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A biocatalytic approach for the synthesis of Lilybelle® and other fragrances starting from citrus industry by-products

TESI MAGISTRALE IN CHEMICAL ENGINEERING

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This work investigates the synthesis of some odorous compounds, specifically Lilybelle® and Calmusal® starting from citrus industry by-products (Limonene and Citral) using different biocatalytic steps as an alternative for traditional, environmentally impactful ways. Specifically, the study explores the possibility of using enzymes like ADHs and OYEs to perform some of the steps needed to complete the synthesis path. When it is not feasible to perform a specific transformation using enzymes, the most environmentally friendly available solution is chosen. This brings to a great focus on the implementation of selected reaction steps in continuous flow mode, through a comprehensive study, performed with the use of advanced equipment and modern techniques of investigation. Overall, the results are promising. The work demonstrates how the proposed approach is not only feasible but can incorporate many advantages under the environmental, but also toxicological, economical and safety point of view.

In questo lavoro è stata studiata la possibilità di produrre fragranze, in particolare Lilybelle® e Calmusal® partendo da scarti dell'industria degli agrumi (limonene e citrale) e utilizzando vie di sintesi con minore impatto ambientale in alternativa ai metodi tradizionali oggi utilizzati. In particolare, è stata studiata la possibilità di utilizzare enzimi come le ADHs e le OYEs per compiere molti dei passaggi di sintesi richiesti. Quando un determinato passaggio sintetico non era perseguibile per via enzimatica l'opzione a minor impatto ambientale è stata scelta. Questo ha portato ad un ampio studio di differenti reazioni in flusso, utilizzando strumentazione e tecniche di investigazione avanzate. In generale i risultati riportati sono promettenti ed il lavoro dimostra come l'approccio proposto non sia solo possibile ma può portare a importanti vantaggi sotto il profilo ambientale, tossicologico, economico e della salute.

Key-words: biocatalysis, enzymes, flow reaction, Lilybelle®, OYE, limonene

1. Introduction

1.1 Lily-of-the-valley fragrance

Lily-of-the-valley fragrance has been prized for centuries and is widely used in perfumery today. The aroma of lily-of-the-valley is composed of several volatile organic compounds (VOCs), including linalool, geraniol, and alpha-terpineol.[1] These compounds work together to create the characteristic fresh, green, and slightly sweet scent of the flowers. The fragrance has been used as a key ingredient in many famous perfumes over the years, including Diorissimo, Muguet des Bois, and Lily of the Valley[2].



Figure 1 – Representation of lily of the valley flower

Its popularity can be traced back to the Renaissance when it was used to mask unpleasant odors and as a symbol of purity and humility. In the Victorian era, lily-of-the-valley became associated with romance and was often included in bridal bouquets. Despite its popularity, lily-of-the-valley is a challenging fragrance to work with in perfumery. The flowers are delicate and difficult to extract fragrance compounds from, and the resulting fragrance is expensive to produce. In addition, the use of lily-of-the-valley has been restricted due to concerns about the impact of over-harvesting on the environment. To address these challenges, perfumers have turned to synthetic fragrance compounds to replicate the scent of lily-of-the-valley. Since the beginning of the 20th century, those compounds like Cyclamen aldehyde®, Bourgeonal®, and Lyral® have been developed making the scent highly available to the big public [2]. Over the last decade, severe concerns were raised over the safety of the lily-of-the-valley odorants, many of them were found to be toxic for human use and their use has been restricted by many jurisdictions to a minimum. For this reason, fragrance houses spend considerable resources on developing new molecules. This led to the production of a new class of fragrances like Nymphaeal®, Super Muguet®, and Lilflore®. Nowadays the challenges of the industry are the creation of fragrances that are not just safer for human use but that are also produced using

sustainable primary sources and synthetic routes that stay true to the principle of the green chemistry. This includes the use of a catalyst at each stage, the recycling of solvents, and the reduction of waste. With this philosophy fragrances like Lilybelle[®], and Biomuguet[®] were made available. [2]

1.2 Green chemistry

Green chemistry, also known as sustainable chemistry, is a scientific approach that aims to design chemical products and processes that are safer, more efficient, and more sustainable. The concept of green chemistry was first introduced in the early 1990s by Paul Anastas and John Warner[3]. Since then, it has gained momentum and become a global movement in the chemical industry. Traditional chemical processes often involve the use of hazardous substances, the generation of toxic waste, and the consumption of large amounts of energy and resources. These practices not only harm the environment but also pose risks to human health. Green chemistry, on the other hand, focuses on minimizing the use of hazardous substances, reducing waste, and conserving energy, and resources.

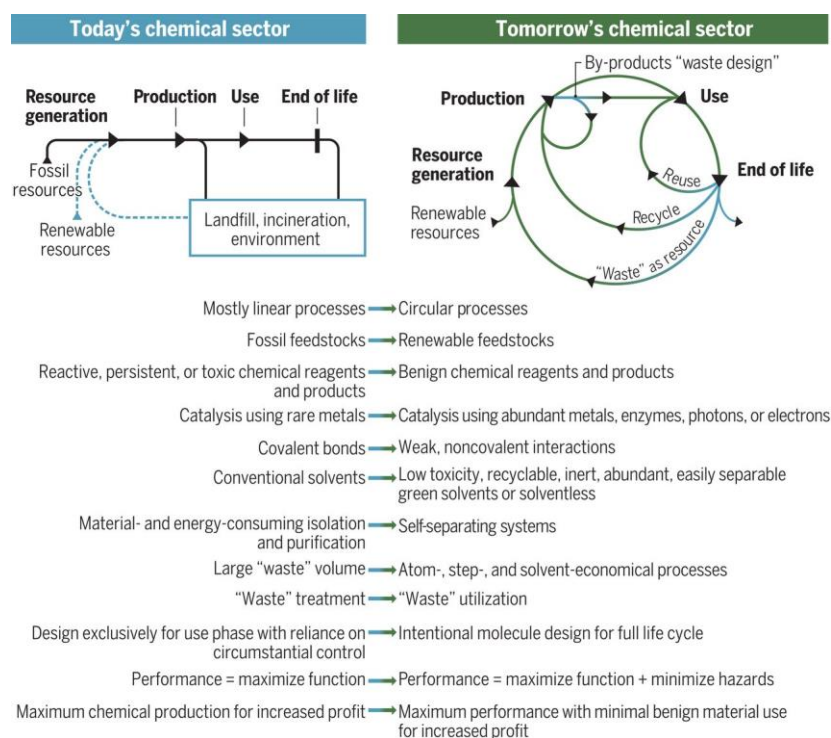


Figure 2 – Green chemistry, a comparison scheme

It is based on 12 principles [3] that provide a framework for designing chemical products and processes that are safe, efficient, and sustainable. These principles are prevention of waste formation, application of atom economy, use of less hazardous chemicals, design of safer chemicals, use of safer solvents and auxiliaries, design of energy-efficient processes, use of renewable feedstocks, reduced use of derivatives, extensive use of catalytic reagents, design solvent for degradation, implementation of real-time analysis for pollution prevention, and use of inherently safer chemistry for accident prevention[4].

1.3 Biocatalysis

Biocatalysis is the use of enzymes to catalyze chemical reactions. Enzymes are naturally occurring proteins that can accelerate chemical reactions by lowering the activation energy required for it to occur. They are highly specific in their action and can perform reactions under mild conditions such as low temperatures and low pressures.[5] Biocatalysis is used in a wide range of applications, including the production of biofuels, pharmaceuticals, food additives, and fine chemicals. It is a more sustainable and environmentally friendly alternative to traditional chemical catalysis because enzymes are typically derived from renewable resources and produce fewer toxic waste products.[5] The advantages of biocatalysis over traditional chemical catalysis include higher selectivity, milder reaction conditions, and lower energy consumption. Biocatalysis also allows for the synthesis of complex molecules that would be difficult or impossible to produce through traditional chemical methods. [6] Advances in genetic engineering and protein engineering have allowed scientists to modify and optimize enzymes for specific industrial applications, such as increasing stability, specificity, and catalytic efficiency.

1.3.1 History

The history of biocatalysis can be traced back to ancient times when humans used microorganisms to produce food and beverages such as cheese and wine. However, the modern history of biocatalysis began in the 19th century when scientists started to study enzymes and their catalytic properties. One of the earliest examples of biocatalysis in modern times was the discovery of diastase, an enzyme that catalyzes the breakdown of starch into sugar. This discovery was made by French chemist Anselme Payen in 1833[7]. Diastase was the first enzyme to be isolated and purified, and it paved the way for further research into the use of enzymes in chemical reactions. In the early 20th century, researchers began to explore the use of enzymes in industrial processes. One of the first commercial applications of biocatalysis was the use of enzymes to produce glucose from corn starch. This process was developed by the US firm Corn Products Refining Company in the 1920s.[8] In the following decades, the use of biocatalysis continued to grow. Enzymes were used to produce a wide range of products, including food additives, detergents, and pharmaceuticals. However, the use of enzymes in industrial processes was limited by the low stability and specificity of many enzymes. In the 1980s, advances in genetic engineering and protein engineering revolutionized the field of biocatalysis. Researchers were able to modify enzymes to improve their stability, specificity, and catalytic efficiency. This led to the development of new enzymes and the optimization of existing ones, making biocatalysis a more attractive option for industrial processes. Today, biocatalysis is used in a wide range of applications, from the production of biofuels to the synthesis of fine chemicals and pharmaceuticals.[9]

1.3.2 Old Yellow Enzyme

In the vast world of biocatalytic enzymes one class has been identified as of high interest in the synthesis of fine organic compounds, the so-called Old Yellow Enzymes (OYE) [10]. They are a family of oxidoreductase enzymes that are known for their characteristic yellow color and their ability to catalyze a wide range of redox reactions. These enzymes have been extensively studied due to their potential for use in biocatalytic applications. One of the most interesting aspects of OYEs is their stereospecificity, which makes them ideal for bioreduction reactions. The regioselectivity of OYEs makes them particularly useful for the reduction of ketones to

produce alcohols.[11] OYEs can be engineered to preferentially produce one enantiomer over the other, providing a valuable tool for the synthesis of chiral compounds that are widely used in the pharmaceutical, agrochemical, and fine chemical industries. The OYE-mediated bioreductions are well-understood, and their general mechanism has been extensively studied. The mechanism involves a two-step reaction sequence, which is illustrated in Figure 3. An activating group, typically one of the electron-withdrawing groups (EWGs), forms a tight H-bond interaction with two donor residues (His191 and Asn194 in OYE1). This interaction positions the substrate and makes it more electronically reactive to the 1,4-hydride addition. The addition of hydride occurs at the β -position from the FMNH₂ prosthetic group, followed by proton transfer to the α -position from an acidic residue nearby (Tyr196 in OYE1). This leads to the formal hydrogenation of the C=C bond with anti-stereospecificity. According to a ping-pong bi-bi mechanism, after the product's release, a molecule of nicotinamide cofactor NAD(P)H binds to reduce FMN back to FMNH₂, thus restoring the catalytically active form and initiating a new reduction cycle. Therefore, to recycle NAD(P)⁺, biocatalytic applications of ERs require a suitable cofactor regeneration system coupled with an inexpensive reducing agent, such as the combination of glucose and a glucose dehydrogenase or a formate dehydrogenase. [12]

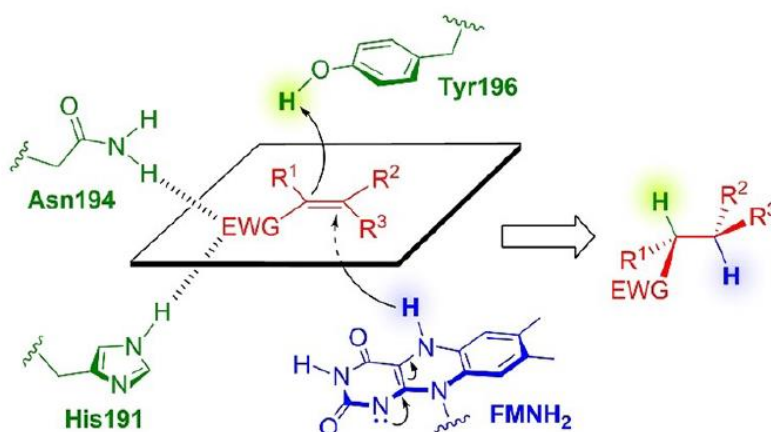


Figure 3 – OYEs working mechanism

1.3.3 Biocatalysis and green chemistry

Green Chemistry is all about finding more sustainable and environmentally friendly ways to produce chemicals. Biocatalysis is a promising approach that aligns well with these principles, as it offers a way to develop more eco-friendly chemical pathways.[13] In general biocatalysis can:

- Reduce waste production using subproducts as feedstock.
- Incorporate more atoms into the final product.
- Work under mild and non-toxic conditions, making it a safer and less hazardous synthesis option and allowing us to use more environmentally friendly solvents,
- Decrease energy consumption and decreasing our dependence on non-renewable resources,

- Eliminate the need for protection/deprotection steps, which is important for reducing derivatization.

In addition, biocatalysts are highly efficient and selective catalysts. Moreover, they are biodegradable and can contribute to designing products that are easier to degrade.

1.4 Flow Chemistry

Continuous flow chemistry is an emerging field that has attracted considerable attention in recent years due to its numerous advantages over batch chemistry, such as improved reaction selectivity, reduced waste generation, and increased safety.[14] Continuous flow organic chemistry has been successfully applied in various areas of organic synthesis, including the development of novel chemical processes, the synthesis of complex molecules, and the production of fine chemicals and pharmaceuticals. It has also been used in the development of green chemistry processes, such as the use of renewable resources and the reduction of waste generation. Continuous flow organic chemistry has been shown to improve reaction yield, reduce reaction time, and enable the use of hazardous reagents and conditions.[15] To standardize flow chemistry protocols, it is essential to establish a range of input parameters that have been shown to work well for a given reaction. This can be done through systematic studies, in which the effects of varying individual input parameters are evaluated. The optimal input parameters can then be established through a combination of experimental data and computational modeling. Once the optimal input parameters have been identified, they can be used as a starting point for conducting future reactions under similar conditions. This approach can help to reduce variability between experiments and increase the reproducibility of results. It is also important to consider the scalability of input parameters in flow chemistry. Input parameters that work well at a small scale may not be applicable when scaling up to industrial processes. Therefore, it is crucial to consider this aspect in the development phase. [15]



Figure 4 - 10 mL standard coiled tubular reactor

1.4.1 Design of Experiment - DoE

Design of Experiments (DOE) is a statistical approach to experimentation that allows researchers to systematically identify and evaluate the effects of various factors on a response variable[16]. The goal of DOE is to optimize the reaction response by identifying the most important factors and determining their optimal level. There are several steps involved in designing a successful DOE. The first step is to identify the response variable, which is the outcome of interest, for example, the yield of a chemical reaction. The next step is to identify the factors that may influence the response variable. These can be independent or manipulated, physical, such as temperature or pressure, or process ones, such as flow rate. Once the response variable has been identified, the next step is to determine the levels at which the factors will be set during the experiment. This is an important step since the results of the experiment will depend on this. The levels should be chosen to represent the range of values that are expected in the real-world application of the experiment. The fourth step is to choose an appropriate experimental design. There are several different types of experimental designs, including full factorial designs, fractional designs, and response surface designs. The choice of design will depend on the number of factors being studied, the number of levels for each factor, and the resources available for the experiment. Once the experimental design has been chosen, the next step is to conduct the experiment and collect the data. It is important to ensure that the experiment is conducted in a controlled environment to minimize the effects of extraneous variables. Once the data has been collected, the next step is to analyze them using statistical methods. The results of the analysis will provide insights of the effects of each factor on the response variable and help to identify the optimal levels for each factor. Finally, the results of the analysis can be used to develop a predictive model that can be used to optimize the response variable in real-world applications. The model can be used to determine the optimal levels for each factor and predict the expected response variable under different conditions.[16]

1.5 Lilybelle®

Lilybelle, *i.e.* 3-[(4R)-4-(Propan-2-yl)cyclohex-1-en-1-yl]propanal (**1**), is one of the most appreciated Lily of the Valley fragrances [17]. It is described as “Floral, Lily of the Valley, Aldehydic, Green, Cyclamen, airy transparent accents. It provides a green aldehydic element to powdery notes. It is refreshing and it provides lift in general.”[18]. The most important specificity of Lilybelle® (**1**) is in the starting material used to its preparation: (*R*)-(+)-Limonene (**2**). Limonene is a colorless liquid hydrocarbon that is classified as a cyclic monoterpene. It is found naturally in the peels of citrus fruits such as oranges, lemons, limes, and grapefruits, and is a major component of their essential oils. Limonene is extracted by vapor distillation or solvent extraction from the waste of the fruit juice industry. It is used in a wide range of applications, including as a solvent for cleaning products and degreasers, a flavoring agent in food and beverages, and as a fragrance ingredient in cosmetics and perfumes. It is also used in the production of resins, adhesives, and plastics[19].

1.5.1 Symrise Synthesis

Lilybelle® was patented by Symrise in 2011 [17]. In the first stage of the synthesis proposed, the isopropenyl moiety of limonene was hydrogenated selectively with Raney nickel. After epoxidation, the resulting oxirane was rearranged to an allyl alcohol by refluxing in xylene in

the presence of aluminum triisopropoxide. Acetalization with butyl vinyl ether and heating to 200°C in a high boiling solvent such as Malotherm (dibenzyl toluene) finally provided Lilybelle® (**1**) via a Claisen rearrangement. Symrise claimed a responsible and sustainable source, (*R*)-limonene (**2**), as a starting material accessible from orange peels. The synthetic route stays true to the principles of green chemistry, which includes the use of catalysts at almost every stage of the synthesis, the recycling of solvents, and restrictions of waste production in its manufacturing process[2], [17]. Lilybelle® was developed with the aim of producing a Lily-of-the-valley odorant with a reduced environmental impact using the principles of green chemistry and starting from sustainable resources. However, its synthesis process still relies on high energy consumption, the use of dangerous reagents, and the generation of hazardous waste. Therefore, finding a more sustainable and eco-friendly synthesis method for Lilybelle® is considered of highly interest.

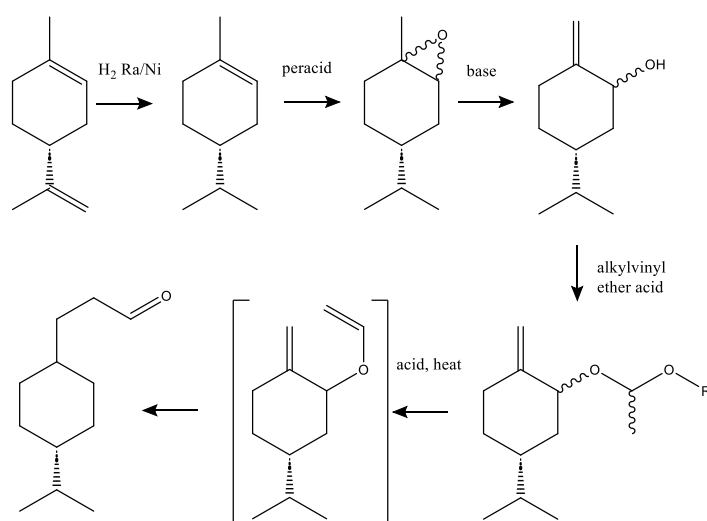


Figure 5 – Symrise patented synthesis of Lilybelle®

2. Aim of the work

In this work, biocatalysis was employed, whenever possible, to increase the sustainability of the synthetic approach to Lilybelle® with less energy input, fewer toxic chemicals, and less hazardous waste. A key factor that can help in the synthesis of Lilybelle® (**1**) is OYE (Old Yellow Enzyme). OYE is a versatile enzyme that can catalyze the reduction of carbon carbon double bond, suitably activated by an electron withdrawing group. This enzymatic capability has been shown to be effective in optimizing the synthesis of complex molecules similar to Lilybelle® (**1**) [20].

The synthetic process that was investigated in this work is shown in Figure 6. Firstly, (*R*)-(+)-Limonene (**2**) was oxidized to (*R*)-4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)methanol (**3**) also known as perillyl alcohol, thanks to the use of the cytochrome P450 CYP153A6 from *Mycobacterium* sp. strain HXN-1500. Then, an alcohol dehydrogenase (ADH) was used to oxidize alcohol **3** to (*R*)-4-(prop-1-en-2-yl)cyclohex-1-ene-1-carbaldehyde (**4**) or perilla

aldehyde. Next, two carbon atoms were added to perilla aldehyde **4** thanks to a methodology which is well established in organic synthesis: the olefination of Horner-Wadsworth-Emmons was employed to obtain methyl (*R,E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)acrylate (**5**) which was then reduced to (*R,E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)prop-2-en-1-ol (**6**) using diisobutylaluminium hydride (DIBAL). The resulting alcohol **6** was oxidized to (*R,E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)acrylaldehyde (**7**), by means of the reaction with MnO₂. After that, OYE3 was employed to hydrogenate the carbon carbon double bond conjugated to the aldehydic moiety to obtain (*R*)-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)propanal (**8**). Finally, the isopropenyl double bond was hydrogenated using a Pt/C as a catalyst in a continuous reactor to obtain Lilybelle® (**1**).

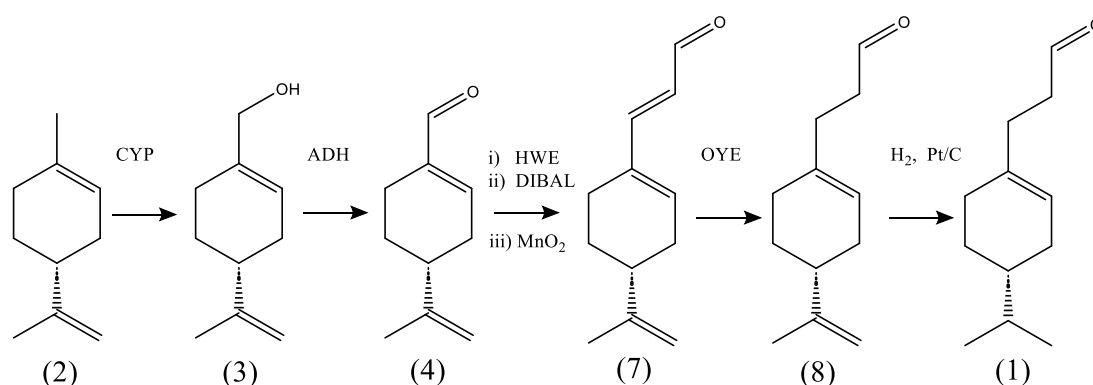


Figure 6 – Overall synthesis scheme of Lilybelle®

To further explore the potential application of OYEs in the reduction of unsaturated aldehydes, we also evaluated the use of citral, a 40:60 mixture of (*Z*)-3,7-dimethylocta-2,6-dienal (**9a**) and (*E*)-3,7-dimethylocta-2,6-dienal (**9b**) to produce Calmusal® (*E*,-6,10-dimethylundeca-5,9-dienal (**10a**) and (*Z*)-6,10-dimethylundeca-5,9-dienal (**10b**), which is another type of odorous compound, patented by Givaudan [21]. The proposed process involves adding two carbon atoms to citral with the Horner-Wadsworth-Emmons olefination, followed by reduction to alcohol and MnO₂ oxidation, to produce dehydrocalmusal, a mixture of (*2E,5E*,-6,10-dimethylundeca-2,5,9-trienal (**11a**) and (*2E,5Z*)-6,10-dimethylundeca-2,5,9-trienal (**11b**) (some traces of (*2Z,5E*,-6,10-dimethylundeca-2,5,9-trienal were detected), followed by an enzymatic step to convert **11** into Calmusal (**10**).

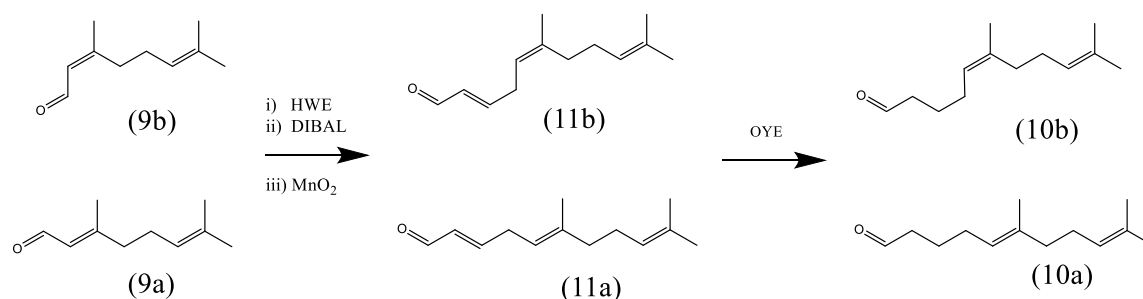


Figure 7 – Overall synthesis scheme of Calmusal®

3. Results and discussion

3.1 Oxidation of Limonene

The use of limonene as a starting material to produce different molecules is a well-established practice. However, the synthetic method for producing (+)-perillyl alcohol from (+)-limonene involves several steps, including hydroboration, oxidation, and dehydration. In the process (+)-Limonene is submitted to epoxidation with meta-chloroperbenzoic acid, followed by treatment with LDA to isomerize the epoxide to the corresponding allylic alcohol. The latter is then converted into the acetate derivative, that underwent Claisen rearrangement in the presence of Pd(PPh₃)₃. Final hydrolysis afforded (+)-perillyl alcohol in modest overall yield. [22]

Although this method is well established, it does not comply very well with the principles of green chemistry, as it uses multiple passages and toxic chemical reactants. A more environmentally friendly approach could be the use of biocatalysis to achieve the same result. Of inspiration was the work of Cannazza et al. [23], where it was shown that recombinant CYP153A6-*E. coli* cells were capable of regioselective hydroxylation of limonene and α -pinene. The hydroxylation reactions produced different products depending on the substrate and reaction conditions used. For example, hydroxylation of limonene at the C-6 position was favored at higher pH values, while hydroxylation at the C-3 position was favored at lower pH values [23]. The study demonstrated that the whole cells of recombinant CYP153A6-*E. coli* have potential as a biocatalyst for the regioselective hydroxylation of monoterpenes, which could have applications in the production of high-value chemicals [23]. Since this methodology is well described in the literature and since it is possible to purchase (+)-perillyl alcohol at a relatively cheap price from the chemical market, we have decided, for reasons of time, not to perform this test in our lab and to start the experimental synthesis from the second step.

3.2 From (+)-perillyl alcohol to (+)-perillyl aldehyde via alcohol dehydrogenase

The oxidation of alcohols to aldehydes is a common practice in chemistry. The use of commercial alcohol dehydrogenases (ADHs) for this step is often described in the literature [17], and it has the great advantage to control the reaction, avoiding further oxidation to carboxylic acid. To test the possibility of using this methodology for our purposes a screening experiment was set up, to oxidize alcohol **3** to aldehyde **4** using different commercially available ADHs. The screening was performed by adding the reactants and substrate according to the procedure in several Eppendorf flasks and letting the tests under shaking for 4h at 30°C. After the incubation time the samples were tested by GC-MS to assess the conversion as reported in the graph below:

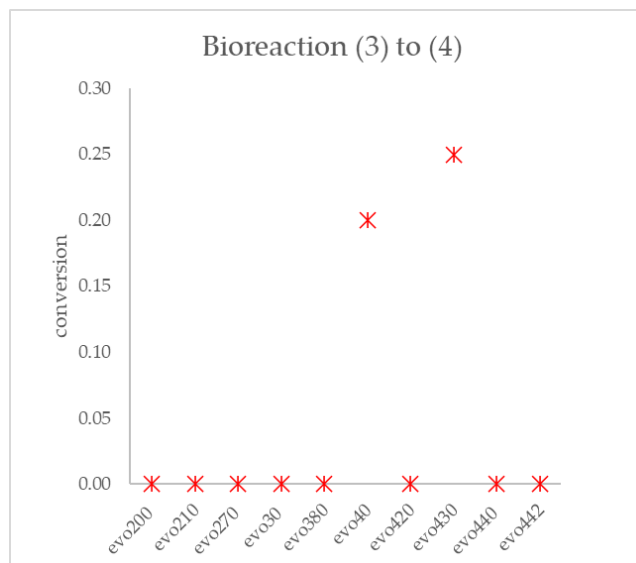


Figure 8 – Conversions of enzymatic dehydrogenation with the ADHs of the commercial kit by EVOXX

As can be seen, evo40 and evo430 were the 2 best-performing ones, while the others did not show any reactivity. Further work will be developed to optimize the reaction conditions and increase the conversion yields.

3.3 Horner Wadsworth Emmons Olefination

Horner Wadsworth Emmons olefination is a chemical reaction used to create carbon-carbon double bonds in organic molecules. This reaction involves the reaction of a phosphonate ester with an aldehyde or ketone, resulting in the formation of an α,β -unsaturated ester. The phosphonate ester, after being deprotonated by a base, acts as a nucleophile, attacking the carbonyl carbon of the aldehyde to form an intermediate, which undergoes a rearrangement to afford the desired α,β -unsaturated ester product. [24][25]

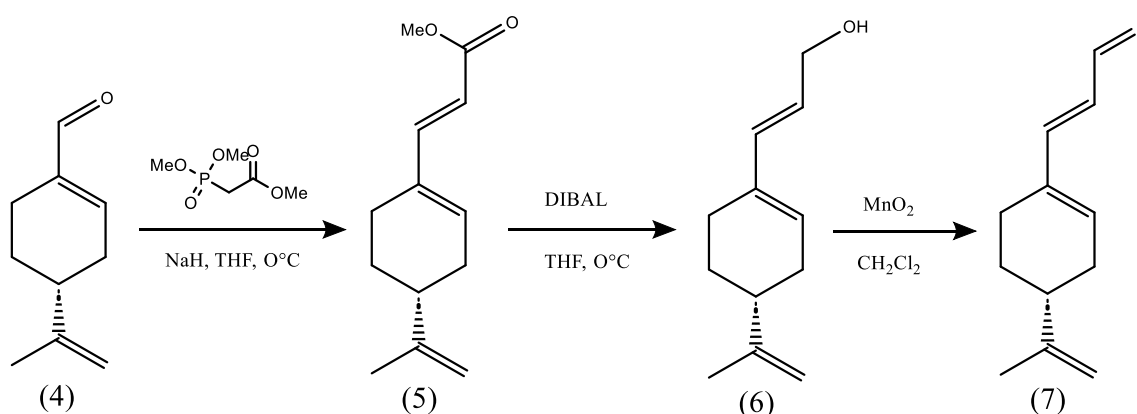


Figure 9 – Olefination reaction on perillaldehyde

One of the first steps taken was to apply this reaction to compound 4. To achieve this, sodium hydride was mixed in tetrahydrofuran together with trimethyl phosphonoacetate. Afterward

compound **4** was added according to the procedure, and the mixture was left reacting. Reaction conversion was followed using TLC and gas chromatography–mass spectrometry (GC-MS). When 100% conversion was reached the workup of the reaction was performed and the resulting sample was tested for ^1H NMR and ^{13}C NMR for molecule characterization resulting in the expected product **5**.

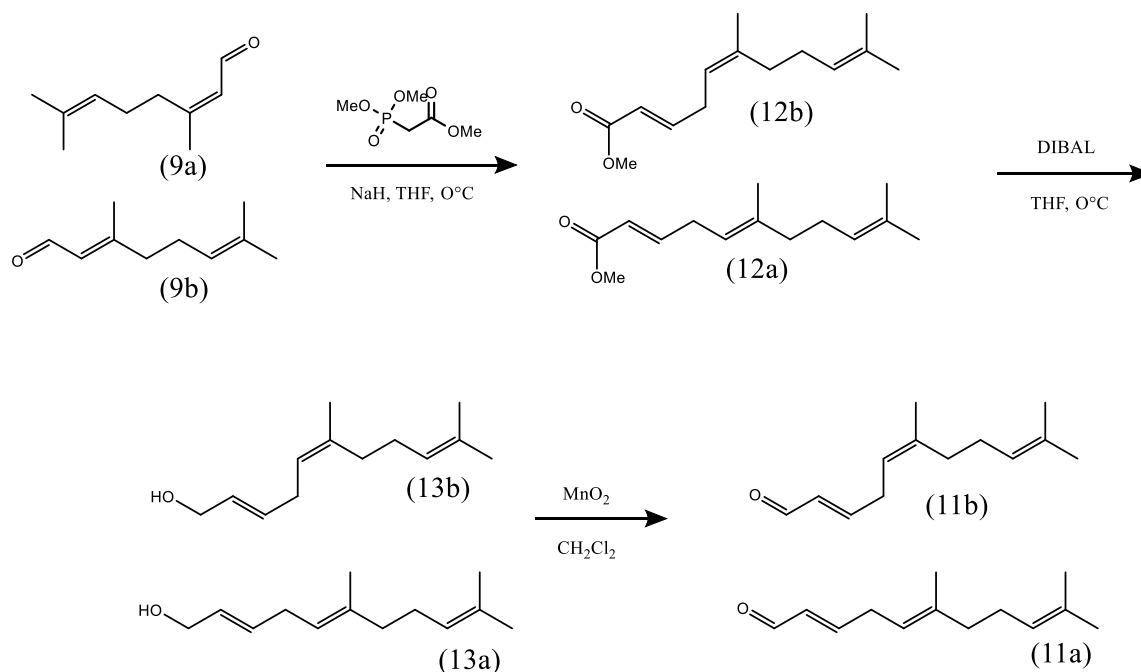


Figure 10 - Olefination reaction of citral

The same methodology was applied to derivative **9**. After complete conversion compound **12** was obtained, this sample was also tested for ^1H NMR and ^{13}C NMR.

The next step of the experiment consisted of reducing **5** to **6**. To achieve this, DIBAL was used as it can efficiently reduce α - β unsaturated esters to the corresponding allylic alcohols. [25] After dilution of **5** in THF, DIBAL was added, and the reaction was left under stirring and then tested with TLC and GC-MS. The reaction was worked up according to the procedure and tested for GC-MS, ^1H NMR, and ^{13}C NMR for molecule characterization. The same methodology was followed to **12** to obtain **13**.

To complete the reaction path and obtain compound **7**, one last step was required. Product **6** needed to be oxidized to **7** with the use of MnO₂ which is a common oxidant used in organic synthesis[26]. Alcohol **6** was diluted in dichloromethane and MnO₂ was added. The reaction was followed using GC-MS and TLC. After removal of the used MnO₂ and concentration, a yellow oily liquid was obtained. GC-MS, ^1H NMR, and ^{13}C NMR confirmed the production of the required aldehyde. The same procedure was followed with alcohol **13** obtaining aldehyde **11** as ^1H NMR and ^{13}C NMR demonstrate.

3.4 Hydrogenation through OYEs

3.4.1 Preliminary screening reactions

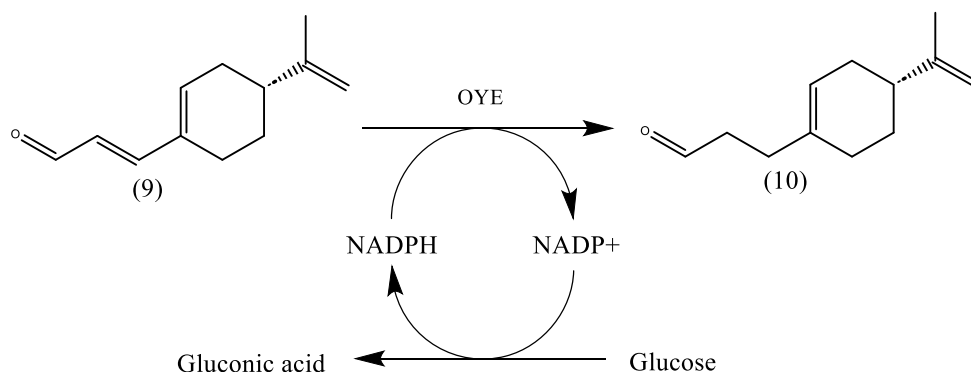


Figure 11 – Enzymatic hydrogenation of **9** with OYE

One of the key steps in the synthesis under investigation is the selective hydrogenation of the alkene bond conjugated to the aldehydic moiety of derivative **7** with the use of OYEs to produce derivative **8**. These enzymes are able to catalyze the hydrogenation of alkene bonds substituted by electron withdrawing groups, by promoting the addition first of a hydride, then of a proton to the C=C bond. The catalytic mechanism of OYEs is well-understood: the enzyme-bound flavin is first reduced at the expense of NAD(P)H cofactor (reductive half-reaction), then a hydride is transferred from the reduced FMNH₂ to the electronically activated C_β position of the alkene substrate. The in situ regeneration of the reduced nicotinamide cofactor can be easily achieved in vitro applications by using an enzymatic recycling system, e.g., that using a NAD(P)H-dependent glucose dehydrogenase (GDH) and glucose as a co-substrate. To maximize the probability of achieving a good conversion, different OYEs were used. The first screening was made with OYE1, OYE2, and OYE3 both on compound **7** and **11**. The enzymes, the substrate, and the co-substrate were suspended in an aqueous buffer with the help of a co-solvent and left under shaking for 24 h at 30°C. After incubation, the samples were tested with GC-MS to assess the conversion of **7** to **8**.

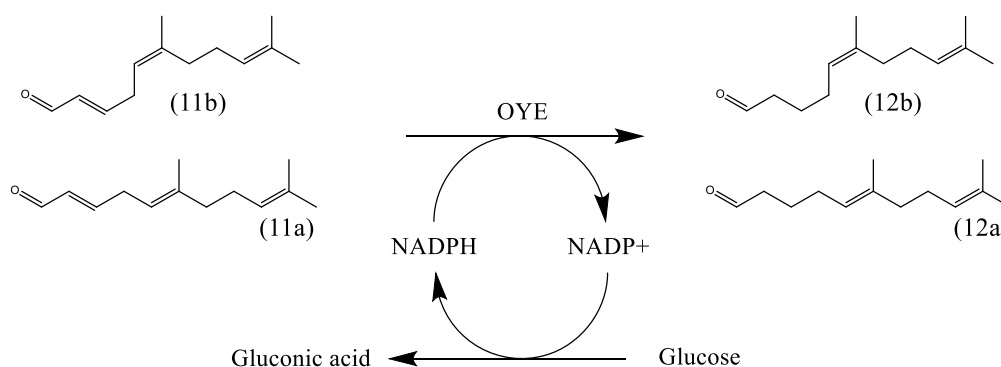


Figure 12 - Enzymatic hydrogenation of **11** with OYE

The same procedure was applied to **11**. The results with the latter were however not satisfactory due to an issue with the solubility of the compound in the aqueous medium. This resulted in a series of organic phase droplets attached to the wall of the vial. We didn't obtain

any useful results by GC-MS. Nevertheless, since the good results with **7** and OYE3, a decision was taken to proceed forward to the preparative step with both the compounds, keeping in mind that the issue of the very solubility of **11** in water still needed to be assessed.

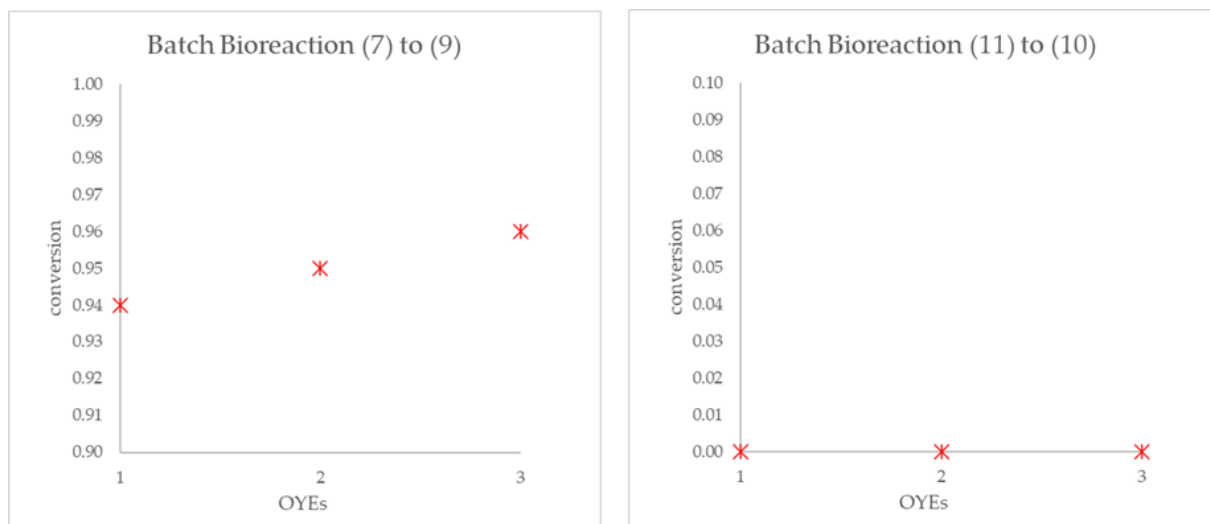


Figure 13 – Conversion results with different OYE of **7** and **11**

3.4.2 Preparative reactions

Screening reactions were tested on a small amount of substrate (5 μmol each vial). In order to obtain enough final product to be able to collect a sample for NMR, a scale-up of the reaction was set up. Since OYE3 was the best performing one, we decided to focus our attention on it. The test was performed on about 0.6 mmol of **7**, the reaction was set up and tested after 24h. Since conversion was not complete, a second addition of enzymes was done to push the conversion of **8** which finally resulted in 96% yield. Product **8** was extracted and tested for GC-MS and ^1H NMR.

As far as reaction with **11** was concerned to being able to achieve satisfactory results, different tests were made to find the best balance between the low solubility of the compound in water and the better performance of OYEs in aqueous conditions. The best setup among the many different runs was found to be using a 10% over the total weight of reaction of DMSO as cosolvent. This allowed us to achieve a 96% conversion of **10** that was then tested for GC-MS, ^1H NMR, and ^{13}C NMR.

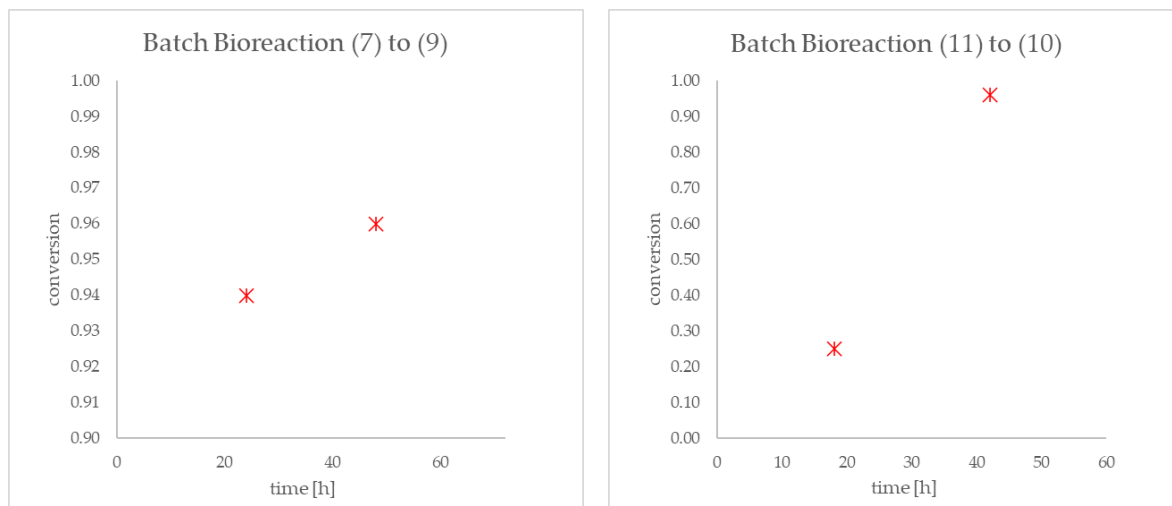


Figure 14 – Conversion results at different times on **7** and **11**

3.4.3 Flow reactions

Previous results demonstrate the possibility of reducing the α - β double bond of **7** and **11** using biocatalytic methods obtaining good conversion levels. Now the attention was moved towards the challenge of optimizing the reaction, to reduce the time needed to achieve a satisfactory conversion and increasing the concentration of substrate in the reaction medium. Increasing the overall productivity of the reaction means making it more sustainable from an environmental and potentially economical point of view. To achieve this, our attention was moved to the possibility of performing the reaction earlier described in a continuous manner. Continuous flow chemistry has many advantages, it is able to increase conversion, reduce reaction time and allow better overall control of the reaction thanks to the ability to easily manipulate different parameters like flow rate, temperature, and pressure.

To find the optimized condition for the conversion of **7** to **8** a new experiment was set up. A reaction medium was prepared according to the procedure later described. Then the reaction was carried out in a continuous tubular reactor, employing two different flow rates: 100 $\mu\text{L}/\text{min}$, and 50 $\mu\text{L}/\text{min}$, respectively.

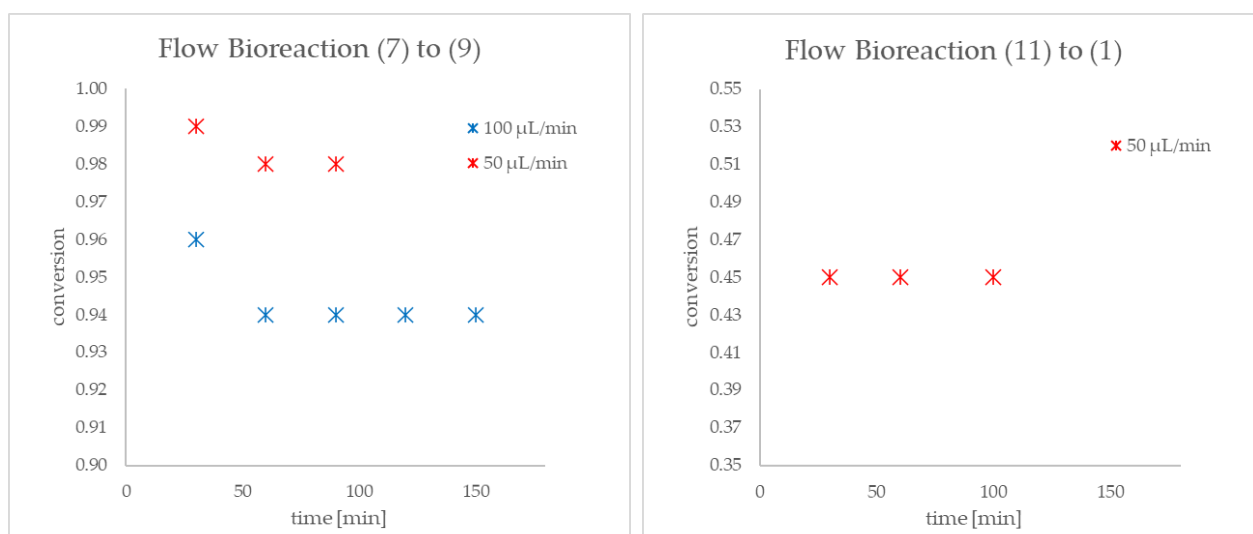


Figure 15 – Conversion results with different flow rate of **7** and **11**

As can be seen the conversion for **7** was quite good with an improvement at working at 50 $\mu\text{L}/\text{min}$ compared to 100 $\mu\text{L}/\text{min}$. A spike in conversion in the first fraction of the samples was observed, this is probably due to the fact that the first units of solution entered in contact with the solvent used to wash the reactor (water) increasing the dilution of the solution, a factor that is known to increase the ability of OYEs to convert substrate.

On the other hand, the use of **11** as substrate proved to be more difficult and conversion remained significantly lower than **7**. This could be explained once again by the low solubility of **11** in aqueous solution. The suspect is that **11** once inside the reactor would get out of solution and would deposit on the walls of the coil. This is suggested by the fact that, during the experiment with this substrate, an increase of pressure at the reactor HPLC pump was registered suggesting some kind of blockage in the system.

In general, the use of the reactor for the reaction of **7** to **8** was of great success, making us able to reach a high level of conversion in much reduced time (4h total) compared to batch tests (24h+). As far as the **11** is concerned, further investigation and optimization of the process is needed.

3.5 Continuous reactor Hydrogenation

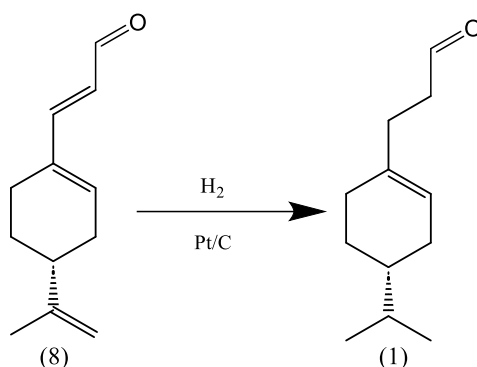


Figure 16 – Flow catalytic hydrogenation of **8** to Lilybelle®

The final step required to achieve Lilybelle was the selective hydrogenation of the isopropyl double bond in compound **8** to obtain derivative **1**. The inspiration for this step was taken from a Symrise patent [17], where a similar hydrogenation process was performed at the beginning of the synthesis on molecule **2**. This is probably done, in the industrialized process, since it is more efficient to perform the hydrogenation step earlier in the synthesis, to reduce separation costs later. However, we decided to postpone the hydrogenation step until the end, where the amount of material to be manipulated was necessarily lower, in order to limit the amount of metal catalyst employed in the synthetic path. The high selectivity of this heterogeneous hydrogenation step using Pt/C catalyst allowed us to achieve satisfactory results for our purposes. Overall, we completed the chemical synthesis of Lilybelle.



Figure 17 – Catalytic hydrogenations reactor

The hydrogenation reaction was performed using a reactor as the one shown in Figure 17. This instrument allows for a wide range of conditions to be set in terms of flow rate, pressure, temperature, and catalyst used. The reactor comes equipped with columns of various sizes packed with suitable catalysts, such as Pt/C, Raney Ni, and Pd/C, among others. For the reaction, hydrogen gas is provided by a high-efficiency electrolysis cell, making the process simple, safe, and environmentally friendly. Due to the numerous factors that could be easily adjusted, the reaction could be replicated with ease and a large number of tests could be performed in a relatively short time using only small amounts of product. Therefore, we believe that a Design of Experiment (DoE) technique would have been the best approach to find the best-performing conditions. This technique would have allowed us to systematically explore and optimize the reaction parameters, thereby maximizing the efficiency and yield of the reaction. However, since only a small amount of our target compound **8** was available, we performed the DoE on commercially available and relatively cheap perilla aldehyde instead, which is very similar in terms of molecular structure. We then used the best-performing conditions from this experiment to reduce **8** and produce Lilybelle®.

3.5.1 Hydrogenation of perilla aldehyde

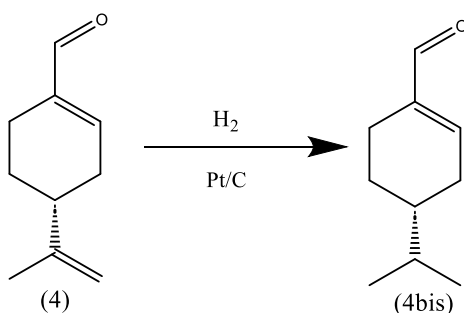


Figure 18 – Flow catalytic hydrogenation of **4** to **4bis**

The first DoE was performed to assess the best conditions in terms of solvent, flow rate, and concentration. For each factor, we selected a "0" point as a probable value for the reaction's success, and then chose +1 and -1 points as values that were at a sensible and rational distance

from the middle point, to understand the reaction's behavior. We selected data points of 0.1, 0.2, and 0.3 ml/min for flow rate, 0.1125, 0.075, and 0.15 M for concentration, and 100% Hexane, 50:50% Hexane-AcOEt, and 100% AcOEt for the solvent. We performed a full factorial design requiring 11 replicates, following the procedure described below. Each time, we reported the conversion of our product and obtained the following results:

Table 1 – results of DoE n°1

	Flowrate [mL/min]	Concentration [M]	Solvents fraction			Conversion	
1	0.2	0.1125	Hex	0.5	AcOEt	0.5	0.76
2	0.1	0.075	Hex	0	AcOEt	1	0.94
3	0.1	0.15	Hex	0	AcOEt	1	0.93
4	0.3	0.15	Hex	1	AcOEt	0	0.58
5	0.3	0.15	Hex	0	AcOEt	1	0.62
6	0.3	0.075	Hex	0	AcOEt	1	0.75
7	0.1	0.075	Hex	1	AcOEt	0	0.9
8	0.1	0.15	Hex	1	AcOEt	0	0.82
9	0.3	0.075	Hex	1	AcOEt	0	0.6
10	0.2	0.1125	Hex	0.5	AcOEt	0.5	0.82
11	0.2	0.1125	Hex	0.5	AcOEt	0.5	0.77

We analyzed the data using the statistical software Minitab® and found that flow rate had the most significant impact on conversion among the three parameters tested. Specifically, a flow rate of 0.1 ml/min resulted in significantly higher conversions compared to the other scenarios, likely due to the longer contact time between the reagent and the catalyst. Regarding the solvent, hexane performed the best on average, although the Pareto chart (Figure 20) showed a small overall correlation with conversion. Lower concentrations also had higher average conversion rates, although the concentration parameter was not as impactful overall (Figure 19). The experiment yielded high product conversions and provided insights on optimizing the reaction conditions.

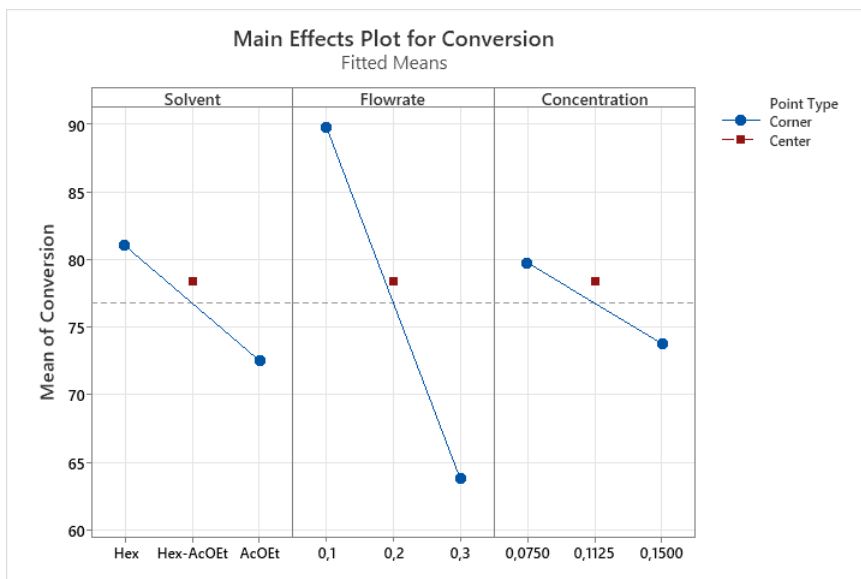


Figure 19 – Plot conversion of DoE n° 1

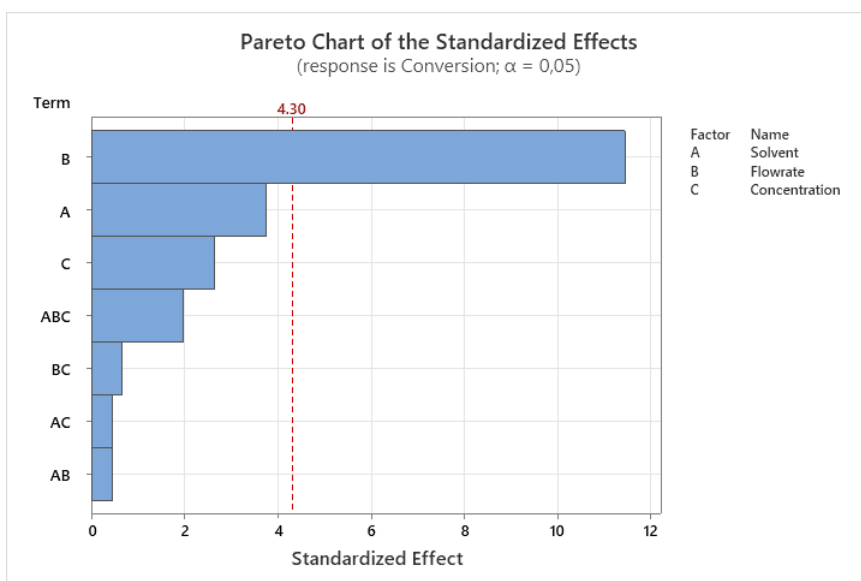


Figure 20 – Pareto chart of DoE n°1

To further develop and improve the process, we conducted a second DoE to determine the optimal working pressure. The full factorial design included three pressure conditions (10, 20, and 30 bar) and three flow rate conditions (0.1, 0.2, and 0.3 ml/min), and we performed seven replicates according to the described methods. We assessed conversion using GC-MS and obtained the following results:

Table 2 – Results of DoE n°2

	Flow rate [ml/min]	Concentration [M]	Pressure [bar]	Solvent	Conversion	Number	Flow rate [ml/min]
1	0.1	0.15	10	AcOEt	0.67	1	0.1
2	0.2	0.15	20	AcOEt	0.67	2	0.2
3	0.2	0.15	20	AcOEt	0.63	3	0.2
4	0.1	0.15	30	AcOEt	0.80	4	0.1
5	0.3	0.15	10	AcOEt	0.76	5	0.3
6	0.3	0.15	30	AcOEt	0.59	6	0.3
7	0.2	0.15	20	AcOEt	0.60	7	0.2

Our analysis in Minitab® revealed that increasing the pressure had generally a positive impact on conversion, likely due to the increased adsorption of hydrogen gas into the catalyst at higher pressures. However, the statistical analysis (Figure 21) indicated a low correlation between pressure and conversion. Results on flowrate were in line with previous results.

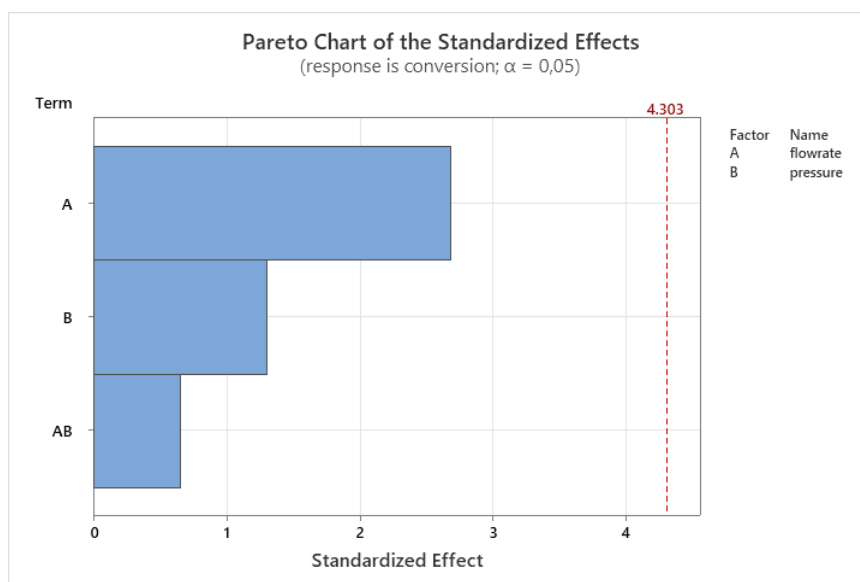


Figure 21 – Pareto charts DoE n°2

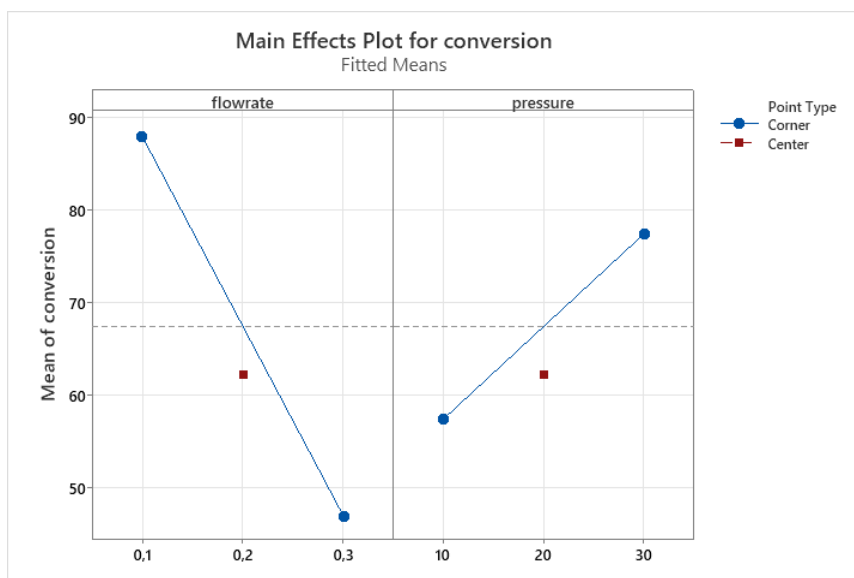


Figure 22 – Plot conversion DoE n°2

3.5.2 Final Hydrogenation to Lilybelle®

Thanks to the information gathered in the previous section, we built enough knowledge to perform the hydrogenation of **8** to Lilybelle®. Since we realized that the most impacting factor on conversion was flow rate, we set the reactor to the best-performing value of 0.1 ml/min. As far as pressure was concerned, 30 bar showed, in general, to be better performing pressure value and we set concentration to 0.05 M. This was for two reasons, first we demonstrate that a small concentration was helping conversion, and secondly, due to the small amount of **8** available, a smaller concentration made the setup of the experiment easier. After letting the reactor run as the usual procedure, we tested the result on GC-MS and were able to obtain 40% conversion on Lilybelle®. The results were confirmed thanks to a ^1H NMR test on the final sample.

4. Materials and methods

4.1 General Methods

Chemicals and solvents were purchased from Merck Life Science s.r.l. (Milan, Italy) and used without further purification. TLC analyses were performed on Macherey Nagel pre-coated TLC sheets Polygram® SIL G/UV254 purchased from Chimikart s.r.l. (Naples, Italy). The continuous flow reactions were performed using an E-Series Integrated Flow Chemistry system from Vapourtec (Alfatech s.p.a., Genoa, Italy) 10 mL standard coiled tubular reactor (ambient to 150°C temperature). H-CUBE MINI PLUS used for hydrogenation reactions was provided by ThalesNano Inc. (Budapest, 7 Zahony Street, Graphisoft Park). ^1H NMR and ^{13}C NMR spectra were recorded on a 400 MHz spectrometer in CDCl_3 solution at r.t. unless otherwise specified. The chemical shift scale was based on internal tetramethylsilane. GC-MS analyses were performed using an HP-5MS column (30 m × 0.25 mm × 0.25 μm , Agilent

Technologies Italia s.p.a., Cernusco sul Naviglio, Italy). The following temperature program was employed: 50°C / 10°C min⁻¹ / 250°C (5 min) / 50°C min⁻¹ / 300°C (10 min).

4.2 Alcohol dehydrogenase

To carry out the dehydrogenation reaction using ADHs of EVOXX kit, a 5 mL Eppendorf vial was prepared with the following components: 16 µL of acetone, a 500 mM solution of **3** in DMSO, 10 µL of a 10 mM solution of NADH, 10 µL of a 10 mM solution of NADP⁺ in water, 904 µL of pH 7 phosphate buffer (50 mM), and 50 µL of a 5 mg/ml solution of ADH. The same amounts of substrate and cofactor were added to all other vials, but with a different type of ADH according to the table below. The vials were closed and incubated at 30°C for 24 hours with shaking at 150 rpm. After incubation, 600 µL of ethyl acetate (AcOEt) was added to each vial and the solutions were centrifuged at 5000 rpm for 3 minutes. The organic phases were separated, dried with a small amount of Na₂SO₄, and analyzed by GC-SM.

Table 3 – List of ADHs used

EVOxxx used
evo200 50 µL 5 mg/ml
evo270 50 µL 5 mg/ml
evo440 50 µL 5 mg/ml
evo40 50 µL 5 mg/ml
evo210 50 µL 5 mg/ml
evo380 50 µL 5 mg/ml
evo430 50 µL 5 mg/ml
evo420 50 µL 5 mg/ml
evo442 50 µL 5 mg/ml
evo30 50 µL 5 mg/ml

4.3 Olefination of Aldehydes

4.3.1 Horner Wadsworth Emmons

The procedure followed for the Horner Wadsworth Emmons Olefination consisted in adding to 240ml of THF anhydrous, 1.1 equivalent of NaH (60% in oil) and 1.05 equivalent of trimethylphosphone acetate dropwise. The reaction mixture was left under stirring at 0°C in N₂ atmosphere for 30min. At the same temperature, drop by drop, 1 equivalent of **4** or **9** was added to the reaction. The reaction was followed with TLC (n-esano/EtOAc 8:2). When complete conversion was reached, the reaction mixture was treated with HCl 1M and water.

The organic phase was extracted with EtOAc (3X50 mL) and then treated with Na₂SO₄. A sample was tested over gas chromatography–mass spectrometry (GC-MS). After concentration at low pressure, a sample was diluted in CDCl₃ and ¹H NMR and ¹³C NMR were performed for characterization.

(4) (*R*)-4-(prop-1-en-2-yl)cyclohex-1-ene-1-carbaldehyde

¹H NMR (400 MHz, CDCl₃) δ 9.36 (s, 1H), 6.76 (m, 1H), 4.74 – 4.62 (m, 2H), 2.49 – 2.31 (m, 2H), 2.27 – 2.10 (m, 2H), 2.14 – 1.96 (m, 1H), 1.84 (m, 1H), 1.69 (t, *J* = 1.2 Hz, 3H), 1.46 – 1.30 (m, 1H).

¹³C NMR (101 MHz, CDCl₃) δ 193.59, 150.36, 148.14, 141.10, 109.38, 40.56, 31.58, 26.21, 21.42, 20.53.

GC-MS: *t_R* = 14.136 min, MS (EI) *m/z* = 150 (60), 135 (104), 122 (112), 108 (67), 91 (118), 79 (244), 68 (266), 53 (112).

(5) methyl(*R,E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)acrylate

¹H NMR (400 MHz, CDCl₃) δ 7.27 (d, *J* = 15.8 Hz, 1H), 6.17 – 6.11 (m, 1H), 5.73 (d, *J* = 15.8 Hz, 1H), 4.77 – 4.66 (m, 2H), 3.70 (s, 2H), 2.35 – 1.96 (m, 6H), 1.93 – 1.84 (m, 1H), 1.57 – 1.35 (m, 1H), 1.32 – 1.04 (m, 2H), 0.88 – 0.77 (m, 1H).

¹³C NMR (101 MHz, CDCl₃) δ 168.05, 148.94, 147.73, 138.15, 134.63, 128.27, 127.06, 114.70, 109.22, 51.46, 40.73, 31.86, 29.78, 27.06, 24.55, 23.81, 20.79, 14.17.

GC-MS: *t_R* = 21.243 min, MS (EI) *m/z* = 260 (55), 191 (13), 175 (16), 163 (18), 147 (30), 79 (244), 68 (266), 53 (112).

(12) methyl (2*E*,5*E*,-and(2*Z*,5*E*,-6,10-dimethylundeca-2,5,9-trienoate (39:57 *Z*:*E* ratio)

¹H NMR (400 MHz, CDCl₃) δ 7.49 (m, 1H), 5.91 (d, *J* = 11.6 Hz, 1H), 5.69 (dd, *J* = 15.1, 10.4 Hz, 1H), 5.07 – 4.97 (m, 2H), 3.74 (d, *J* = 11.2 Hz, 1H), 3.69 – 3.61 (m, 4H), 2.22 (h, *J* = 7.0 Hz, 1H), 2.16 – 2.00 (m, 5H), 1.86 – 1.75 (m, 5H), 1.60 (s, 5H), 1.53 (s, 4H), 1.29 – 1.03 (m, 2H), 0.95 – 0.70 (m, 1H).

¹³C NMR (101 MHz, CDCl₃) δ 168.00, 149.98, 149.84, 141.16, 140.89, 132.49, 132.13, 124.11, 123.31, 123.24, 118.42, 118.20, 53.18, 53.12, 52.59, 51.27, 51.25, 50.87, 40.24, 33.80, 32.84, 32.45, 30.28, 29.66, 26.87, 26.28, 25.60, 25.58, 24.36, 17.62, 17.61, 17.32.

GC-MS: *t_R* = 19.921 min, MS (EI) *m/z* = 208 (20), 140 (62), 125 (43), 109 (10), 91 (10), 81 (72), 68 (145), 53 (13).

GC-MS: *t_R* = 20.877 min, MS (EI) *m/z* = 208 (12), 140 (32), 125 (50), 109 (10), 91 (10), 81 (72), 68 (220), 53 (20).

4.3.2 DIBAL reduction

To reduce **5** or **12** 1 equivalent of substrate was diluted in THF anhydrous at 0°C in N₂ atmosphere. DIBAL (sol 25% in toluene 1.2 equivalent) was added at 0°C and left under agitation for 30 min. When the reaction was complete, the mixture was treated with HCl 1M and water. The organic phase was extracted with EtOAc (3X50 mL) and then treated with Na₂SO₄. A sample was tested by GC-MS. After concentration at low pressure and dilution in CDCl₃, ¹H NMR and ¹³C NMR were performed for characterization.

(6) (*R,E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)prop-2-en-1-ol

¹H NMR (400 MHz, CDCl₃) δ 7.37 – 7.20 (m, 1H), 7.19 – 7.10 (m, 1H), 6.23 (d, *J* = 15.7 Hz, 1H), 5.81 – 5.61 (m, 2H), 4.78 – 4.69 (m, 4H), 4.16 (d, *J* = 6.1 Hz, 2H), 3.78 – 3.51 (m, 1H), 2.44 – 2.24 (m, 3H), 2.16 (m, 4H), 2.04 (q, *J* = 4.0 Hz, 1H), 1.98 – 1.75 (m, 2H), 1.74 (s, 3H), 1.73 (s, 1H), 1.61 – 1.36 (m, 2H), 1.26 (d, *J* = 5.3 Hz, 1H), 1.25 – 0.99 (m, 1H), 1.02 – 0.77 (m, 2H).

¹³C NMR (101 MHz, CDCl₃) δ 149.66, 137.92, 134.87, 134.72, 129.29, 129.12, 128.58, 128.31, 127.04, 126.72, 126.56, 125.40, 124.87, 108.88, 63.86, 41.25, 31.37, 30.80, 29.81, 27.74, 27.48, 25.06, 24.01, 22.79, 21.53, 20.88, 14.21.

GC-MS: *t*_r = 19.715 min, MS (EI) *m/z* = 178 (17), 149 (22), 147 (37), 134 (62), 119 (65), 105 (75), 91 (133), 79 (154), 78 (23), 67 (90), 53 (35).

(13) (*2E,5E*,-and(*2Z,5E*,-6,10-dimethylundeca-2,5,9-trien-1-ol (45:54 *Z*:*E* ratio)

¹H NMR (400 MHz, CDCl₃) δ 6.38 (m, 1H), 5.77 (d, *J* = 10.8 Hz, 1H), 5.64 (ddt, *J* = 15.6, 9.6, 6.1 Hz, 1H), 5.08 – 4.98 (m, 1H), 4.09 (t, *J* = 5.3 Hz, 2H), 2.11 (dd, *J* = 8.3, 6.0 Hz, 1H), 2.00 (td, *J* = 13.7, 5.1 Hz, 5H), 1.69 (s, 2H), 1.61 (s, 3H), 1.53 (s, 3H), 1.28 – 0.95 (m, 2H), 0.95 – 0.73 (m, 1H).

¹³C NMR (101 MHz, CDCl₃) δ 140.00, 139.75, 131.93, 131.71, 129.43, 129.28, 129.02, 128.34, 128.22, 128.09, 126.96, 125.30, 124.72, 123.90, 123.89, 63.74, 63.72, 39.91, 32.47, 30.32, 29.70, 26.82, 26.54, 25.67, 23.79, 17.66, 16.65.

GC-MS: *t*_r = 18.261 min, MS (EI) *m/z* = 180 (15), 162 (22), 147 (), 133 (7), 119 (79), 105 (30), 91 (82), 79 (78), 69 (66), 55 (28).

GC-MS: *t*_r = 19.131 min, MS (EI) *m/z* = 180 (10), 162 (20), 147 (25), 133 (7), 119 (112), 105 (55), 91 (132), 79 (80), 69 (112), 55 (60).

4.3.3 Oxidation with manganese dioxide

To oxidize **6** and **13** to **7** and **11** respectively, the following procedure was followed: **6** or **13** was diluted in 80 mL of DCM and put under stirring. 5 equivalents of activated MnO₂ were added and reaction progress was monitored by TLC till complete conversion. The mixture was filtered over celite and concentrated at low pressure. Samples were tested for ¹H NMR and ¹³C NMR.

(7) (*R,E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)acrylaldehyde

¹H NMR (400 MHz, CDCl₃) δ 9.51 (d, *J* = 7.8 Hz, 1H), 7.07 (d, *J* = 15.7 Hz, 1H), 6.29 (d, *J* = 5.5 Hz, 1H), 6.02 (dd, *J* = 15.6, 7.8 Hz, 1H), 4.75 – 4.62 (m, 3H), 2.43 – 2.23 (m, 4H), 2.23 – 2.11 (m, 5H), 2.11 (d, *J* = 9.1 Hz, 1H), 1.95 – 1.87 (m, 1H), 1.71 (s, 4H), 1.50 (m, 2H).

¹³C NMR (101 MHz, CDCl₃) δ 194.27, 155.60, 148.62, 140.55, 135.23, 128.74, 127.28, 126.19, 109.71, 109.38, 60.40, 40.51, 32.05, 26.89, 24.61, 23.73, 20.78, 14.23.

GC-MS: *t*_r = 19.966 min, MS (EI) *m/z* = 176 (22), 133 (25), 105 (23), 91 (27), 79 (37), 68 (35), 53 (12).

(11) (2*E*,5*E*,-and(2*Z*,5*E*,-6,10-dimethylundeca-2,5,9-trienal (33:66 Z:E ratio)

¹H NMR (400 MHz, CDCl₃) δ 9.49 (t, *J* = 8.1 Hz, 1H), 7.31 (td, *J* = 14.7, 11.5 Hz, 1H), 6.08 (d, *J* = 11.5 Hz, 1H), 6.07 – 5.94 (m, 1H), 5.07 – 4.98 (m, 1H), 2.28 (t, *J* = 7.6 Hz, 1H), 2.19 – 2.04 (m, 4H), 1.87 (d, *J* = 3.6 Hz, 3H), 1.68 – 1.44 (m, 9H), 1.20 (d, *J* = 8.9 Hz, 2H), 0.83 – 0.74 (m, 1H).

¹³C NMR (101 MHz, CDCl₃) δ 193.96, 152.96, 152.87, 148.49, 148.29, 132.88, 132.46, 130.00, 129.76, 124.67, 123.75, 123.06, 122.98, 40.51, 33.07, 29.68, 29.36, 26.82, 26.25, 25.66, 25.64, 24.73, 17.68, 17.59, 14.09.

GC-MS: *t_r* = 17.762 min, MS (EI) *m/z* = 178 (2), 163 (4), 120 (6), 110 (8), 95 (18), 69 (30), 53 (4).

GC-MS: *t_r* = 18.577 min, MS (EI) *m/z* = 178 (4), 163 (3), 120 (2), 110 (25), 95 (24), 69 (50), 53 (5).

4.4 Enzymatic Hydrogenation

4.4.1 Preliminary screening

To carry out the hydrogenation through OYE1, a 5 mL Eppendorf vial was prepared with 860 μL of phosphate buffer (pH 7 - 50mM), 20 μL of a 1000 mM solution of glucose in distilled water, 30 μL of a 40 U/mL GDH solution in water, 10 μL of a 10 Mm solution of NADP+ in water and 10 μL of a 500 mM 7 solution in DMSO and 70 μL of a solution of a 1.9 mg/ml solution of OYE1 in water. A second vial was prepared with 30 μL of 4 mg/ml solution of OYE2 and a third one with 40 μL of 3.5 mg/ml solution of OYE3. The amount of buffer was adjusted each time so that the total volume was always approximately 1 mL (respectively 860, 900 and 890 μL). The vials were closed and left under shaking at 150 rpm at 30°C for 24. After the incubation 600 μL of AcOEt, was added in each vial, the solutions were centrifuged at 5000 rpm for 3 min, the organic phases were separated, dried with a small amount of Na₂SO₄ and tested for GC-MS. The exact same quantities were used with 10 μL of 500mM solution of **11** in DMSO. After the same work up, all were tested for GC-MS.

Reported quantities for screening reaction with OYEs:

Table 4 – OYEs screening summary tables

OYE 1	OYE1 1.9 mg/ml (70 μL)	7 500mM DMSO (10 μL)	NADP+ (10Mm 10 μL)	GDH-MB 40U/mL (30 μL)	Glc 1000 mM (20 μL)	Phosphate buffer pH 7 50mM (860 μL)
OYE 2	OYE2 4 mg/ml (30 μL)	7 500mM DMSO (10 μL)	NADP+ (10Mm 10 μL)	GDH-MB 40U/mL (30 μL)	Glc 1000 mM (20 μL)	Phosphate buffer pH 7 50mM (900 μL)
OYE 3	OYE3 2.8 mg/ml (40 μL)	7 500mM DMSO (10 μL)	NADP+ (10Mm 10 μL)	GDH-MB 40U/mL (30 μL)	Glc 1000 mM (20 μL)	Phosphate buffer pH 7 50mM (890 μL)
OYE 1	OYE1 - 1.9 mg/ml (70 μL)	11 500mM DMSO (10 μL)	NADP+ (10Mm 10 μL)	GDH-MB 40U/mL (30 μL)	Glc 1000 mM (20 μL)	Phosphate buffer pH 7 50mM (860 μL)
OYE 2	OYE2 - 4 mg/ml (30 μL)	11 500mM DMSO (10 μL)	NADP+ (10Mm 10 μL)	GDH-MB 40U/mL (30 μL)	Glc 1000 mM (20 μL)	Phosphate buffer pH 7 50mM (900 μL)
OYE 3	OYE3 - 2.8 mg/ml (40 μL)	11 500mM DMSO (10 μL)	NADP+ (10Mm 10 μL)	GDH-MB 40U/mL (30 μL)	Glc 1000 mM (20 μL)	Phosphate buffer pH 7 50mM (890 μL)

4.4.2 Preparative reactions

To carry out the preparative hydrogenation through OYE3, a 50 mL vial was prepared with 4393 μL of Phosphate buffer pH7 - 50mM, 409 mg of D-glucose, 2000 μL of a 40U/mL GDH solution in water, 7 mg of NADP+, 0.567 mmol of **7** in 150 μL of isopropyl alcohol and 2450 μL of 3.9 mg/ml OYE3 in water. The vial was closed and left under shaking at 150 rpm at 30°C. After 24h an additional 2450 μL of 3.9 mg/ml OYE3, 6 mg NADP+, 500 μL of 40U/ml GDH-MB, 200 mg Glucose and 1000 μL of buffer were added. Then the reaction was left under shaking at the same condition as before for others 24h. The organic solution was extracted in AcOEt, dried with a small amount of Na₂SO₄ and tested for GC-MS and ¹H NMR. To carry out the hydrogenation with **11** a 50 mL vial was prepared with 7650 μL of Phosphate buffer pH7 - 50mM, 165 mg of Glucose, 450 μL of a 40U/mL GDH solution in water, 7 mg of NADP+, 0.227 mmol of **7** in 1000 μL of DMSO and 500 μL of 4.3 mg/ml OYE3 in water. The vial was closed and left under shaking at 150 rpm at 30°C. After 24h an additional 850 μL of 34.4 mg/ml OYE3, 6 mg NADP+, 500 μL of 40U/ml GDH-MB, 70 mg Glucose and 1000 μL of buffer were added. Then the reaction was left under shaking at the same condition as before for others 24h. The organic solution was extracted in AcOEt, centrifuged at 12000 rpm for 5 min and dried with a small amount of Na₂SO₄ and tested for GC-MS and ¹H NMR and ¹³C NMR.

Table 5 – OYEs batch summary tables

0h	OYE3 - 3.9 mg/ml (2450 μL)	7 (0.567 mmol)	NADP+ (7 mg)	GDH-MB 40U/mL (2000 μL)	Glc (409 mg)	Phosphate buffer pH 7 50mM (4393 μL)	iPrOH (150 μL)
24h	OYE3 - 3.9 mg/ml (2450 μL)		NADP+ (6 mg)	GDH-MB 40U/mL (500 μL)	Glc (200 mg)	Phosphate buffer pH 7 50mM (1000 μL)	
0h	OYE3 - 4.3 mg/ml (500 μL)	11 (0.227 mmol)	NADP+ (7 mg)	GDH-MB 40U/mL (450 μL)	Glc (165 mg)	Phosphate buffer pH 7 50mM (7650 μL)	DMSO 10% (1000 μL)
24h	OYE3 - 4.3 mg/ml (850 μL)		NADP+ (6 mg)	GDH-MB 40U/mL (500 μL)	Glc (70 mg)	Phosphate buffer pH 7 50mM (1000 μL)	

(8) (*R*)-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)propanal

¹H NMR (400 MHz, CDCl₃) δ 9.78 – 9.67 (m, 1H), 4.64 (d, *J* = 6.7 Hz, 2H), 3.76 – 3.66 (m, 1H), 3.62 (dd, *J* = 11.3, 5.6 Hz, 1H), 2.50 – 2.36 (m, 2H), 2.27– 2.19 (m, 4H), 2.11 (d, *J* = 5.1 Hz, 1H), 2.05 – 1.95 (m, 3H), 1.94 – 1.83 (m, 1H), 1.74 (d, *J* = 10.4 Hz, 1H), 1.69 (s, 2H), 1.47 – 1.36 (m, 3H), 1.23 – 1.13 (m, 3H).

GC-MS: *t_r* = 19.966 min, MS (EI) *m/z* = 178 (2), 134 (19), 119 (14), 105 (8), 91 (30); 79 (16), 68 (32), 53 (8).

(10) (*E*,-and(*Z*))-6,10-dimethylundeca-5,9-dienal (37:49 *Z*:*E* ratio)

¹H NMR (400 MHz, CDCl₃) δ 9.69 (t, *J* = 1.7 Hz, 1H), 5.07 – 4.96 (m, 2H), 2.43 – 2.33 (m, 2H), 2.26 (q, *J* = 7.1 Hz, 2H), 2.04 – 1.87 (m, 4H), 1.58 – 1.50 (m, 5H), 1.48 – 1.28 (m, 1H), 1.28 – 1.21 (m, 1H), 1.17 (d, *J* = 14.7 Hz, 2H), 0.98 – 0.74 (m, 2H).

¹³C NMR (101 MHz, CDCl₃) δ 202.62, 202.53, 136.96, 136.85, 131.78, 131.48, 124.09, 124.05, 122.81, 122.02, 44.17, 43.94, 39.60, 31.91, 29.69, 29.37, 26.57, 26.43, 25.68, 25.65, 23.32, 20.86, 20.69, 17.66, 17.62, 16.02.

GC-MS: *t_r* = 15.557 min, MS (EI) *m/z* = 180 (2), 165 (3), 137 (20), 93 (25), 69 (140), 53 (30).

GC-MS: *t_r* = 15.969 min, MS (EI) *m/z* = 180 (2), 137 (30), 93 (55), 69 (200), 53 (59).

4.4.3 Continuous flow enzymatic reactions

The continuous enzymatic flow reactions were performed using a standard coiled tubular reactor. To exploit this technology and test it to out purposes, a flask was fill with: 18065 μL of Phosphate buffer pH7 - 50mM, 400 mg of Glucose, 600 μL of a 40U/mL GDH solution in water, 200 μL of a 10 mM solution of NADP⁺ in water, 200 μL of a 500 mM solution of **7** in DMSO and 535 μL of 4.2 mg/ml OYE3 in water. The vial was then taken a 0°C to avoid the starting of the reaction. The inlet tube of the reactor was placed inside the vial. The reactor internal pump was set to 100 μL/min and the reactor temperature to 30°C. At the outlet of the coil a clean flask was inserted to collect the reacted solution. The solution (water) that came out for the first 1:40 h was discarded as this is the time for the dead volume of the 10 ml coil. Then samples were taken, collecting the solution in flasks, and changing them every 30 min until (40 the last one) until all the solution has passed through. The 5 samples were then extracted with AcOEt, dried over Na₂SO₄, and tested at GS-MS to assess the conversion. A second test was carried out with the same set up using the following quantities: OYE3 - 4.2 mg/ml (217 μL), **7** DMSO 500 mM (100 μL), NADP⁺ 10 mM (100 μL), GDH-MB 40U/mL (300 μL), Glucose 1M (200 μL), Phosphate buffer pH 7 50mM (9000 μL). This time the reactor was attached to an HPLC pump set to 50 μL/min. The coil was set to 30 °C and the experiment was performed as described earlier. Three samples were collected, extracted in AcOEt, dried on Na₂SO₄ and tested at GC-MS to assess conversion. One last test was made this time using **11**. The exact same procedure was followed as the other two with the following quantities: OYE3 - 4.2 mg/ml (535 μL), **11** DMSO 500 mM (200 μL), NADP⁺ 10 mM (200 μL), GDH-MB 40U/mL (600 μL), Glucose 1M (400 μL), Phosphate buffer pH 7 50mM (18065 μL).

4.5 Continuous reactor Hydrogenation

The H-Cube Mini Plus hydrogen reactor from Thales Nano was used to perform various catalytic reactions. Before each use, preparatory steps were taken, including filling the water

tank reservoir with Milli-Q® water, cleaning all circuits with isopropyl alcohol, and filling the pump cleaning system with a 50% mixture of isopropyl and Milli-Q® water. After multiple cleaning cycles and ensuring no residual material was left in the reactor, a 35 mm catalyst cartridge was inserted into the allotted space. The reactor inlet tube was then inserted into a flask containing the solvent to be used for the reaction. It is important to note that all solvents and reaction mixtures were filtered through nylon filters. The reactor was set to the desired reaction parameters, including pressure, temperature, and flow rate (hydrogen pressure was set automatically by the system), and started. At this point, the reactor initiated a preparatory cycle that ended with the stabilization of the reactor at the correct conditions. The solvent flask was then replaced with the reaction mixture flask. After the dead volume time (which depended on the different flow rates), samples were collected and brought to the GC-MS test. For all the reactions performed, 35 mm Pt/C cartridges were used provided by Thales Nano. All reactions were performed at ambient temperature on 10 ml samples. All other reaction parameters are already described in results sections.

(1) (*R*)-3-(4-isopropylcyclohex-1-en-1-yl)propanal

¹H NMR (400 MHz, CDCl₃) δ 4.66 – 4.61 (m, 2H), 4.05 (q, *J* = 7.1 Hz, 1H), 3.76 – 3.59 (m, 1H), 3.57 (m, 1H), 2.72 – 2.31 (m, 3H), 2.23 (dq, *J* = 13.3, 8.0 Hz, 2H), 2.01 (s, 1H), 1.97 (s, 1H), 1.93 (s, 1H), 1.90 – 1.70 (m, 3H), 1.64 – 1.00 (m, 8H), 0.80 (m, 6H).

GC-MS: *t_r* = 17.626 min, MS (EI) *m/z* = 180 (2), 162 (1), 136 (4), 91 (14), 69 (13), 53 (4).

5. Conclusions

This work aimed to understand the possibility of producing Lilybelle® and Calmusal® starting from (+)-*R*-Limonene (**2**) and Citral (**9**), respectively, using a synthesis pathway that could include biocatalytic steps as much as possible. We believe that the results achieved were quite promising since we were able to synthesize both. At the heart of the work there is the confirmation that OYEs are efficient and flexible enzymes able to hydrogenate a great number of compounds, including those under investigation. Thanks to this class of enzymes, we were able to analyze a wide spectrum of conditions that allowed us to identify the best ones to achieve our goal. In terms of conversion great values were achieved and thanks to the further optimization of the synthesis in flow condition, reaction times were kept low. As far as Calmusal® is concerned, some issues were encountered especially since the compound has a very low solubility in an aqueous medium. Despite this being a common issue among these kinds of reactions, the high flexibility of OYEs still allowed us to obtain the final product adjusting the recipes to our needs. Great attention was also paid to the possibility of performing this reaction in continuous mode. This is very important since this allowed us to increase considerably the efficiency of our process, providing precious information about a hypothetical future scalability of it. The focus on flow reactions was not limited only to the enzymatic ones, but also to heterogeneous catalytic ones. The use of a reactor like the one employed is an innovative and advanced way of synthesis investigation that can provide a great amount of information in a time-efficient way. Nevertheless, some points of

investigation are still open: a lot of work still needs to be done in the use of alcohol dehydrogenases with the substrate employed in this work. We were able to perform just a very preliminary screening with partial and not completely satisfactory results. A systematic study, both on the best enzymes and on the best reaction conditions, could be an interesting starting point for future research. Overall, we believe that the demonstrated possibility of producing complex, useful, and extremely costly compounds starting from cheap subproducts and through environmentally friendly procedures is an exciting reality. Still, a lot of work needs to be done to make this process able to fully compete with the traditional methods but many works, among which this represents an infinitesimal and marginal part, show how the future is promising.

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