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EXECUTIVE SUMMARY OF THE THESIS

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

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1. Introduction

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 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.

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)** [1].

The synthetic process that was investigated in this work is shown in Figure 1. 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 MnO_2. 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)**.

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 [2]. The proposed process involves adding two carbon atoms to citral with the Horner-Wadsworth-Emmons olefination, followed by reduction to alcohol and MnO_2 oxidation, to produce dehydrocalmusal, a mixture of (2*E*,5*E*,-6,10-dimethylundeca-2,5,9-trienal **(11a)** and (2*E*,5*Z)*-6,10-dimethylundeca-2,5,9-trienal **(11b)** (some traces of (2*Z*,5*E*)-6,10-dimethylundeca-2,5,9-trienal were detected), followed by an enzymatic step to convert **11** into Calmusal **(10)**.

3. Results and discussions

For the first step of the synthesis of inspiration was the work of Cannazza et al. [3], where it was shown that recombinant CYP153A6-*E. coli* cells were capable of regioselective hydroxylation of limonene and α-pinene. Since this methodology is well described in the literature and since it is possible to purchase (+)-perillyl aldehyde (**4**) at a relatively cheap price from the chemical market, we have decided, for reasons of time, to use most of our resources to optimize the experimental synthesis from the third step.

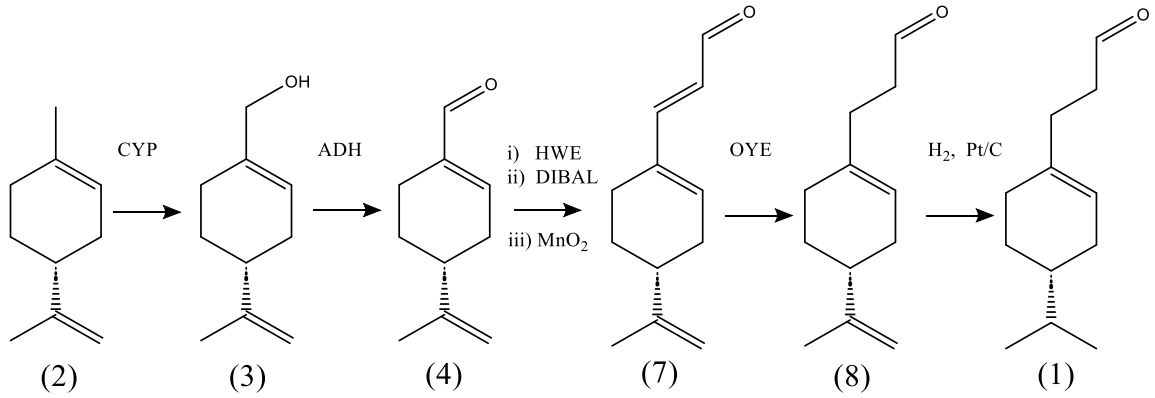

Figure 1 – Overall synthesis scheme of Lilybelle®

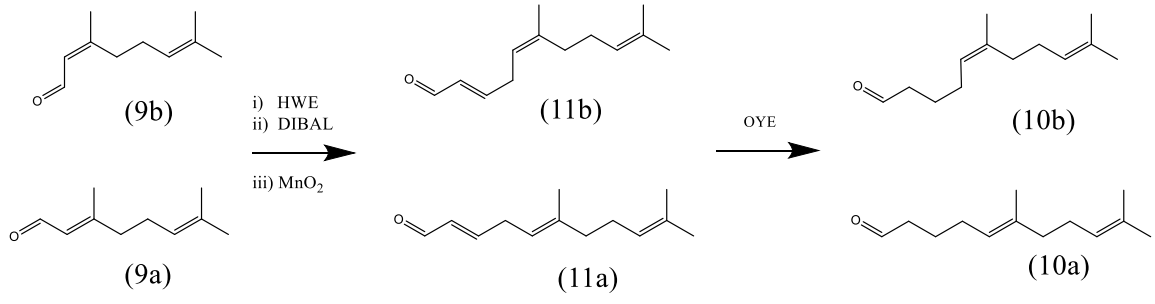

Figure 2 – Overall synthesis scheme of Calmusal®

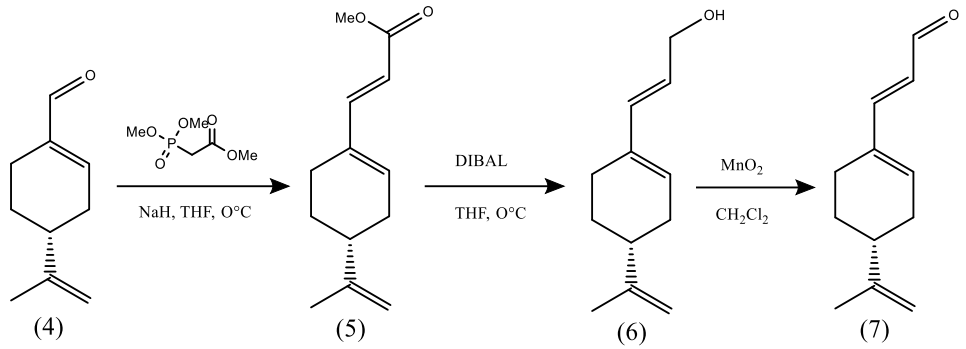

Figure 3 – Olefination reaction on perillaldehyde

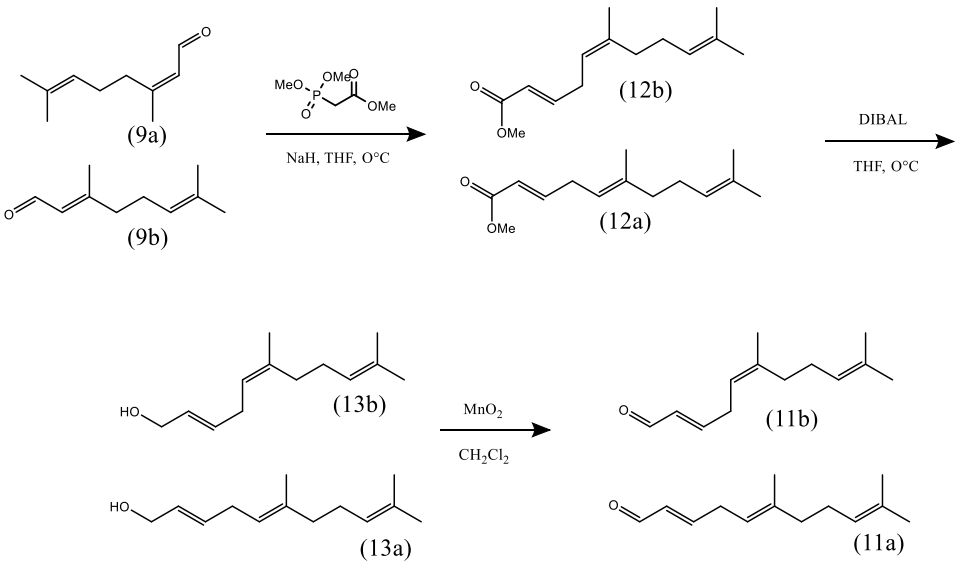

Figure 4 - Olefination reaction of citral

3.1 Horner Wadsworth Emmons

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. [4][5] This methodology was successfully applied to compounds **4** and **9** to obtain **5** and **12.**

The next step of the experiment consisted of reducing **5** and **12** to **6** and **13**. To achieve this, DIBAL was used as it can efficiently reduce α-β unsaturated esters to the corresponding allylic alcohols. [5] To complete the reaction path and obtain compound **7** and **11** MnO_2, which is a common oxidant used in organic synthesis, was employed with good results [6].

3.2 Hydrogenation through OYEs

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. To maximize the probability of achieving a good conversion, different OYEs were used. Overall OYE3 was identified as the best performing one as it was able to hydrogenate with good level of conversion both compounds **7** and **11.**

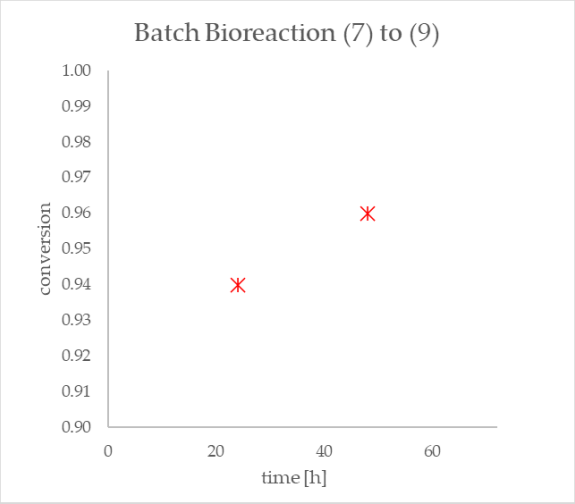

Figure 5a – Conversion results at different times on **7** with OYE3

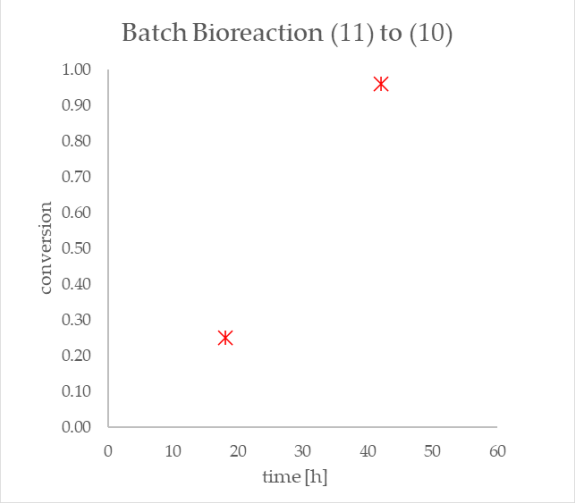

Figure 6b – Conversion results at different times on **11** with OYE3

Since we demonstrated that OYEs were able to successfully produce the desired products, we decided to try to optimize the reactions using a continuous flow coil reactor. A great deal of tests were done in order to find the best performing conditions, and overall, the results were promising, allowing us to obtain high values of conversion (over 95%) in much shorter times (less than 4 hours).

3.3 Continuous reactor Hydrogenation

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 [7], where a similar hydrogenation process was performed at the beginning of the synthesis on molecule **2**. 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.
To do so Design of Experiments (DoE) techniques were used testing the catalyst with different substrates in a great variety of condition. Thanks to the information gathered, we were able to build 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. After letting the reactor run were able to obtain Lilybelle® with 40% conversion.

4. 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.

5. Bibliography

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