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Laboratory Experiment

Introduction to Preparative Chromatography: Description of a Setup with Continuous Detection

Karoline Gangestad Primdahl,* Frederik André Hansen, Eirik Johansson Solum, Jens Mortansson Jelstrup Nolsøe, and Marius Aursnes*

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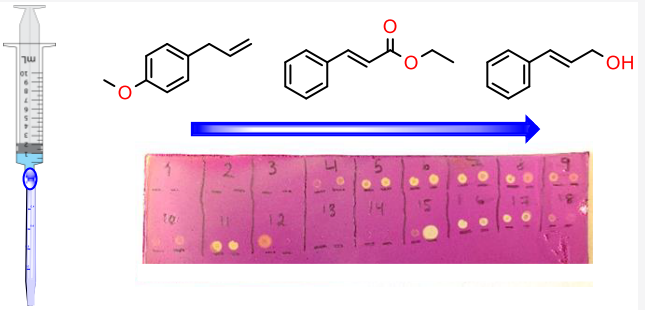
ABSTRACT: Preparative chromatography is one of the most common and important purification techniques in organic chemistry and is employed in laboratories across the globe. In order to facilitate student proficiency with the method, a unique approach for the separation of estragole, ethyl cinnamate, and cinnamyl alcohol using microscale preparative flash column chromatography and gradient elution has been developed. During the chromatographic purification, a chemically impregnated TLC plate is used to provide as close to real-time elution information as possible, in this manner mimicking the effect of a UV detector. This straightforward yet powerful technique greatly aids in the visualization process, gives valuable information regarding the ongoing elution process, and minimizes confusion and errors.



KEYWORDS: *Chromatography, Separation Science, Thin-Layer Chromatography, Microscale Lab, Laboratory Instruction, Hands-On Learning, General Chemistry, Analytical Chemistry, Organic Chemistry*

INTRODUCTION

Purification of fine chemicals by preparative chromatography is a routine task in the organic chemistry laboratory. While numerous advances have been made since Still's seminal publication on flash column chromatography,[1] especially in the context of automated systems using prepackaged columns and UV detectors, the classic approach is still in widespread use in research laboratories around the world.[2−5] The low-tech nature also means that the technique is widely accessible and may be carried out using standard laboratory equipment. Additionally, and of special importance in this context, the process is highly educational, with an exposed setup in which all internal workings are observable—unlike that of an HPLC system, which typically operates in a much more "black box" manner.[6,7] As a consequence of this, the described chromatographic purification experiment will also be helpful for preparing students for more advanced practices and theory related to chromatography through practical experience.

There are other benefits in introducing the students to the art and science of "running a column" by performing microscale flash column chromatography. Obviously, the amounts of resources needed, such as silica gel, solvent, and compound matrix, are comparably small, making the process far more affordable. Additionally, since the preparation and elution of a column is fast, students have the time to make *mistakes* during the laboratory course—*productive failures* being an important part of learning. Should, for example, the first attempt to pack a column yield unsatisfactory results, it may simply be discarded, while the student gives it another attempt with adjustments based on the newly accrued experience. Furthermore, all of the important practical and theoretical aspects are equally well retained and conveyed on this scale.

After working with numerous students at different levels and with varying backgrounds, it became evident that many lacked sufficient hands-on training to be competent with the technique. In light of this, an experiment to provide undergraduate students with direct experience related to all aspects of flash column chromatography has been developed. The *practical pedagogical goals* for the students were defined as follows: learn how to (i) pack and prepare the column, (ii) mix the mobile phase, (iii) load the material onto the column, (iv) monitor the elution process, and (v) evaluate the results. The last point involves identifying which fractions contain which products and give a qualified assessment regarding the obtained purity. In addition, the following *theoretical learning goals* were set: After completion of the laboratory course, the students should know that preparative chromatography is a (i)

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separation technique that occurs in a (ii) column as a result of the varying degrees of (iii) intermolecular interactions between the compounds in question and the (iv) solid phase as they are transported by the (v) mobile phase. Furthermore, they should know that (vi) gradient elution instead of (vii) isocratic elution is used to aid in the separation and that ethyl acetate serves as a (viii) modifier to increase the (ix) strength of the mobile phase and be able to rationalize the elution order on the basis of the major (x) retention mechanisms.

Furthermore, it was deemed crucial for the experiment to be safe, affordable, and easily adjustable in terms of complexity according to the level of prior experience. On the latter point, this may be achieved by judicious control of the amount of information given to the students beforehand, which in turn will influence the degree of independent experimental exploration that is needed to achieve satisfactory results (see the Supporting Information). The laboratory course may be carried out in 3−4 hours, with each student performing the task individually.

The experiment must also be *realistic* and *sufficiently challenging* to prepare students for real-lab purification tasks. In order to achieve this, a number of techniques and methods have been incorporated that, when taken as a whole, offer a uniquely pedagogical introduction to the subject.

First, we describe a safe protocol for the assembly of a chromatographic system that minimizes exposure: both the compound matrix and the stationary phase are given out in a premade format. Second, emulating larger-scale flash column chromatography, we have developed a pressurized device that eliminates the risk of backdraft associated with the use of a pipet bulb. Third, we describe a regime and a detection system that involves the use of a chemically impregnated TLC plate to monitor the chromatographic process in a close-to-real-time elution, allowing spot visualization to be performed without interrupting the elution process—thereby circumventing the drawbacks with conventional go-to UV detection. Fourth and finally, we describe a chromatographic separation of three compounds (estragole, ethyl cinnamate, and cinnamyl alcohol; Figure 1) on a milligram scale using microscale preparative

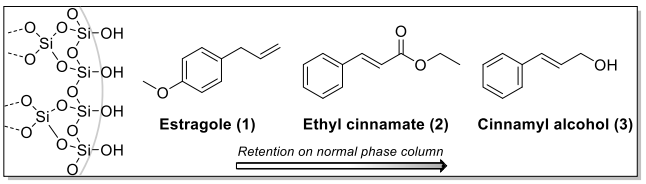

Figure 1. Compound matrix to be separated using normal-phase column chromatography and simplified view of the silica gel stationary phase.

flash column chromatography and gradient elution. The use of a common scaffold with incremental functional variation serves the purpose of conveying the physiochemical parameters that dictate the chromatographic behavior of related compounds and illustrates the scope of the technique; from a pedagogical point of view, the experimental design puts an emphasis on the intellectual process to unify theory and practice. Furthermore, the above-mentioned compounds are all affordable and readily available from reputable chemical dealers.

It should be stressed that even though this purification experiment has been designed to teach preparative chromatography to undergraduate students, the techniques and methods described have been extensively used by the authors in synthetic campaigns in which microscale operations were frequently encountered during the final steps. One recent example involves a key step in the synthesis of the specialized proresolving mediator resolvin E4 (Figure 2).[8] The crude material was purified by microscale flash column chromatography to furnish 7 mg of the desired product.

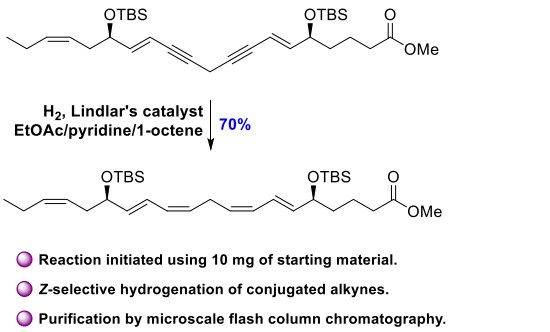

Figure 2. Hydrogenation reaction in the synthesis of resolvin E4, in which the crude reaction mixture was purified by the described techniques.

EXPERIMENTAL PROCEDURE

The standard way of pressurizing a Pasteur pipet flash column with the aid of a pipet bulb is inherently flawed because of the high risk of inadvertently drawing both solvent and silica gel into the bulb when removing it. Alternatives to this approach have been presented before,[9,10] but the one included here is especially facile, using standard equipment present in all organic chemistry laboratories. A suitably sized septum was pierced with an awl or similar tool, through which the tip of a disposable 10 mL syringe was introduced, giving a simple device that is well-suited for pressurizing a Pasteur pipet column (Figure 3). This greatly mitigates the above-mentioned risk and allows for smooth, even elution.

Upon first entering the chemical laboratory, students experience first-hand that chemistry is the science of the occult, the concealed, the hidden. Thus, in order to address this issue and provide a visual aid containing as close to real-time elution information as possible, mimicking the effect of a UV detector, the following technique was adopted: A TLC plate is marked, dipped in a $KMnO_4$ staining solution, and briefly dried using a heat gun. Then, as the elution process is taking place, a small amount of the mobile phase is continuously spotted onto the impregnated TLC plate using a capillary tube and quickly shows up as yellow spots when it contains oxidizable compound material (Figure 4, right). This detection system allows the students to see what is happening, tying it together with the chemical structures and relevant functional groups as well as the information obtained from the initial TLC analysis (Figure 4, left). Since it greatly aids in the visualization process, it also strengthens the cerebral travel between the microscopic and macroscopic worlds—a mental process that is of paramount importance in the training of scientists.[10]

HAZARDS

As always in a laboratory setting, a lab coat, safety glasses, and gloves must be worn at all times. Because of the use of organic solvents and compounds, the experiment must be conducted in

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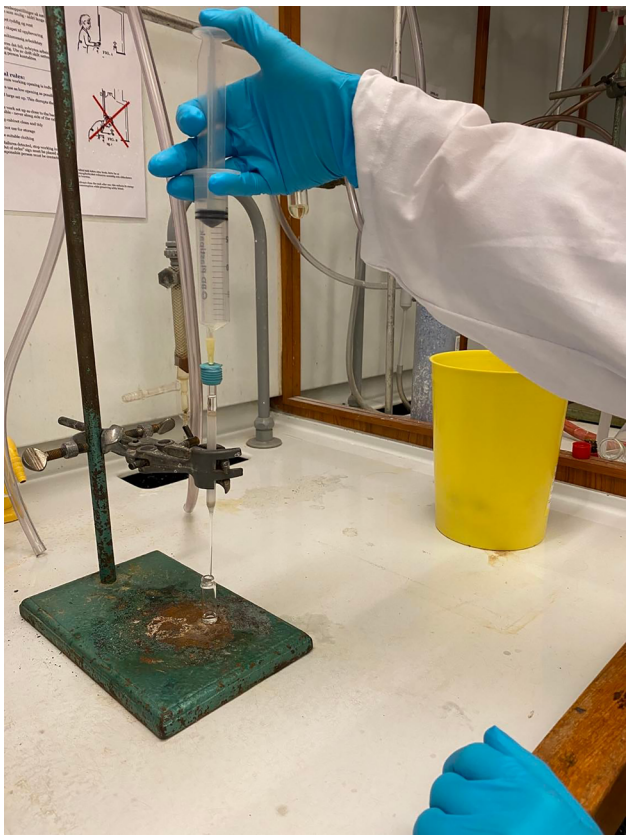

Figure 3. Eluting the column using a syringe fitted with a septum.

a fume hood. The use of *n*-heptane instead of *n*-hexane is important because the former solvent is not metabolized in vivo to neurotoxic 2,5-hexanedione and has a significantly improved safety profile. The combined compound matrix consisting of estragole, ethyl cinnamate, and cinnamyl alcohol should be weighed into small, stoppered vials (~20−25 mg per vial) in a fume hood in advance by the supervisors and must be handled only inside a fume hood by the students to avoid exposure. In light of the inhalation hazard associated with working with silica gel, special care must be taken for students with varying degrees of experience. Hence the slurry should be premade by the instructors in vials with screw caps. Generally, great care should be taken to ensure that all material that might constitute a risk is placed inside the fume hoods before the course begins, and a waste container should also be present inside said fume hood. Finally, the students must be carefully supervised and observed throughout the course.

RESULTS

The column was packed by adding a premade slurry of silica gel in *n*-heptane to a Pasteur pipet charged with a small amount of cotton and sand and then applying pressure using the described elution device until the height of the column was close to the upper indent of the pipet. A small amount of sand was then added to the top of the column to serve as a protective layer. The compound matrix was added onto the column next, and *n*-heptane was used to start the elution. Fractions were collected in small vials (Figure 5), and the elution process was continuously monitored by spotting a $KMnO_4$-impregnated TLC plate. Once the least-retained compound had completely eluted, a mobile phase consisting of 5% EtOAc in *n*-heptane was made in a 10 mL graduated cylinder using squeeze bottles filled with the above-mentioned solvents, and elution and monitoring were continued. Once the second compound had passed through the column, the polarity of the mobile phase was increased to 20% EtOAc in *n*-heptane,

Figure 5. Collected fractions in vials from the flash column chromatography purification. The inverted septum is used to keep track of the next fraction to be spotted onto a TLC plate.

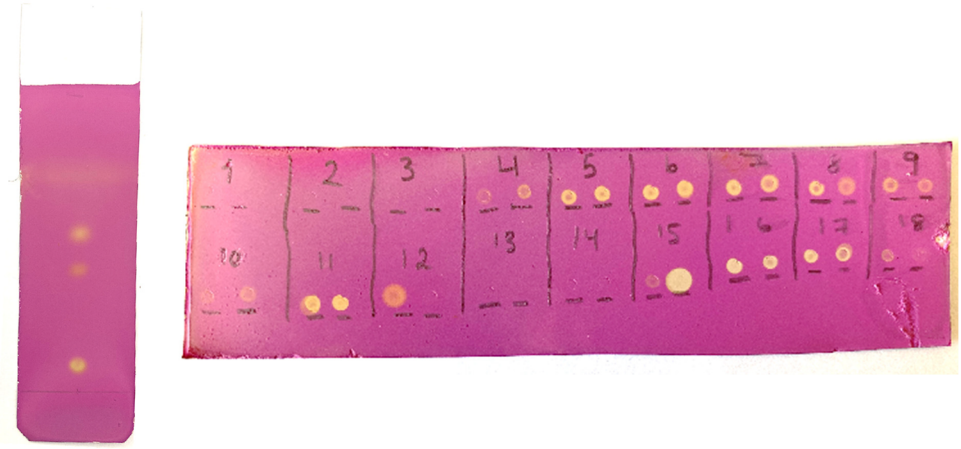

Figure 4. TLC analysis of the matrix in 20% EtOAc in *n*-heptane (left) and a premarked, impregnated TLC plate used for continuous monitoring of elution progress (right).

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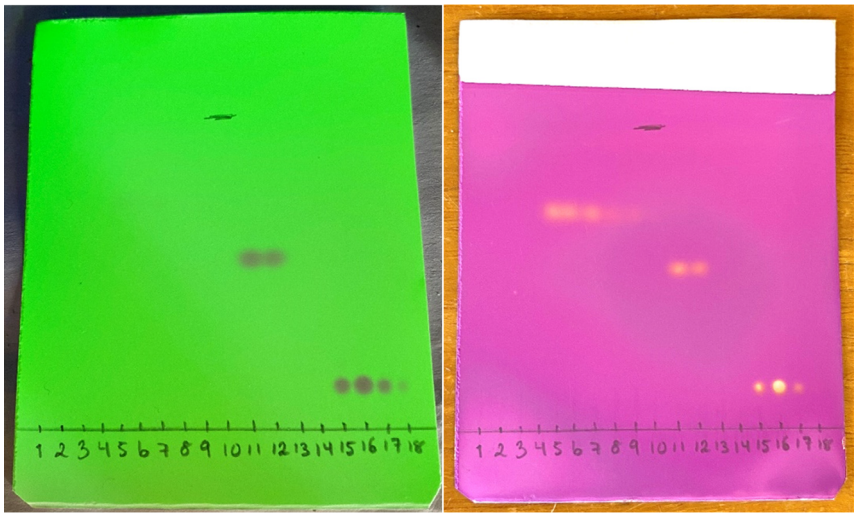

Figure 6. TLC plate (i) under UV light and (ii) after it was stained with $KMnO_4$. Estragole is not seen well under UV light (254 nm).

and elution was performed until the third and final compound was out of the column.

The logic applied when monitoring the elution is described using fraction 12 as an example: The vial was filled until roughly half-full, and then this solution was sampled and spotted onto the $KMnO_4$-impregnated TLC plate on the first horizontal line under the 12 mark with the aid of a capillary tube, showing up as a yellow spot (Figure 4). Elution was continued until the vial was full and then a drop of the mobile phase was sampled from *the tip of the Pasteur pipet* and placed on the second placeholder line. Since no spot was observed, this confirmed that the second compound had now cleared the column completely (Figure 4). If no spot had been observed from the solution in the half-filled vial, then the second sample would also have been collected from the vial instead of the tip of the pipet. This reasoning was used throughout the purification procedure.

A marked TLC plate was then prepared, and each fraction was spotted onto it. Then the plate was developed in a TLC chamber using 20% EtOAc in *n*-heptane. Once development was complete, the plate was evaluated under UV light (254 nm). Thereafter, the plate was dipped into $KMnO_4$ stain and treated with a heat gun until spots appeared (Figure 6).

Finally, a pure fraction containing each of the three compounds was used together with standards of estragole, ethyl cinnamate, and cinnamyl alcohol to confirm the order and identities of the eluted compounds using qualitative TLC analysis and the cospot technique. The resulting TLC plate was first examined under UV light and then developed using $KMnO_4$ staining with concomitant heating.

Naturally, spectroscopic identification techniques such as UV−vis, IR, and NMR spectroscopy may be used as well. An example of the latter is the following: After completion of the column chromatography separation experiment, the students were tasked with providing vials with pure material for the three separated compounds based on their final TLC analysis of the collected fractions. The decision about which fractions to include was left entirely up to their judgment, and this led to educationally important discussions among the students. The fractions received for estragole, ethyl cinnamate, and cinnamyl alcohol were combined in three separate flasks, concentrated in vacuo, and subjected to ^{1}H NMR analysis (see the Supporting Information).

From two rounds of laboratory courses consisting of 20 students each day, the instructors collected the developed TLC plates showing all of the fractions, and hence the individual results of the performed separation, for assessment purposes. The plates were analyzed and grouped into four main categories consisting of (i) complete separation, (ii) minor coelution, (iii) major coelution, and (iv) failed separation (Figure 7). Of note, when coelution did occur, it was generally with the two least retained compounds, estragole and ethyl cinnamate.

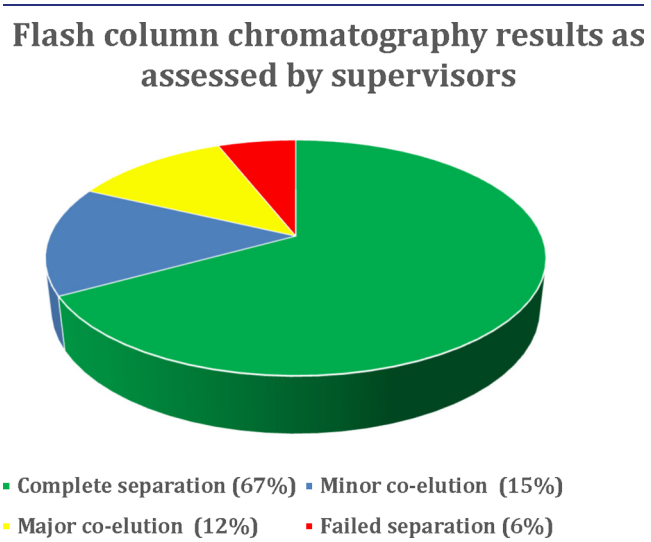

Figure 7. Visual representation of the obtained flash column chromatography results as assessed by supervisors.

For the theoretical assessment, the participants were asked to fill out a postlab question form (provided in the Supporting Information). A total of 95% of the participants were able explain the differences between analytical and preparative chromatography and tell which categories the column separation and TLC separation belong. Similar results were seen for the question regarding what constitutes the stationary phase and the mobile phase in the experiment. With regard to the elution order, 70% were able to rationalize the results to a sufficient degree on the basis of the functional groups present

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in the molecules and their interactions with the silica gel surface. When asked about the reason for changing the composition of the mobile phase, 66% of the students were able to answer this question satisfactorily.

CONCLUSIONS

The described experiment has been used to introduce students to preparative chromatography. The exercise has been well-received, and the results have been good, with the majority achieving separation of the three compounds present in the matrix. Practical solutions to typical challenges and problematic areas, such as pressurizing the column, avoiding issues with the column cracking or breaking, deciding whether the desired compounds have eluted, handling silica gel, etc., have been presented.

Embracing a vision that the protocol should be operationally simple and illustrate a tactile aspect of a basic analytical principle, the format nonetheless aimed at transcending the notion of a mere introductory technique. Thus, the skill set acquired at the end of the outlined exercise even forms a part of the rudiments required in an advanced laboratory environment. By directing the students' attention to the physiochemical descriptors that set the molecules apart from each other, the exercise also serves to demonstrate how the interrelation between functional groups forms the basis of chromatographic separation. Consequently, to come up with a qualified opinion about the sequence of elution, the students learn how the chromatographic practitioner must look to the underlying electronic signatures of the sample constituents to understand how different parameters affect the relative retention. Within the purpose of introducing students to chromatography as a part of the canon integral to practical laboratory work, the overreaching goal of the protocol has been of a holistic nature, pairing the manual aspect with the theory taught in organic chemistry.

In terms of practical skills learned, the students are introduced to a small-scale laboratory operation for purification. By extending the principle of partition to include the differential affinity of related components in a solute phase toward a solid phase, the experiment lets the students observe in real time the basis of chromatography in two relevant configurations, namely, column and thin-layer chromatography. As the exercise is divided into two interlocking tasks that involve the separation and identification of sample components, the students learn the distinction between preparative and analytical chromatography. Also, while the TLC is performed with one eluent system only, a series of eluent systems are applied during the column chromatography. Hence, the students become acquainted with isocratic and gradient elution as well as solvent strength. An accompanying didactic aspect of both operations is the attention to the starting bandwidth and to provide the students with an explanation of the underlying diffusion that causes smudging of spots. It is also opportune to acquaint the students with nondestructive and destructive detection methods, contrasting the visualization by UV and $KMnO_4$ staining used in the exercise. In this way, the students learn about physical and chemical methods of detection in an analytical setup.

In terms of application of theoretical skills, the students come to appreciate how the incremental variation of functional motifs changes the overall polarity of related structures. The basis of chromatographic separation is the ability of a given solute molecule to interact with a given stationary phase. In the described protocol, the students experimentally observe the significance of nonpermanent chemical bonds through (Lewis) acid/base interactions and how this can be exploited to distinguish between different structures.

The described course was developed over several years with the basis in our interactions with undergraduate students in need of practical and theoretical competence in preparative column chromatography. This process has involved approximately 35 students at three different universities, and the vast majority of these students were supervised in the ensuing time, allowing direct evaluation of the acquired skill set, retention of knowledge, and ability to independently apply the methods taught in other, nonrelated separations. The approach and methods used herein, starting with a TLC analysis and then preparing and eluting the column using gradient elution and a detection system, were to a large degree implemented by the students going forward.

Indeed, the technique with continuous real-time monitoring using chemically impregnated TLC plates has been widely adopted by those exposed to the method, and different tactics have been utilized in congruence with the obtained information, one example being the use of different fraction volumes depending on the status of the elution process. Other adaptations have also been seen, such as TLC plates impregnated with the boron-specific curcumin stain, which turns red when the mobile phase carries with it an organoboron compound (with slight, intermittent heating) as a result of the formation of a boron−curcumin complex.[11]

In conclusion, the experiment is affordable and straightforward to perform and constitutes a highly educational introduction to the important area of chromatography. Moreover, the outlined experiment complements the theoretical aspects covered in introductory organic chemistry courses, establishing a link between functional group characteristics and polarity through variation of structural motifs adorning a common scaffold. Thus, in the experimental setup, pointing out that the stationary phase constituted by silica gel is in fact a polyol helps students see that retention is a case of affinity caused by the extent of self-similarity, i.e., an example of the "like dissolves like" rule. This should provide the students with a premonition of the sequence of elution and concretely demonstrate the concept of (Lewis) acid/base interactions as a result of electron density.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.1c00917.

Procedure with detailed step-by-step instructions, illustrative photographs, NMR spectra, and postlab questions (PDF)

AUTHOR INFORMATION

Corresponding Authors

Marius Aursnes − *Department of Pharmacy, Section for Pharmaceutical Chemistry, University of Oslo, 0316 Oslo, Norway;* orcid.org/0000-0002-9960-0254; Email: marius.aursnes@farmasi.uio.no

Karoline Gangestad Primdahl − *Department of Pharmacy, Section for Pharmaceutical Chemistry, University of Oslo, 0316 Oslo, Norway;* orcid.org/0000-0001-7060-5900; Email: k.g.primdahl@farmasi.uio.no

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Authors

Frederik André Hansen − *Department of Pharmacy, Section for Pharmaceutical Chemistry, University of Oslo, 0316 Oslo, Norway;* orcid.org/0000-0002-1666-0447

Eirik Johansson Solum − *Faculty of Medicine and Health Sciences and Department of Chemistry, Faculty of Natural Sciences, Norwegian University of Science and Technology, 7491 Trondheim, Norway*

Jens Mortansson Jelstrup Nolsøe − *Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1432 Ås, Norway;* orcid.org/0000-0003-4862-5096

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jchemed.1c00917

Notes

The authors declare no competing financial interest.

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