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Synthesis, Characterization, and Bioactivity of 3-Substituted Coumarins as an Undergraduate Project

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SYNTHESIS, CHARACTERIZATION, AND BIOACTIVITY OF 3-SUBSTITUTED COUMARINS AS AN UNDERGRADUATE PROJECT

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ABSTRACT

Coumarins are an important class of phytochemicals, a chemical defense presumed to be secreted by plants. More recently, Coumarins have gathered popularity for their basis in anti-cancer agents. This paper dives into the organic synthesis of two 3-substituted coumarins from o-vanillin using the Knoevenagel condensation reaction. The 3-substituted coumarin were characterized using melting point analysis, ^{1}H-NMR, and UV-Vis spectroscopy. In addition, the anticancer activity of synthesized 3-substituted coumarin compounds were assayed against topoisomeraseIIα, which is the target enzyme of FDA approved anti-cancer drug etoposide since it is an active enzyme in cancer cell replication. The demonstrated procedures can be used as an undergraduate senior project that traverses through several classes that chemistry majors take.

Keywords

Coumarins, topoisomerase, o-vanillin, anti-cancer activity, undergraduate project, bathochromic shift

INTRODUCTION

Coumarin comes from the French term, Coumarou, meaning Tonka bean. It was first isolated in 1820 as a natural product. Due to its sweet-smelling, hay-like odor, it has been used in perfumes since 1882. Coumarin is a phytochemical; a chemical compound that is naturally occurring in the plant kingdom. Phytochemicals are presumed to be produced by plants as a chemical defense to discourage predation. It is speculated to have potential activity against cancer and metabolic or degenerative diseases. They are a family of benzopyrones and bear the typical framework. The unique and versatile oxygen-containing heterocyclic structure sets an important place in medicinal chemistry aside for coumarins. Within the coumarin ring, there is a large-conjugated system with electron-rich and charge-transport properties. This is vital in the interaction of this scaffold with other molecules and ions. There has been much headway in coumarin-based anti-coagulant, anti-viral, anti-parasitic, anti-fungal, antioxidant, anti-neurodegenerative, anti-inflammatory, anti-diabetic, and anti-cancer agents (Matos et al., 2015).

With much of the excitement surrounding coumarin, this paper dives into the synthesis and characterization of two, 3-substituted coumarins Ethyl 8-methoxycoumarin-3-carboxylate **1** and 3-acetyl-8-methoxychromen-2-one **2** using *o*-vanillin as shown in figure 1 and how the synthesis relates to the basic reactions that are taught in the organic chemistry sequence. It also explores the anti-cancer activity; where topoisomeraseIIα is assayed with the coumarin compounds synthesized. TopoisomeraseIIα was chosen to investigate the anticancer activity due to it being a target of several important classes of anticancer drugs.

Topoisomerases are integral to the survival of an organism that plays essential roles in several fundamental DNA processes due to the function of preventing DNA from

tangling and excessively supercoiling during replication. TopoisomeraseIIα is an enzyme active during cell replication, especially in cancer cells (Nitiss et al., 2009). A drug that targets and inhibits topoisomeraseIIα can reduce DNA relaxation causing malfunctions in DNA replication and stop cell division and cause cell death. One such known drug that targets topoisomeraseIIα is etoposide. Etoposide kills cancer cells by stabilizing the cleavage complex that is a transient intermediate of the topoisomeraseIIα catalytic cycle and thus stopping the catalytic cycle of the enzyme (Baldwin et al., 2005).

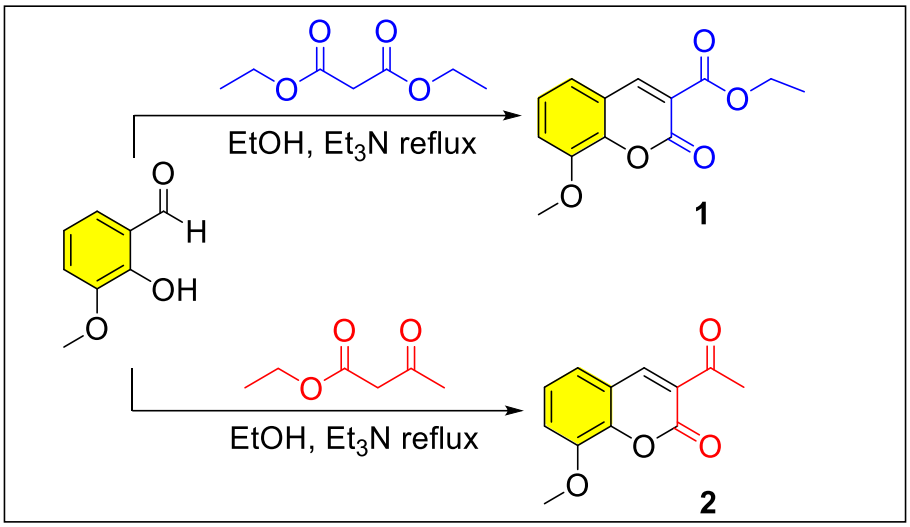

Figure 1. Synthesis of ethyl 8-methoxycoumarin-3-carboxylate **1** and 3-acetyl-8-methoxychromen-2-one **2**.

MATERIALS & METHODS

All chemicals used in the synthesis and characterization were purchased through Sigma Aldrich St. Louis, MO unless specified otherwise.

Synthesis of ethyl 8-methoxycoumarin-3-carboxylate 1:

To a 50 mL round bottom flask, 0.8 g (0.00526 moles) of 3-methoxysalicylaldehyde, 0.93 grams (0.0058 moles) of diethyl malonate, 0.5 mL (0.0036 moles) of triethylamine, and 5 mL of absolute ethanol was added. The mixture was refluxed for 1 hour. After one hour, the reaction was allowed to cool to room temperature then about 15 mL of water was added to the round bottom flask. The flask was covered with parafilm and placed in the fridge for 2 days. The solid was collected via vacuum filtration. The solid was then dissolved in heated 95% ethanol and then placed in the fridge for 3 days to allow recrystallization to occur. The crystals were collected via vacuum filtration and air dried to obtain **1** as yellow crystals with melting point range of 76-79°C. 0.58 g (0.0023 moles) were obtained corresponding to 44.23% yield. ^{1}H NMR (60 MHz, $CDCl_3$) δ 8.49 (s, 1H), 7.36 – 7.02 (m, 3H), 4.42 (q, J = 7.1 Hz, 2H), 3.97 (s, 3H), 1.40 (t, J = 7.1 Hz, 3H).

Synthesis of 3-acetyl-8-methoxychromen-2-one 2:

2 was synthesized in the same way using 0.9 mL (0.0071 moles) ethyl acetoacetate. Melting point range of 164-171 °C. 0.69 g (0.0031 moles) of **2** were obtained

corresponding to 59.8% yield. ^{1}H NMR (60 MHz, $CDCl_3$) δ 8.47 (s, 1H), 7.27 - 7.18 (m, 3H), 4.00 (s, 3H), 2.75 (s, 3H).

UV-Vis spectrophotometry:

An OceanOptics USB2000 UV-Vis was blanked using a quartz cuvette with 95% ethanol. UV-Vis spectra for the two synthesized compounds in the 300-450 nm range were obtained using the quartz cuvette: 6.684 x 10^{-4} M O-Vanillin in 95% ethanol and 6.687 x 10^{-4} M Coumarin product in 95% ethanol. The data was collected and plotted on SigmaPlot.

Topoisomerase Relaxation Assay:

A 50.0mM stock drug solution was prepared by dissolving the synthesized coumarin product in 100% DMSO. A 5.0, 4.0, 3.0, 2.0, and 1.0 mM solution were then created in 10% DMSO. Microcentrifuge tubes were then prepped with 2µL of each solution labeled A through E with 5.0 mM being added to A, 4.0 mM to B, 3.0 mM to C, 2.0mM to D, and 1.0mM to E. One microcentrifuge tube was labeled NT for no topoisomerase and had 2 µL 10% DMSO added as a negative control. Two microcentrifuge tubes have designated no drug (ND) for no coumarin drug added and each had 2µL 10% DMSO added as positive controls. All microcentrifuge tubes were kept on ice during the duration of use. A master mix (MM) was created using 34 µL 5x reaction buffer (17µL buffer A and 17µL buffer B) (TopoGen Buena Vista, CO) , 5.78µL kDNA (383 ng/µL) (TopoGen Buena Vista, CO), 109.98 µL diH_2O, and 4.25 µL of 1.0 mg/mL topoisomeraseIIα, purified in house at Tennessee Tech, in a microcentrifuge tube. Before the topoisomeraseIIα was added 18µL of the MM was added to NT. After the TopoIIα was added to the MM 18µL was added to each tube following 30-second intervals and moved to a dry bath set at 37°C. Each tube was on a dry bath for 30 minutes before being removed, having 5µL 5x stop buffer (TopoGen Buena Vista, CO) added, and moved to ice. The assay was performed in duplicate for both coumarin compounds.

Agarose Gel Electrophoresis:

A 1% agarose gel was created in 100mL 1x TBE buffer using 1.0 g agarose medium-low EEO (Acros Organics Fair Lawn, NJ) and allowed to sit with a twenty-tooth comb and once solidified it was stored in a 1x TBE buffer in the gel electrophoresis apparatus. Once all tubes were moved back on ice after the topoisomerase relaxation assay, 15 µL of each solution were added to wells on the prepared gel and 10 µL linear marker (TopoGen Buena Vista, CO) added to well 1. The gel was then run for 90 minutes at 120 volts and 250 milliamps. Once the gel was run, it was stained using 10 µL SYBR green in 100 mL 1x TBE and for 20 minutes on a shaker. The gel was then imaged using an Accuris Smartdoc equipped with an orange filter.

RESULTS

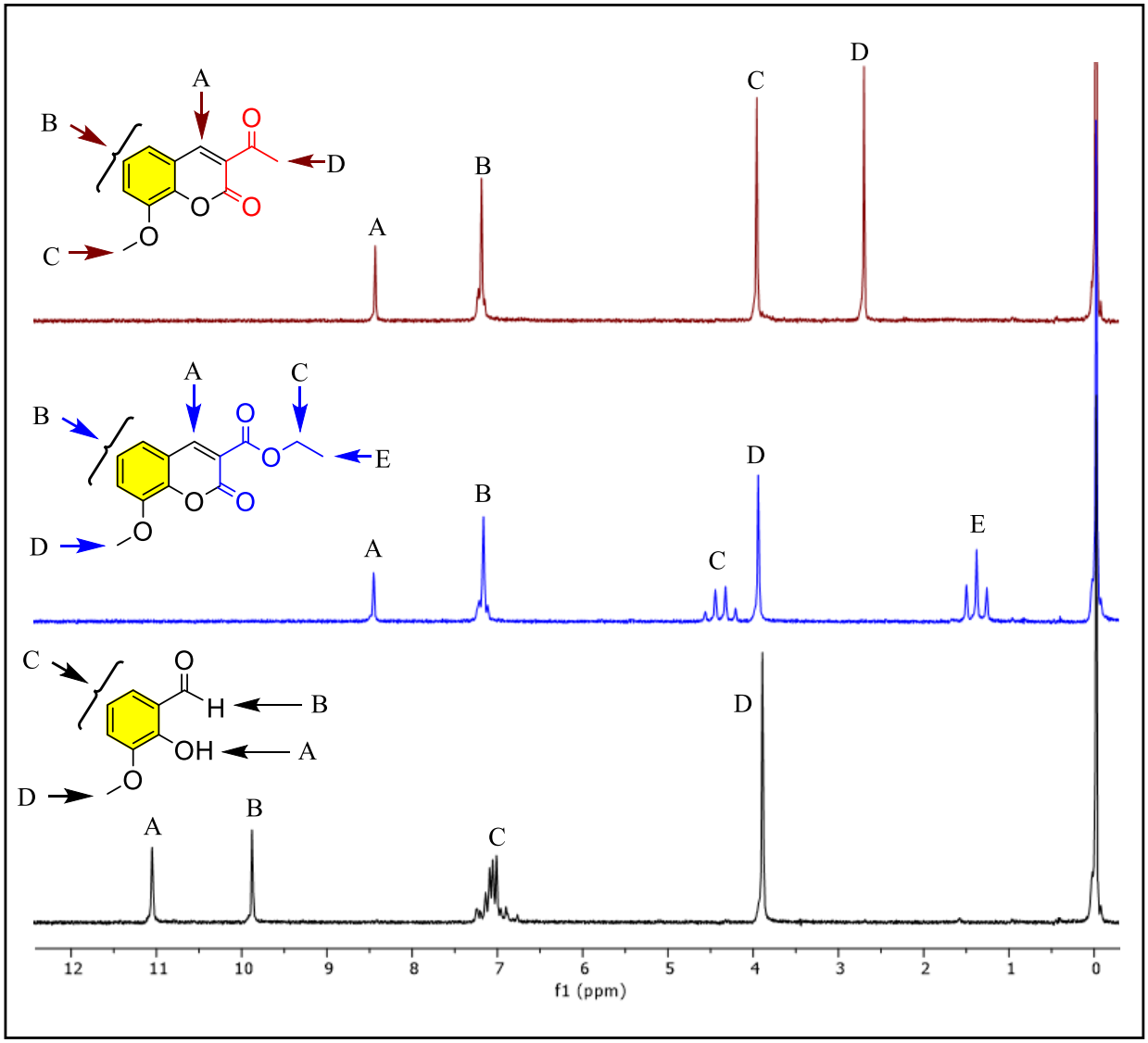

Figure 2: Stacked ^{1}H NMR spectra showing successful conversion of *o*-vanillin (bottom spectrum) to ethyl 8-methoxycoumarin-3-carboxylate **1** (middle spectrum) and 3-acetyl-8-methoxychromen-2-one **2** (top spectrum).

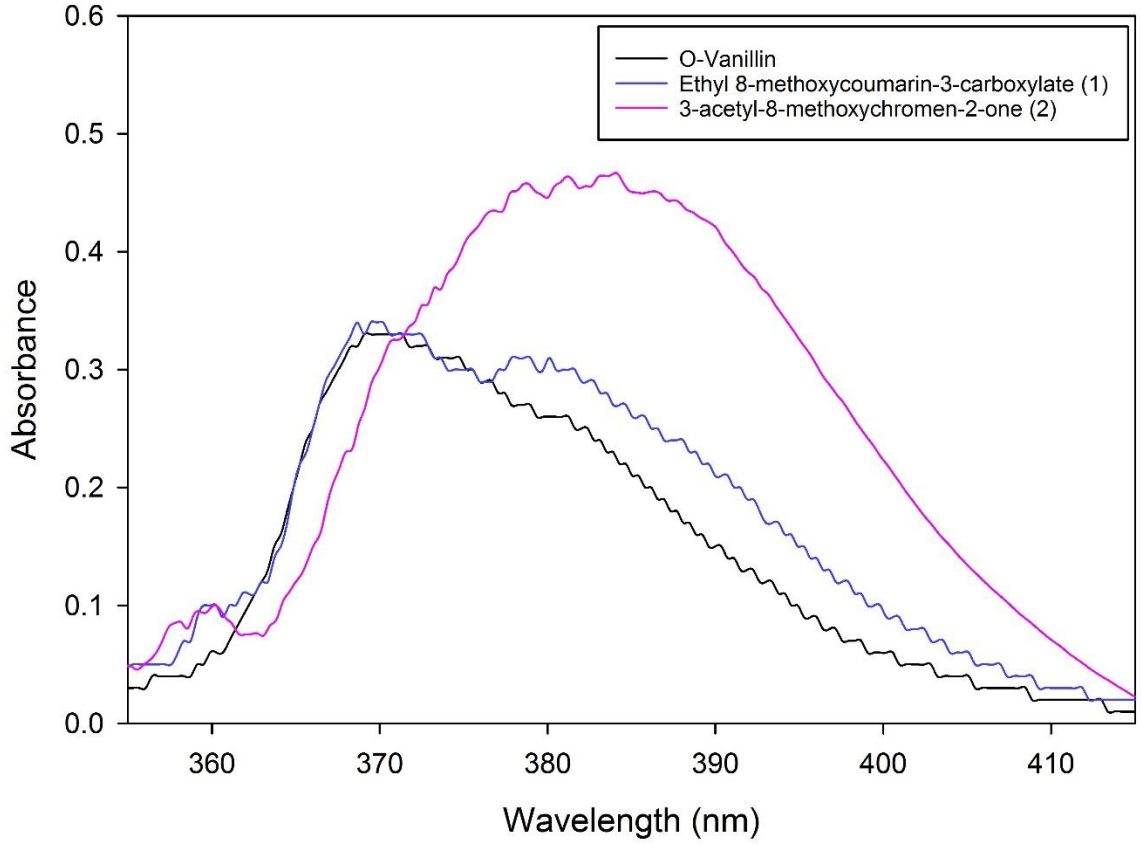

Figure 3: Comparison of the of UV-Vis spectra of starting material (o-vanillin) and the two synthesized coumarins. The spectra show a bathochromic shift in the absorbance peaks when the product is formed.

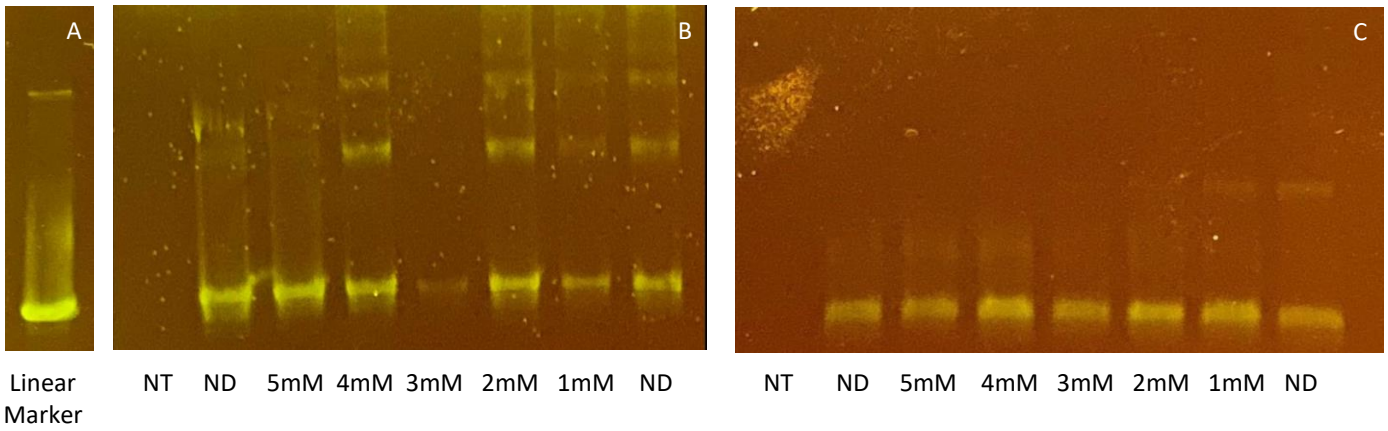

Figure 4: A) Linear marker; B) TopoisomeraseIIα assay with Ethyl 8-methoxycoumarin-3-carboxylate; C) TopoisomeraseIIα assay with 3-acetyl-8-methoxychromen-2-one, NT – no topoisomeraseIIα added (negative control), ND – no coumarin added (positive control), rest of the lanes indicate the concentration of the coumarin product added to each reaction tube to inhibit the topoisomeraseIIα enzyme.

DISCUSSION

Ethyl 8-methoxycoumarin-3-carboxylate **1** and 3-acetyl-8-methoxychromen-2-one **2** were synthesized by a slight variation of the Knoevenagel condensation reaction (Horning et al., 1955). Briefly as shown in figure 5, the enolate ion **4** formed by reaction of triethylamine (Et_3N) and diethyl malonate or ethyl acetoacetate underwent direct addition to the carbonyl carbon of *o*-vanillin **3**. Taking a more in depth look into this step we see it is just a slightly modified Claisen condensation, also known as the Acetoacetic-ester condensation with the use of Et_3N as the base instead of sodium ethoxide (NaOEt) (Bruice, 2015). The resulting tetrahedral intermediate **5** underwent proton transfer followed by dehydration. The resulting conjugated phenolate ion **6** underwent intramolecular direct addition to give the cyclic tetrahedral intermediate 7 which collapsed to afford ethyl 8-methoxycoumarin-3-carboxylate **1** or 3-acetyl-8-methoxychromen-2-one **2**. It is important to note that the reactions taught during the Organic Chemistry sequences are not abstract but used in synthesis of biologically or pharmaceutically relevant molecules. Through this project students will appreciate the applications of the basic synthetic steps they learned during class.

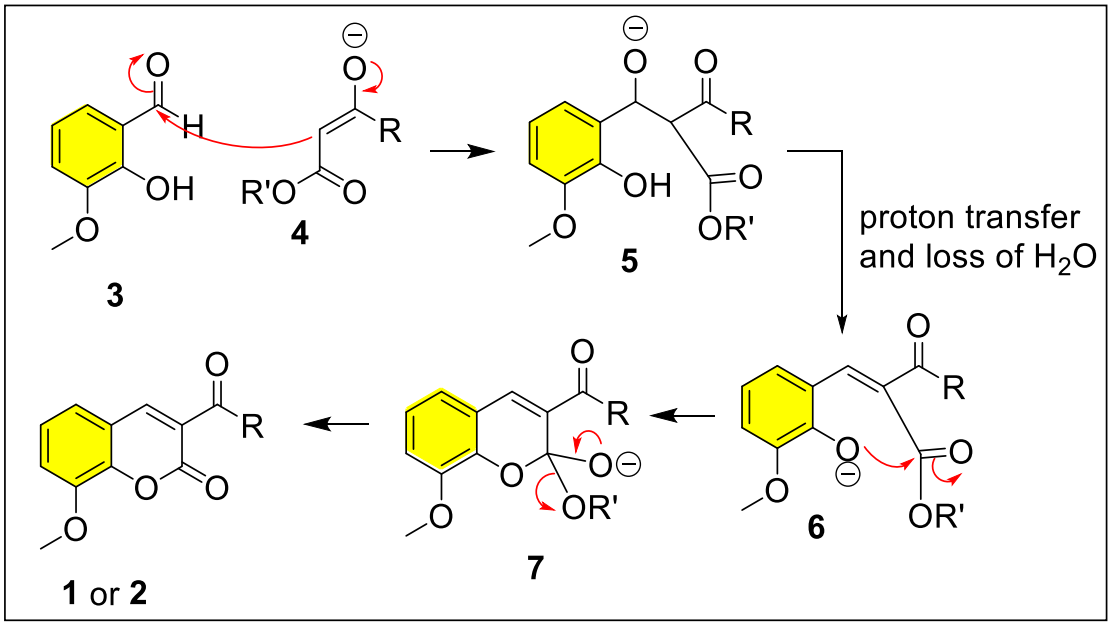

Figure 5: Proposed mechanism for the formation of ethyl 8-methoxycoumarin-3-carboxylate **1** or 3-acetyl-8-methoxychromen-2-one **2.**

Analysis of the ^1H NMR spectra of 3-substituted coumarin product **1** and **2** in comparison with the starting material *o*-vanillin shown disappearance of the peak due to hydroxy proton and the peak due to aldehyde proton (singlets at around 11 ppm and 10 ppm (see figure 2 bottom spectrum)) consisted with transformation of *o*-vanillin to products **1** and **2**. The analysis of the UV-Vis spectra also showed a red shift from reactants to products, which is indicative of a more conjugated structure. The molar extinction coefficient for **1** was determined to be $2.0*10^{-3}$ $M^{-1}cm^{-1}$ with a λ_{max} of 369.44 nm and the molar extinction coefficient of **2** was determined to be $1.234*10^{-3}$ $M^{-1}cm^{-1}$ with a λ_{max} of 384.13 nm. Both **1** and **2** showed peaks around 375-385 nm which was not observed in the starting compound o-vanillin.

Coumarins exhibit high biological activity while maintaining low toxicity. Because of these traits, it is often observed as an anticancer agent. It is even used in treating, prostate cancer, renal cell carcinoma and leukemia, and it's claimed that they also have the ability to counteract the side effects caused by radiotherapy (Küpeli et al., 2020). The synthesized coumarins were tested for the ability to inhibit topoisomeraseIIα which is an active enzyme in cancer cells and the target of some of the existing anticancer therapeutics.

However, at the concentrations tested for each product during this experiment (up to 5 mM), topoisomeraseIIα was not successfully inhibited. Comparing the linear marker which is used as a positive enzyme activity indicator (figure 4-A) it can be clearly seen that even in the presence of the coumarins the enzyme was fully active. It can clearly be observed that there's no difference in enzyme activity between no addition of coumarin (ND, figure 4-B and 4-C) and addition of coumarin (labeled with different concentrations). This result only indicates that the synthesized coumarins are not active against tomoisomeraseIIα. Further studies are needed to establish the activity, most importantly a study against a cancer cell line.

Synthesis and characterization of coumarins is particularly applicable as an undergraduate research project for senior chemistry students, as it brings together knowledge gained in organic chemistry, biochemistry, and analytical chemistry classes. Using the fundamentals of organic chemistry to synthesize coumarin, this experiment walks through the synthesis, characterization, and bioanalysis of a chemical compound. This can also be utilized as a project that can connect the individual chemistry labs together for the student. For example, coumarins can be designed and synthesized during organic chemistry lab (second year of curriculum), characterized during analytical chemistry lab (third year of curriculum), assayed for bio-activity (fourth year of curriculum) to give the students an idea of how each lab plays an important role in the sequence.

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