (s, 1H, H27). ^{13}C NMR (500 MHz, CDCl3) δ: 165.73 (C1), 158.70 (C4), 150.60 (C8), 143.77 (C24), 137.27 (C3), 135.66 (C20), 129.10 (C10), 129.02 (C13), 126.38 (C18), 125.41 (C12), 118.97 (C11), 117.90 (C22), 114.69 (C25), 109.16 (C9), 105.64 (C17), 78.16 (C6), 61.78 (C14), 59.29 (C16), 41.14 (C23), 37.44 (C15), 23.16-22.66 (C26-C27). HRMS (EI): m/z calcd for $C_{22}H_{24}N_5O_2$ 390.1930, found 390.1932.

Hazards

Protective clothing, goggles, and gloves should be worn at all times during this experiment. Dichloromethane is harmful if swallowed or inhaled; may be harmful by skin contact. Chloroform is harmful if swallowed or inhaled; may be harmful by skin contact. Flasks should be inspected for cracks before rotary evaporator/lyophilzer use.

Results and Discussion

This experiment was validated by two undergraduates working in our laboratory. Both students were successful in fermenting and isolating roquefortine C from *P. crustosum*. Mycelial material per 9 by 13 inch tray was approximately 70 grams prior to lyophilization and 7.5 grams following lyophilization. Students were able to isolate about 125 mg of purified roquefortine C per tray.

The polarity of roquefortine C allows for straightforward extraction and purification. Roquefortine C exhibits high solubility in polar aprotic solvents such as chloroform, acetone, and dichloromethane, which permits its extraction from the mycelial material. Additionally, the polarity of roquefortine C also allows for it to be easily purified from less polar metabolites.

This particular laboratory experiment allows for instruction in and discussion of several scientific disciplines. In the field of fungal metabolism, for example, students will learn that carbon and nitrogen sources are needed to construct the amino acid building blocks of small molecules and proteins. The carbon and nitrogen sources may be identified by the students as sucrose and sodium nitrate, respectively, from the list of reagents that comprise the Czapek-Dox broth growth media. By examining roquefortine C's biosynthesis (Ali et al., 2013; Garcia-Estrada et al., 2011; Kosalkova et al., 2015; Ries et al., 2013), students can note that the secondary metabolite roquefortine C is comprised of three primary metabolite building blocks: tryptophan, histidine, and dimethylallyl pyrophosphate (Scheme 1).

Experiments in fermentation also allow for the discussion of biosynthesis and methods, such as gene silencing, that are used to elucidate biosynthetic pathways. Additionally, observing changes in the production of secondary metabolites in response to various environmental stimuli can provide clues to the role of the product in the biology of the fungus.

Experience in structural characterization can be obtained through spectroscopic analysis of roquefortine C and other structurally related compounds. The skeleton of roquefortine C is that of an indole alkaloid of the 2,5-diketopiperazine type. Roquefortine C also contains an unusual *E*-dehydrohistidine moiety, which is known to undergo facile isomerization under photochemical conditions to give the 3,17 Z-isomer, isoroquefortine C (Scott et al., 1979). Structural features such as the reverse prenyl methyl groups can be readily identified by ^{1}H NMR and roquefortine C can be differentiated from its precursor, roquefortine D, and from its *Z*-photoisomer, isoroquefortine C, by ^{1}H NMR (structures shown in Figure 2). Additionally, advanced students may perform ^{1}H-^{1}H NOESY (Nuclear Overhauser Effect Spectroscopy) experiments to observe a strong NOE correlation between H2 and H17 in roquefortine and a very weak or absent NOE correlation between H2 and H17 in isoroquefortine C.

Scheme 1: *Biosynthesis of Roquefortine C*

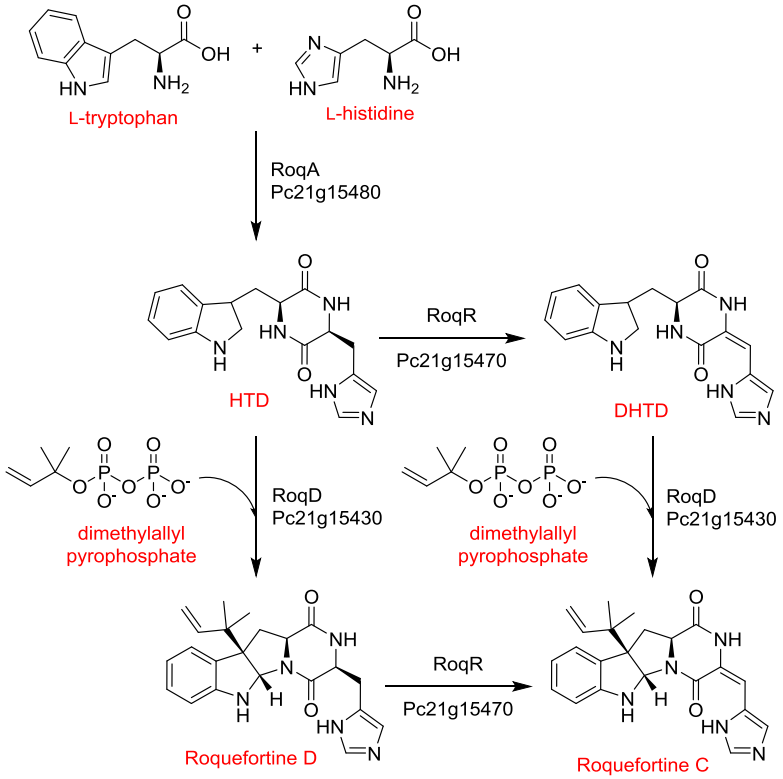
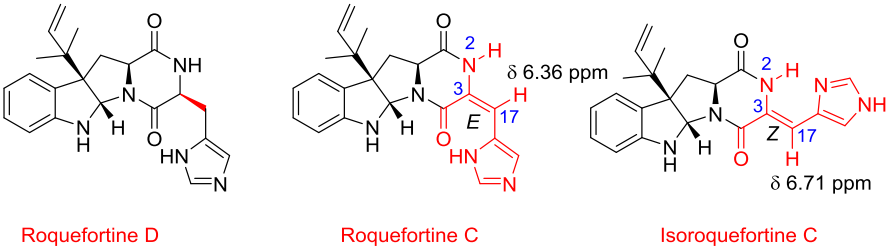


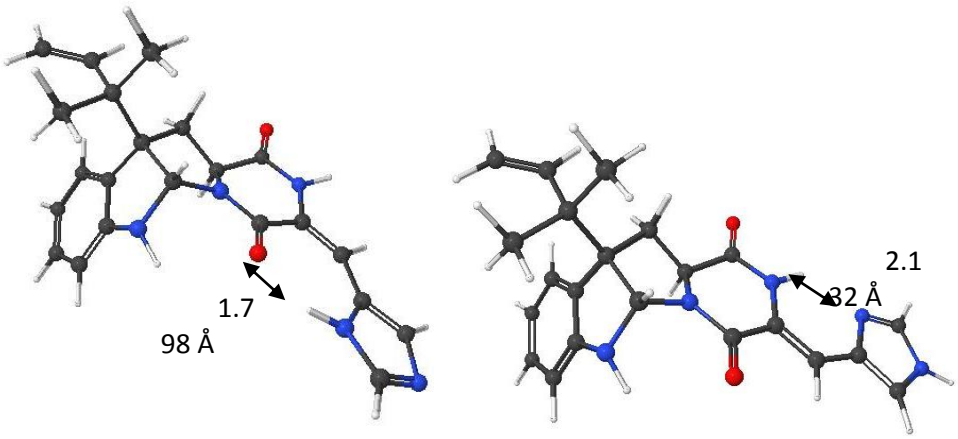
Figure 2: *Structures of Different Roquefortines*



Spectroscopic analysis of roquefortine C can also be used to demonstrate differences in physical properties between isomers. The stereochemistry of the 3,17 double bond in roquefortine C and isoroquefortine C allows for two different intramolecular hydrogen bonds. This difference in structure produces compounds with different activity towards hepatic cytochrome P450s, exemplifying the importance of stereochemistry in determining bioactivity (Aninat et al., 2001). As a consequence of the hydrogen bond between the imidazole N-H and the carbonyl of the diketopiperazine, the two imidazole nitrogens are not equivalent, and roquefortine C exhibits a strong efficient Fe-N interaction with hepatic cytochrome P450s and microperoxidase, whereas isoroquefortine is rapidly oxidized. The energy-minimized structures of the two compounds (Figure 3) clearly show the differences between them; the tertiary

nitrogen of the imidazole is free in roquefortine C but involved in a hydrogen bond in isoroquefortine C.

Figure 3: *Geometry-optimized Structures of Roquefortine C (Left) and Isoroquefortine C (Right). Calculations Performed at HF 631-G Level of Theory.*



Conclusions

The experiment discussed illustrates the use of fermentation in the production of organic compounds. It also introduces students to the indole alkaloid roquefortine C and to the use of spectroscopic methods to characterize novel compounds and their use in differentiating specific structural units.

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