JOURNAL OF CHEMICAL EDUCATION

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Laboratory Experiment

Biginelli Reaction and β-Secretase Inhibition: A Multicomponent Reaction as a Friendly Educational Approach to Bioactive Compounds

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Cite This: https://doi.org/10.1021/acs.jchemed.0c01298

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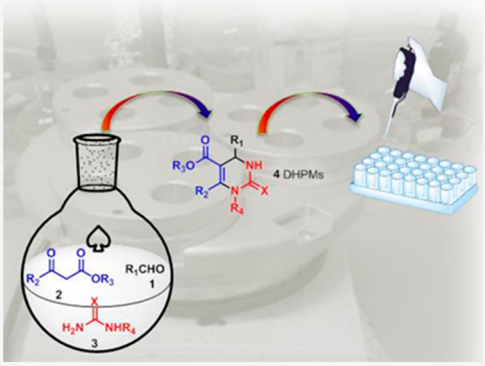
ABSTRACT: Multicomponent reactions (MCRs) represent very interesting tools to reach eco-friendly and sustainable transformations in organic chemistry. In particular, the Biginelli reaction furnishes a very easy approach to the synthesis of a library of biological active compounds in an academic course. Here we describe the realization of several experiments involving the synthesis of potential inhibitors of β-secretase by the Biginelli reaction. All of the obtained compounds were tested with a FRET fluorimetric assay. The experiments were proposed to students either at entry level or during advanced laboratory courses of organic and bioorganic chemistry. The learning objectives at the advanced level were to introduce the students to the practice of combinatorial synthesis and to the evaluation of biological activity of combinatorial libraries by enzyme inhibition assays. The meeting of the learning objectives was probed first by analyzing their daily performance in the laboratory and their increasing proactive attitude, and the contents of their final presentations. The resulting marks obtained by the students were compared with the average evaluation of their career. Second, the students were asked to evaluate the course and their own experience, and the outcome of their evaluation was compared with that of the teachers.



KEYWORDS: *High School/Introductory Chemistry, First-Year Undergraduate/General, Graduate Education/Research, Organic Chemistry, Hands-On Learning/Manipulatives, Combinatorial Chemistry, Enzymes*

INTRODUCTION

Multicomponent reactions (MCRs) are well-known processes that have been revisited and developed in the past 30 years; they often appear as very intriguing solutions for a chemistry that respects both the fundamental principles of green chemistry, such as the atom economy and/or the use of eco-friendly solvents, and the 17 goals that the United Nations (UN) has in 2015 identified as important for building a sustainable world.[1]

By definition,[2] MCRs simultaneously engage three or more components, resulting in products that incorporate (preferably all) atoms of the starting materials[3] in their framework. Hence, MCRs represent a great opportunity in combinatorial chemistry because they permit the synthesis of a library of new molecules potentially active as drugs. Furthermore, MCRs are also realizable by the use of microwave apparatus[4] and/or solid-phase synthesis[5] with an increase of chemical yields.

Among the MCRs, there is the Biginelli reaction and in this work we focused on it because, in our opinion, it offers the opportunity to propose interesting experiments, useful in chemical education for undergraduate students and for more advanced courses.

The described experiments were carried out for five years at different levels. The first one was offered as a beginner experience in a laboratory of organic synthesis to high school students, hosted by the University during open days and dissemination events. The same experience was also proposed to first degree undergraduate students during their first course of experimental organic chemistry. At the second level, the experience was upgraded to master's students, in a course of experimental bioorganic chemistry.

The principal goal of this work is stimulating others to explore and set up similar experiments in teaching lab, because we think that the students of any level can either appreciate a green approach to the synthesis or develop interest for the opportunity to easily prepare bioactive compounds.

Received: October 20, 2020
Revised: February 16, 2021

At the first level, other important goals were to try a basic literature research or compare a known proposed experiment with a new greener approach.

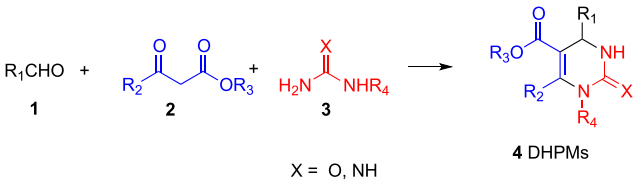
At the second level, the goals were as follows:

- Introducing the students to the synthesis of combinatorial libraries of small molecules, also by means of supported synthesis.
- Giving a first experience on *in vitro* testing of biological activity and screening a library of potentially bioactive compounds.
- Developing the complementary skills of the students, by improving their ability to communicate effectively their results.

Biginelli Reaction

The Biginelli reaction was first reported[6] at the end of the 19th century: it is an acid-catalyzed three-component, one-pot synthesis of 3,4-dihydropyrimidin-2-(1*H*)-ones (DHPMs), carried out using easily accessible starting materials, such as benzaldehyde, ethyl acetoacetate, and (thio)urea, in protic solvents (Scheme 1). Recently,[7] also guanidine has been used instead of urea, enriching the scope of available heterocycles.

Scheme 1. Biginelli Reaction



From the beginning of 1980, this reaction and some other MCRs were also considered as a sustainable method to prepare bioactive products, as reported in an interesting work[8] where the Biginelli reaction was used with undergraduate students to evidence a direct application of the 12 principles of green chemistry, by comparison of traditional and modern approaches.

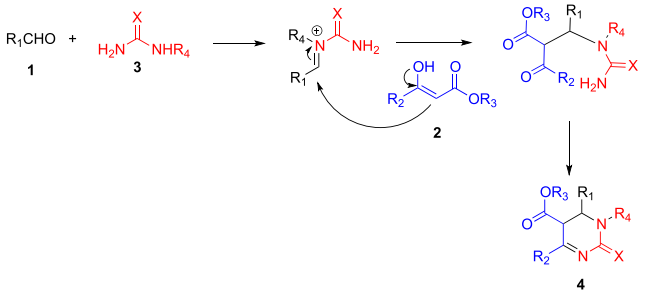
Furthermore, DHPMs are compounds with a broad spectrum of biological activities, such as antiviral, antibacterial, anti-inflammatory, and many others.[9] Moreover, when X is a NH group, these products (DHPMs) share several important features with guanidine inhibitors of β-secretase (BACE1), an aspartyl protease probably involved in the occurrence of Alzheimer's disease.[10] We have recently reported the synthesis and evaluation of a series of such Biginelli products as inhibitors of BACE1.[11] Our research experience in this field could be easily transferred into a didactic experience and allowed the students to design small libraries of target molecules which were potential inhibitors of the enzyme, which is commercially available from major suppliers.

Activity and inhibition tests are well-known and easy to carry out, as the enzyme is very stable if compared with similar proteases. For this reason, it was conveniently exploited to offer a first experience on *in vitro* testing of biological activity to students of bioorganic chemistry.

The Biginelli reaction also provides two other interesting aspects that could be investigated in chemical education: the first concerns the presence of a chiral carbon in the DHPMs (the reaction was recently[12] exploited also in asymmetric version) and the second the reaction mechanism. For both of these aspects a discussion could be stimulated among the students' groups (either for undergraduate or for second level students) by observation of the structure.

Regarding the mechanism, many proposals have been discussed in various experimental and theoretical reports,[13] even if, actually, the data seem to confirm the hypothesis first formulated by Kappe[14] and reported in Scheme 2.

Scheme 2. Mechanism of the Biginelli Reaction



As it can be seen, the reaction proceeds first by iminium ion formation, then by Mannich-type reaction of this ion with the enolic form of the β-keto ester, and finally by cyclization with elimination of a water molecule. Moreover, it is reasonable to expect an effect of substituent on the aldehyde and/or on β-keto ester structure that explains the final chemical yields (40–60%). Such a mechanism has been also verified in a recent work,[15] where a sequence of Cu(I)-catalyzed click-Biginelli reaction was reported.

Hence, with these ideas in mind and starting from the already tested experiment,[16] we organized the experiences for the two levels of students, as reported in the next paragraphs.

OUR EXPERIMENTS AND THEIR OUTCOMES

First Level: In Laboratory

First, we explained the importance of combinatorial synthesis in medicinal chemistry due to the simultaneous preparation of a large library of compounds. As an example, we showed the Merrifield synthesis on support and explained also the possibility to realize an analog procedure in solution.

Then, we explained the importance of eco-friendly research and of the green chemistry principles, particularly in organic synthesis. In this case we show, as reported in a previous work,[8] the Biginelli reaction as a green procedure to the preparation of new potential bioactive compounds.

Finally, a protocol for the realization of the Biginelli reaction was given to each student, stimulating to observe that for each of them a different aldehyde was used.

The aim was the realization of an easy reaction protocol, affording solid compounds in good yield and mild conditions. The reactions were realized simultaneously by all the students using a small scale parallel synthesis apparatus showed in Figure 1A.

In a vial, each student introduced urea (1.25 mmol), ethyl acetoacetate (1.90 mmol), ethanol (0.5 mL), and the aldehyde (equimolar with urea): at this point, before heating to about 60 °C, one drop of 12 M HCl was added.

All of the vials were stirred for about 90 min, until the formation of a precipitate was observed (Figure 1B); then the solid was separated by filtration on a Buchner filter (Figure 1C) affording the desired compound.

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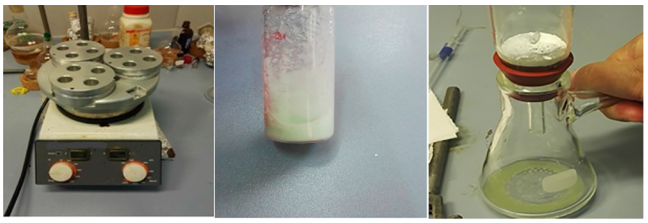


A B C

Figure 1. (A) multiwell heating and stirring plates exploited for the parallel synthesis experiments. (B) Solid product of the Biginelli reaction. (C) Filtration apparatus.

After isolating, the reaction products were characterized by ^{1}H NMR and mass spectra. Then each student had to calculate the chemical yield and measure the melting point; finally, she/he had to verify the structure of the compound obtained by comparison with literature data.

On the basis of the typology of students (graduate or undergraduate), we stimulated a discussion on the observation that the chemical yields were between 40 and 60%, depending on the reactivity of the aldehyde.

At the end of the laboratory activity, we explained that the DHPMs showed various biological activities and prompted the students to search in literature (by using Google Scholar) about their use in medicinal chemistry.

Second Level

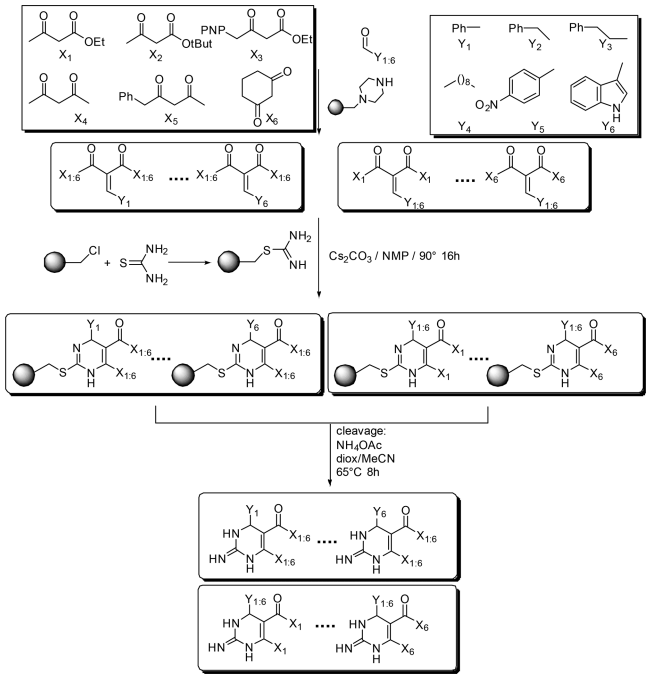
At the second level, groups of students were involved in a combinatorial experiment, to obtain two orthogonal libraries of compounds that were tested in pool for their ability as inhibitors of BACE1. The experience was offered to students in their first year of a MS course in chemistry, within an organic and bioorganic curriculum at the University of Trieste. As B.S. graduates, they had already gained skills in organic chemistry and on the general techniques of organic chemistry laboratory practice.

Their previous experience was in conventional organic synthesis at 100 mg scale, and structural characterization by IR, NMR, and MS. Before the bioorganic chemistry laboratory course, they attended a 50 h course on bioorganic chemistry, focused on catalytic effects in enzyme reactions, enzyme inhibition, and enzyme mimicking. The aims of this laboratory practice were therefore on one side to introduce the students to a lower, milligram scale synthetic activity in a combinatorial fashion and to allow them to experience also an example of evaluation of enzyme inhibition, increasing, by this way, their interest and skills in bioorganic research. The experience was offered to five cohorts of students, along five years, and involved 63 students as a whole.

In this experience, we focused on Biginelli products containing a guanidinium group (Scheme 1, X = N). The reaction was carried out in a different way with respect to the first-level one: this option has been also proposed by Kappe.[17] Rather than a multicomponent reaction, it is a two-step synthesis, where a Knoevenagel reaction is carried out first between the active methylene 1,3-dicarbonyl compound and the aldehyde (Scheme 3).

In the second step, the resulting derivative reacts with Wang resin supported thiourea to furnish the heterocyclic system. According to different cleavage conditions, oxo or imino derivatives can be obtained. This option is also interesting for educational purposes, as it offers the opportunity to experience

Scheme 3. Synthesis of Two Orthogonal Libraries of Biginelli Compounds



resin-supported solid-phase synthesis and can be exploited also to outline a combinatorial experiment.

The starting materials were as follows:

- a set of six 1,3-dicarbonyl compounds X1−X6 that could be representative, with their side chains, of several common isosteres of amino acids often involved in the design and synthesis of proteases inhibitors.
- a set of six aldehydes Y1−Y6 also containing some typical bioisostere group.

At the beginning of the synthesis, the students had to synthesize the catalyst needed for the first step, namely, resin-bound piperazine.[18]

The students were then divided into 6 teams, and a set of 12 sublibraries (two per each team) of products were prepared. The libraries were designed to obtain two (X and Y) orthogonal libraries. The X sublibraries (X1···X6) are resolved in X: each contains six products, deriving from one of the dicarbonyl compounds X1−X6, and an equimolar mixture of all the aldehydes Y1−Y6. Conversely, the Y sublibraries (Y1···Y6) are resolved in Y and each contains also six products, deriving from one aldehyde Y1−Y6 and an equimolar mixture of all of the dicarbonyl compounds X1−X6. In the first step, the Knoevenagel products were obtained in solution. The 12 reactions were carried out simultaneously on a Carousel apparatus for parallel synthesis. The subsequent step was the synthesis of the dihydropyrimidines libraries from the Knoevenagel ones. This protocol is a solid-phase synthesis, involving first the loading of thiourea to a Wang resin. This material was then used for the synthesis of the heterocyclic compounds, and each team had the products bound to the resin by reaction of the immobilized thiourea with their sublibrary of Knoevenagel compounds. The second-level experience was offered within a very compact course, where the students meet for 12 days in the afternoon from 2 to 6 pm. This allowed them also to setup and start several overnight reactions, stopped by the teacher the day after in the morning

https://doi.org/10.1021/acs.jchemed.0c01298
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and then submitted to workup by the students in the afternoon. An example timetable of the activities is reported in the Supporting Information (SI) Table S2, while full experimental details are also reported in the SI.

An interesting point in the synthesis of Kappe is the versatility allowed to remove the products from the solid phase. If the cleavage is performed with ammonium acetate, a guanidinum-like moiety is obtained in the final products. This way was always followed by the students in all of the years, and the final products were therefore two orthogonal libraries made of six sublibraries, each containing six compounds. The sublibraries were then characterized only by ESI-MS, and the molecular ions of the expected products were identified by the students. In this experiment it is not possible to obtain NMR spectra, as the products are obtained only as mixtures and on a very small scale. By the way, the aim of the experience is also to show an example of a protocol perhaps more similar to those followed in nonacademic laboratories, where NMR is often not available, and analyses are carried out mostly by MS. LC-MS would be preferable to a simple MS analysis of the mixtures, but this option was not possible with the available resources. The students were already aware of the outcomes of similar reactions from their previous courses and conscious of the limitations of this approach in comparison with a full characterization of the products. This point was always underlined in the discussions with the students.

The composition of the sublibraries and their mass spectra are reported in the Supporting Information (Scheme S1 and Figure S1). The outcomes were discussed in the classroom, to give details on the interpretation of mass spectra, which show the molecular ions of most of the expected compounds as the MH^+ and/or the MNa^+ ions. Other ions are clearly present in the spectra, belonging to unreacted materials and unknown compounds, and their presence leads to interesting discussions.

At the end, the experience was completed with an *in vitro* assay on β-secretase. This last experience is very instructive as the students can face all of the aspects involved in an enzyme inhibition experiment. The inhibition test involves the measure of enzyme kinetics by a fluorimetric technique,[19] and was carried out in a fluorimetric 96-well plate reader. Alternatively, the enzyme assay can be performed also in a spectrofluorimeter equipped with a 5 mm optical path square cell. In this case, each library is evaluated separately, and in order to shorten the measurement times, the % inhibition measured at a single concentration (typically 50 μM) can be simply measured.

The raw kinetic data were elaborated by the students, and the initial rates of the enzyme reactions were obtained, together with % inhibitions data (Figure 2).

The final discussion involves the students in the evaluation of the results, attempting to cross-evaluate the inhibition data from the two libraries in order to find out the most active compounds and try to rationalize the outcome. In principle, a cross-evaluation matrix could lead to the deconvolution of the libraries and identification of the active compounds. Figure 3 describes this concept.

In some cases, significant activities were found, although no IC_{50} was measured but just the % inhibition was measured. Nevertheless, the discussions were very useful to the students to enter in the world of experimental bioorganic chemistry. During the classroom discussion, the clear limitations of this analysis were underlined, and the evidence that the outcome should be confirmed by repeating the synthesis of the top scored compounds alone, fully characterizing their structures

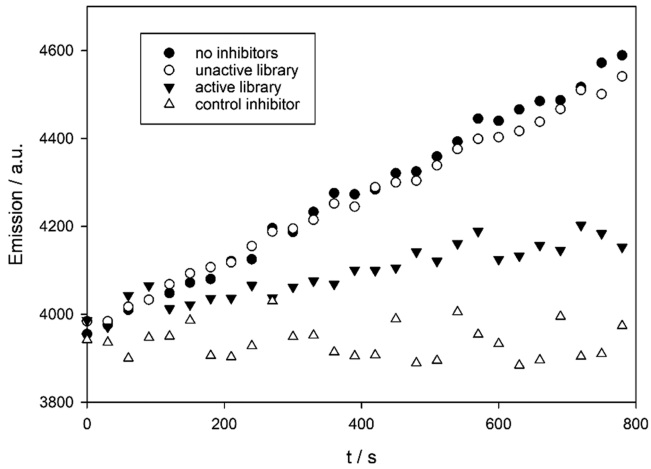

Figure 2. Example of an enzyme inhibition kinetic run obtained with a fluorogenic BACE1 substrate. In this run the libraries were tested at 10 μM theoretical concentration.

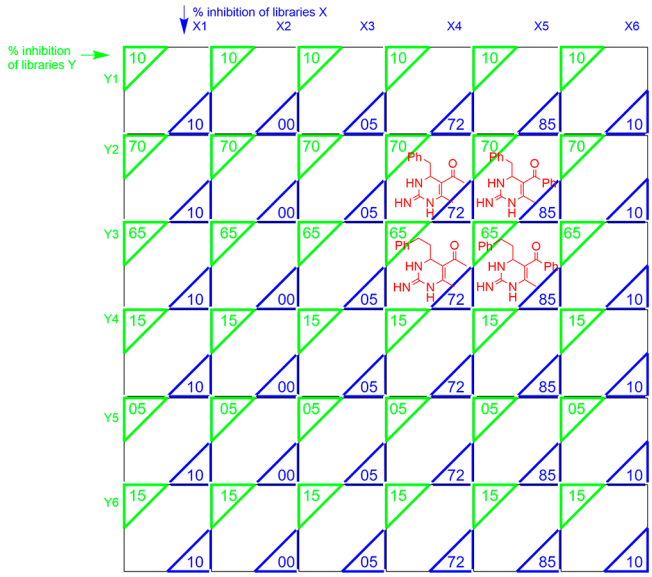

Figure 3. Deconvolution matrix. The % inhibition of the enzyme reaction measured with the Y sublibraries are reported in green, while those obtained with the X sublibraries are reported in blue. A cluster of four top-scorer compound may be identified as the unique compound contained at the intersections X4 ∩ Y2, X4 ∩ Y3, X5 ∩ Y2, and X5 ∩ Y3. Their structures are reported in red.

and activity, was clear to the students. Along the different years, several instructing mishaps occurred to some teams, as losses of resin-bound materials with consequent decreased yields and forgotten reagents in the mixtures. This led to evident false results in the activity measures and allowed highlighting of the potential confusion in the screening of libraries, whose results may reflect not only the activities of the compounds but also unequal yields along the syntheses.

HAZARDS

Benzaldehyde and related reagents (such as 4-methoxybenzaldehyde, 4-nitrobenzaldehyde, 2-thiophenecarboxaldehyde, phenylacetaldehyde, and hydrocinnamaldehyde) causes serious eyes, respiratory, and skin irritation. Undecanal causes skin irritation. Methanol, acetonitrile, tetrahydrofuran, and dioxane are flammable liquids (and vapors) and are irritants for eyes,

https://doi.org/10.1021/acs.jchemed.0c01298
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Table 1. Teachers' Evaluation of the Students (Mark Reported as % of the Maximum Mark)

	Cohort 1	Cohort 2	Cohort 3	Cohort 4	Cohort 5
Number of students	12	13	13	12	13
Average mark %	91.54	97.02	97.06	94.02	93.95

Table 2. Evaluations of the Students on the Contents of the Course (%; 100 = Full Satisfaction)

Question	Cohort 1	Cohort 2	Cohort 3	Cohort 4	Cohort 5	Average
Was my prior knowledge suitable for the contents of this course?	75	96	96	84	84	88
Is the workload of the course adequate?	83	99	94	85	85	91
Do I think this activity had a useful impact on my training?	92	98	100	92	92	95
Was I overall interested in this activity?	82	92	99	85	85	93
Am I overall satisfied with this course?	80	94	92	86	86	94

respiratory tract, and skin. Furthermore, all of the last cited and dichloromethane, too, are suspected of causing cancer. Acetylacetone and 1,3-cycloexanedione are harmful if swallowed and toxic in contact with skin and eyes. Cesium carbonate causes serious eye damage and causes damage to organs (kidney, adrenal gland) in prolonged or repeated exposure if swallowed; it is also suspected of damaging fertility. 1-Methyl-2-pirrolidinone causes skin, eyes, and respiratory irritation; it may harm the unborn child. All of the other reagents and/or solvents are not dangerous.

The hazards of the products are unknown, and all should be handled as hazardous in case of inhalation, skin contact, eye contact, and ingestion. Eye protection and gloves must be used throughout the entire experiment.

EVALUATION

The goals described in the Introduction were achieved in both level activities; furthermore, two other objectives were obtained:

1 To allow one to spread recent important research results for the treatment of the Alzheimer's disease and generally about medicinal chemistry.
2 To synthesize a large library of "small molecules" active against BACE1.

We think that the experience is useful for all types of students, from undergraduate to graduate ones, as well as high school students. In particular, we could verify that in these years the high school students have shown much interest in this type of laboratory activity and about 80% of them discussed successively the activity in their final exam. Moreover, we can think that this positive answer has contributed to the increase of enrollment in the course of studies in chemistry.

In particular, at the second-level experience, its impact on the educational journey of the students was estimated by both our evaluation of their proficiency and their own evaluation of the impact of the course. Our evaluation was carried out during and after the experience. During the experience we have evaluated their interest, attention, ability to discuss with colleagues and teachers about the ongoing experiments, and improvement of practical skills. After the experience, the students had to prepare a public talk to colleagues and teachers, followed by a final discussion. We have decided to ask for this presentation rather than the usual laboratory report, as the students were already experienced in many laboratory courses. Moreover, they already received specific training on literature searching in a dedicated course, and they were enabled to use SciFinder, Scopus, and WoS. The final mark was the average of both the ongoing and final evaluations. The 63 students who had the exam passed with an average mark of 28.41/30 in the Italian mark system, corresponding to 94.71%, with a standard deviation of 7.37%. This outcome was very satisfactory and above the average evaluation of the MS course. The average evaluation for each cohort is reported in Table 1.

The students had the opportunity of giving their own evaluation of the course within the didactic performance evaluation system of the University of Trieste. In this system, they give their mark on 12 questions, on organization, quality of available instruments, ability of the teachers, and contents of the course. The students must report their satisfaction in a blind way before receiving their marks from the teachers, and their evaluation is not disclosed to the teachers before the end of all of the exam sessions of the year. Their average marks on the last point questions are reported in Table 2.

The evaluations from the students were clearly gratifying, and quite above their average evaluations, thus showing a clear impact of the experience on their education around green and sustainable chemistry in synthesis. We note some correlation between our evaluation of the student performance and their evaluation of the course. The first cohort was the less satisfied and received the lower, although very good, average mark from us. We can acknowledge that this may be due to a non-optimal organization of the first version of the course and to the contents unexpected by the students. Cohorts 2 and 3 received the highest marks and expressed the highest satisfaction, while then both evaluations become stabilized in cohorts 4 and 5.

CONCLUSION

In summary we think that the described experience could be a useful example of how modern chemistry and advanced research could be converted into an appealing education lab. We will stimulate the scientist to try similar experiences with either undergraduate or high school students with the aim to introduce the young people to the research world.

Furthermore, by increasing the difficulty level, the same laboratory activity could be extended to graduate students. During the laboratory activity all of the students show curiosity and interest to understand the correct sequence of manipulations.

We think that to see in the students' eyes the light of the desire to know and deepen understanding is the greatest satisfaction of the teacher.

https://doi.org/10.1021/acs.jchemed.0c01298
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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.0c01298.

Instructions for students and instructors, the experimental procedures, and other experimental details (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the University of Basilicata and the University of Trieste for financial support and all of the students who participated in this activity during the past years.

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