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DOI

[10.3390/catal9060522](https://doi.org/10.3390/catal9060522)

Publication date

2019

Document Version

Final published version

Published in

Catalysts

Citation (APA)

Leemans, L., van Langen, L., Hollmann, F., & Schallmey, A. (2019). Bienzymatic cascade for the synthesis of an optically active O-benzoyl cyanohydrin. *Catalysts*, 9(6), Article 522.
<https://doi.org/10.3390/catal9060522>

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Article

Bienzymatic Cascade for the Synthesis of an Optically Active O-benzoyl Cyanohydrin

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Received: 14 May 2019; Accepted: 10 June 2019; Published: 12 June 2019



Abstract: A concurrent bienzymatic cascade for the synthesis of optically pure (S)-4-methoxymandelonitrile benzoate ((S)-3) starting from 4-anisaldehyde (1) has been developed. The cascade involves an enantioselective *Manihot esculenta* hydroxynitrile lyase-catalyzed hydrocyanation of 1, and the subsequent benzoylation of the resulting cyanohydrin (S)-2 catalyzed by *Candida antarctica* lipase A in organic solvent. To accomplish this new direct synthesis of the protected enantiopure cyanohydrin, both enzymes were immobilized and each biocatalytic step was studied separately in search for a window of compatibility. In addition, potential cross-interactions between the two reactions were identified. Optimization of the cascade resulted in 81% conversion of the aldehyde to the corresponding benzoyl cyanohydrin with 98% enantiomeric excess.

Keywords: Enantioselectivity; enzyme cascade; hydroxynitrile lyase; lipase; hydrocyanation; transesterification

1. Introduction

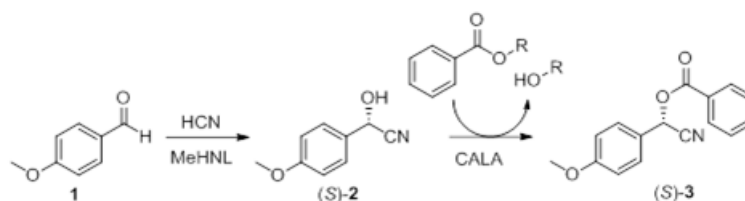
In the past decades, the field of biocatalysis has grown exponentially, becoming an industrially attractive technology for the production of chiral pharmaceutical intermediates and precursors in the synthesis of fine chemicals [1,2]. Recently, considerable progress has been made in developing enzymatic cascades for the production of valuable chemical products [3–6]. These are synthetic processes that combine two or more enzymes in one pot and can be performed in a concurrent (simultaneous) or sequential mode [7]. The combination of individual reaction steps in a one-pot process has several advantages in terms of process efficiency and sustainability, since the solvent consumption and waste generation is generally decreased due to the lower number of work-up steps [5]. Furthermore, the coupling of reactions has the potential to drive equilibria towards the desired products, hence reducing the required excess of reagents [8]. There are, however, some challenges associated with the design of enzymatic cascades, mainly due to enzymes often having different optimum conditions. Furthermore, the presence of side products of certain reactions may lead to enzyme inhibition, which could be avoided by running the reactions in a sequential mode [9,10].

A current challenge in the field of enzymatic cascades is the coupling of a hydroxynitrile lyase (HNL)-catalyzed cyanohydrin synthesis with an acylation catalyzed by a lipase. The immediate acylation of the formed cyanohydrin could prevent the back reaction from taking place and yield a chemically more stable product. In addition, if a lipase with appropriate enantioselectivity is chosen, the enantiopurity of the final product can be enhanced.

However, the one-pot combination of the two enzymes is not trivial, for they have very different requirements. It is well known that water acts as a competing nucleophile in lipase-catalyzed

transesterification reactions [11], whereas HNLs require relatively high water content when working in organic solvents [12–14]. Due to these different water content requirements, the cascade synthesis of acylated cyanohydrins catalyzed by an HNL and a lipase is a challenging task. Hanefeld et al. attempted to couple the hydrocyanation of benzaldehyde with the acetylation of the formed mandelonitrile, in order to shift the equilibrium of the first reaction. The reactions were catalyzed by *Hevea brasiliensis* hydroxynitrile lyase (HbHNL) and *Candida antarctica* lipase B (CALB), respectively. However, they encountered problems due to hydrolysis of the acyl donor and subsequent deactivation of the HbHNL [10]. As an alternative to the bienzymatic synthesis of acylated cyanohydrins, researchers have studied kinetic resolution of acylated cyanohydrins and dynamic kinetic resolution approaches combining an unselective hydrocyanation with an enantioselective transesterification [15–20]. Nevertheless, it has been shown that under certain conditions HNLs can work at low water content [21], opening the possibility for combining the two mentioned enantioselective enzymatic steps.

In the present study, we have selected the hydrocyanation of 4-anisaldehyde (**1**) combined with the benzoylation of the formed cyanohydrin (**2**) to yield 4-methoxymandelonitrile benzoate (**3**) as a model reaction (Scheme 1). The hydrocyanation of 4-anisaldehyde requires a particularly high excess of HCN to drive the equilibrium towards the cyanohydrin, compared to other benzaldehydes [22–24], which is attributed to the positive mesomeric effect of the *para*-methoxy substituent. The direct benzoylation of the cyanohydrin was envisioned as a possible solution to avoid isolating this instable product. The obtained cascade product **3** is interesting, as it can be transformed in a single hydrogenation step into (*S*)-tembamide [25], a naturally occurring *N*-(β -hydroxy) amide for which antiviral (HIV) and hypoglycemic activity have been reported [26,27].



Scheme 1. Proposed cascade synthesis of (*S*)-4-methoxymandelonitrile benzoate.

Herein, *Manihot esculenta* hydroxynitrile lyase (MeHNL)—an *S*-selective enzyme [28] that can catalyze the hydrocyanation of 4-anisaldehyde—was studied. For the benzoylation of the resulting cyanohydrin, *Candida antarctica* lipase A (CALA) was chosen, which is known to accept bulky substrates and displayed preference for (*S*)-4-methoxymandelonitrile benzoate in a prior hydrolase screening (see supporting information Table S1 and Figure S1). Moreover, CALA is a robust industrial enzyme, and was shown to exhibit acyltransferase activity even in aqueous media [29], which we deemed an attractive feature in our envisioned cascade.

2. Results and Discussion

A crucial step in the development of the intended cascade was the identification of a suitable benzoyl donor that would facilitate the CALA-catalyzed transesterification. Several donors were screened in the CALA-catalyzed benzoylation of 4-methoxymandelonitrile (see Figure S2). Of the screened esters, phenyl benzoate afforded the highest reaction rate.

For a successful combination of MeHNL and CALA in the cascade synthesis of (*S*)-4-methoxymandelonitrile benzoate, the enzymatic hydrocyanation and transesterification reactions were subjected to a systematic study of the relevant reaction variables. The effects of temperature and water activity on both reactions were evaluated and a suitable carrier was selected for the immobilization of each enzyme. In a preliminary solvent screening, isopropyl ether was selected as reaction solvent due to its compatibility with both enzymes and the relatively high solubility of the substrates.

2.1. Influence of Reaction Temperature

In general, temperature affects enzymatic stability and has a great effect on the reaction rate of enzyme-catalyzed reactions and their enantioselectivity. The influence of temperature on the enantioselectivity of enzymatic reactions can be explained by a simple theoretical model [30]. Depending on whether the reaction takes place below or above the so-called racemic temperature, a decrease in temperature will increase or decrease the enantioselectivity, respectively.

According to literature, *MeHNL* shows optimal hydrocyanation activity at ~40 °C [31]. A reduction in reaction temperature below 40 °C would decrease enzymatic activity, but is expected to slightly increase its enantioselectivity, since *MeHNL* displays an elevated racemic temperature of 580 °C in the hydrocyanation of phenylpropionaldehyde in dibutylether [14]. Furthermore, decreasing the reaction temperature should reduce the rate of the nonenzymatic hydrocyanation of 4-anisaldehyde to a higher extent than the enzymatic reaction, which would also contribute to increase the enantiomeric excess (e.e.) of the cyanohydrin [32–34]. Finally, the reaction must be performed at a rather low temperature, since the boiling point of HCN is 25.6 °C [35]. Based on this information, hydrocyanation reactions were performed at 5, 10, and 20 °C.

Within the studied temperature range, the hydrocyanation rate increased moderately with the reaction temperature (see Figure 1). On the other hand, the enantioselectivity of the enzymatic reaction slightly increased at lower temperatures, as expected. These results suggest that the optimal reaction temperature, which would afford excellent enantioselectivity with a satisfactory reaction rate, would be 10 °C.

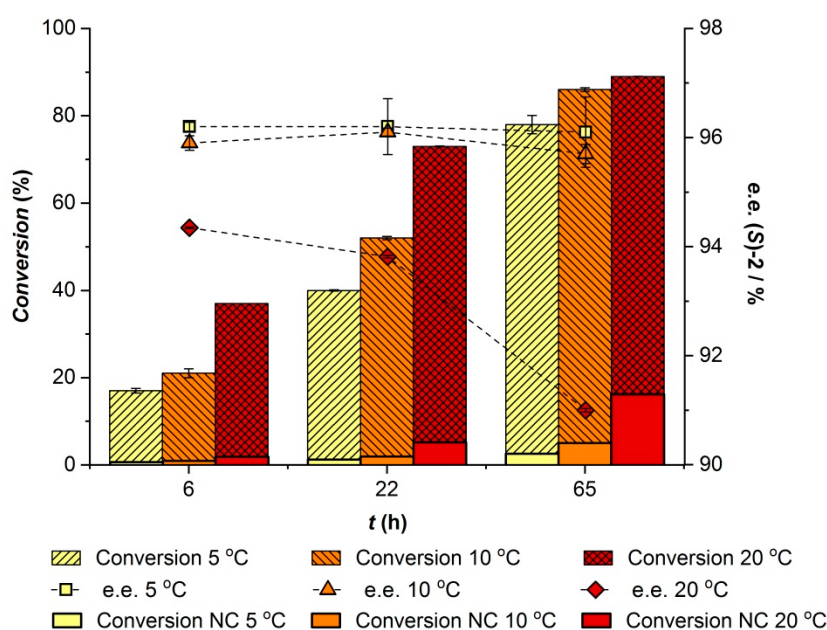


Figure 1. Effect of reaction temperature on the *MeHNL*-catalyzed hydrocyanation of 100 mM 4-anisaldehyde in a biphasic system using 6.5 equivalents of HCN. Negative control (NC) shows the rate of the unselective background reaction.

In the case of CALA, based on the data from kinetic resolution studies, the lipase was expected to show increased enantioselectivity with lower temperatures [36,37]. However, immobilized CALA has been reported to display a temperature optimum of 90 °C [38], thus the conversion will be limited by low reaction rates at low temperatures.

The effect of reaction temperature on the CALA-catalyzed benzoylation of racemic 4-methoxymandelonitrile was studied in isopropyl ether without added water in the range of 5 to 25 °C. The results showed that, when reducing the temperature, the enantioselectivity of CALA was slightly enhanced (E value of 7 at 5 and 10 °C and 6 at 25 °C), although this was at the expense

of a pronounced reduction of reaction rate, as shown in Figure 2. Within the studied temperature range, the CALA-catalyzed benzoylation of 4-methoxymandelonitrile can be achieved in relatively high yields with optimal performance at 25 °C. Although CALA shows preference for conversion of the (*S*)-enantiomer, it is not selective enough to afford an enantiopure product. However, when preceded by an enantioselective hydrocyanation, the transesterification reaction can enhance the enantiopurity of the final product. Therefore, the observed minor effect of reaction temperature on the enantiomeric ratio can be neglected.

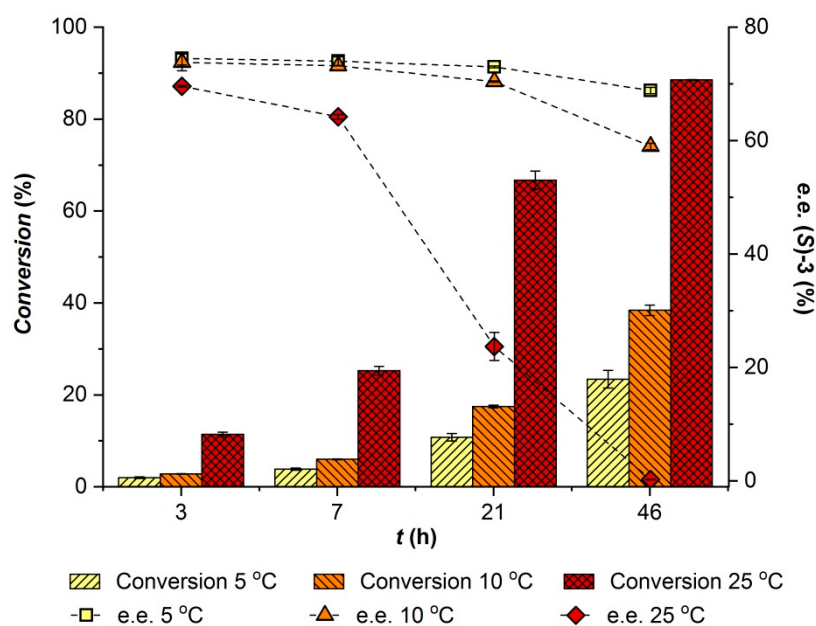


Figure 2. Effect of temperature on CALA-catalyzed benzoylation of 65 mM (±)-4-methoxymandelonitrile using 200 mM phenyl benzoate.

2.2. Selection of Enzyme Carriers

In our designed cascade, we envisioned a low-water organic solvent system for which the immobilization of both enzymes would be required. In organic solvents, free lipases generally express low catalytic activity and they tend to form aggregates which can lead to mass transfer limitations [39]. Adsorption is believed to enhance the catalytic activity of lipases due to hydrophobic interactions between the support and part of the enzyme yielding the active enzyme conformation by opening the lid [40]. Similarly, immobilization of hydroxynitrile lyases can improve their activity and stability towards organic solvents [41], and may lead to higher enantioselectivity [42]. In this study, CALA was adsorbed on Relizyme EXE309, a macroporous bead based on a methyl methacrylate/styrene copolymer that was recommended by the manufacturer for effective lipase adsorption. For MeHNL, adsorption on Celite R-633 was identified as the best immobilization method (see supporting information Table S2).

2.3. Influence of Water Activity

When working with enzymes in organic solvents, their activity is greatly affected by the water content in the reaction medium. This variable is best quantified in terms of thermodynamic water activity (a_w), which is equal in all phases in equilibrium [43]. Water is believed to act as a molecular lubricant, increasing the conformational flexibility of enzymes and, thus, their catalytic activity [44]. The water content also affects enzyme selectivity in a complex way, which involves interactions with the reaction medium and the substrates [14].

In the case of HNLs it has been shown that when working at low water activities, the enzymes are insufficiently hydrated, resulting in activity and selectivity loss [12–14]. Lipases, on the other hand, can generally withstand lower water content [45], and some studies have shown that a_w does

not significantly influence the enantioselectivity of lipases [14,45–47]. Furthermore, water competes with the substrate alcohol (in this case, cyanohydrin) for the nucleophilic attack of the acyl-enzyme intermediate, leading to ester hydrolysis [48].

A prerequisite for the combination of both enzymes in a concurrent mode is to find a suitable a_w range that allows for sufficient *MeHNL* activity and high selectivity, while minimizing ester hydrolysis catalyzed by CALA. In order to evaluate the a_w effect on both enzymatic reactions, a salt hydrate system that could cover a wide range of a_w values was selected: $\text{Na}_2\text{HPO}_4/\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}/\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}/\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. These salt pairs afford water activity values of 0.15, 0.57 and 0.74, respectively, at 20 °C [43]. The hydrocyanation and benzylation reactions were studied independently using immobilized *MeHNL* and CALA and the three salt pairs.

Before evaluating the influence of a_w on *MeHNL*, the effect of the phosphate salt hydrate pairs on the background hydrocyanation reaction was studied. The chemical hydrocyanation of 100 mM 4-anisaldehyde in isopropyl ether was performed using 6.5 equivalents of HCN and 0.5 mmol salt pair per mL of reaction. This revealed that the $\text{Na}_2\text{HPO}_4/\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ pair could convert 32% of the aldehyde in 24 h, whereas the other two salt pairs afforded a much lower chemical background reaction rate with only 4% conversion after 24 h. The negative control, where no salt was added, showed 1% conversion.

The effect of the three water activity values provided by the phosphate salts on the *MeHNL*-catalyzed hydrocyanation of 4-anisaldehyde was then evaluated (see Figure 3). Results at $a_w = 0.15$ are difficult to interpret due to the above-mentioned catalytic effect of the salt. It seems, however, that the enzyme is hardly active at this low water activity. Comparison of the results at the two highest water activity values, where the background reaction is similarly low, shows that the activity and selectivity of *MeHNL* increased with increasing a_w . Hence, to ensure a high e.e. of the resulting cyanohydrin, it is necessary to work at a high a_w for the combination of *MeHNL* and CALA.

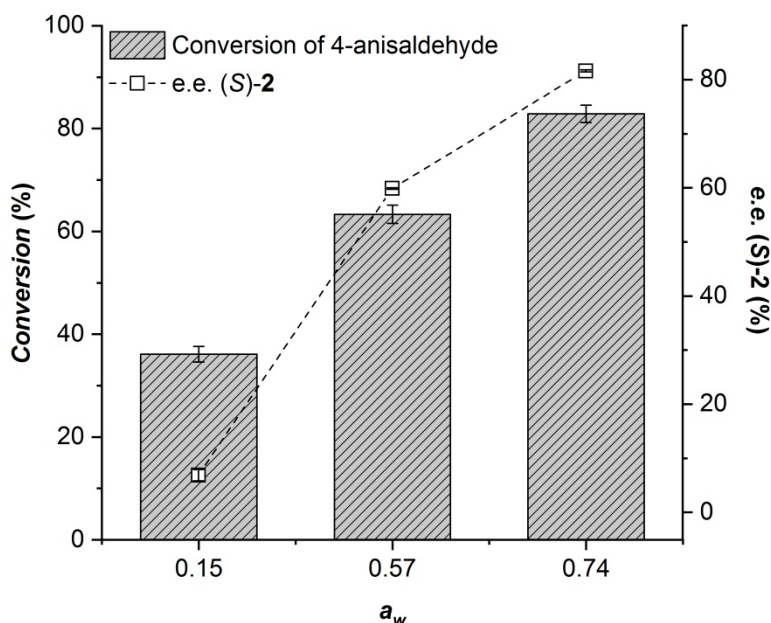


Figure 3. Effect of water activity on the *MeHNL*-catalyzed hydrocyanation of 90 mM 4-anisaldehyde using 6.5 equivalents of HCN. Conversion and enantiomeric excess (e.e.) values taken after 23 h of reaction.

In the case of CALA, at $a_w = 0.57$ and below, no significant difference in the enantioselectivity of the enzyme ($E = 5$) or in the selectivity for transesterification over hydrolysis was observed (see Figure 4). When working at $a_w = 0.74$, however, the hydrolysis rate increased significantly at the expense of the transesterification reaction. Although the enantioselectivity also increased under these

conditions ($E = 9$), it is not a sufficient improvement to compensate for the increased hydrolysis rate. Considering the observed effects of water activity on *MeHNL* and *CALA*, we concluded that it should be possible to combine both enzymatic reactions at $a_w = 0.57$.

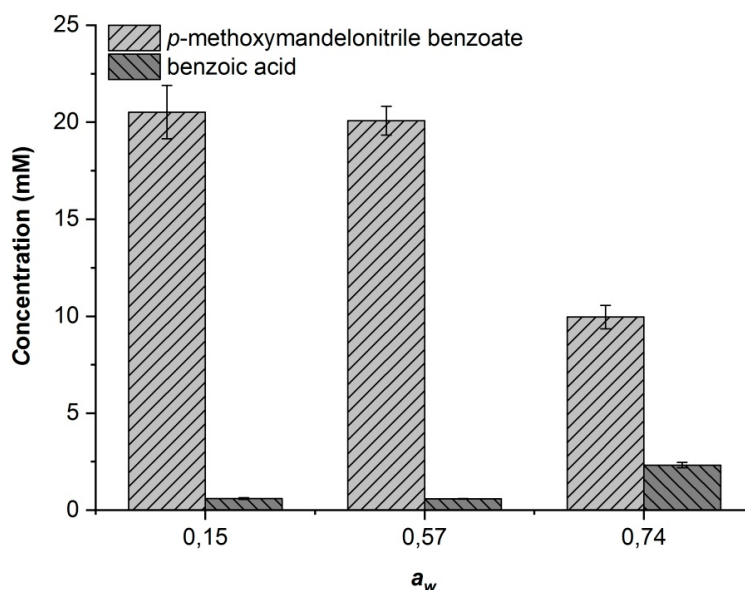


Figure 4. Effect of the different phosphate hydrate pairs on the *CALA*-catalyzed benzoylation of 66 mM ($\pm$)-4-methoxymandelonitrile using 200 mM vinyl benzoate after 22 h of reaction.

2.4. Cross Interactions

When performing reactions in one-pot, the reagents, products, or byproducts of one reaction may affect the other reactions. Thus, for an optimized cascade, the effect of hydrocyanation reagents on the *CALA*-catalyzed transesterification reaction as well as the influence of the benzoyl donor and the transesterification byproduct on the *MeHNL*-catalyzed hydrocyanation reaction was evaluated. Further, the influence of the side product benzoic acid on both transesterification and hydrocyanation reactions was studied.

The influence of 4-anisaldehyde and HCN on the reaction rate and selectivity of the *CALA*-catalyzed benzoylation of 4-methoxymandelonitrile was evaluated. As shown in Figure 5A, both 4-anisaldehyde and HCN exert a moderate negative effect on the benzoylation rate. Moreover, both compounds reduce the selectivity of *CALA* towards transesterification (see Figure 5B). Regarding the enantioselectivity of the benzoylation reaction, it was not significantly affected by the presence of either of the hydrocyanation substrates, with the E values varying between 5 and 6. Similarly, the effect of benzoic acid on *CALA* was studied in a benzoylation reaction of 70 mM ($\pm$)-4-methoxymandelonitrile containing one equivalent of benzoic acid. The reaction rate was 20% slower than that of the control.

The influence of phenyl benzoate and its leaving group, phenol, on the *MeHNL*-catalyzed hydrocyanation of 4-methoxymandelonitrile was studied. Addition of either three equivalents of phenyl benzoate or phenol to the hydrocyanation reaction afforded (*S*)-4-methoxymandelonitrile with the same yield and enantiopurity as the control reaction (data not shown). To evaluate the effect of the side product benzoic acid on the hydrocyanation reaction, an experiment was performed with addition of different amounts of the acid to the reaction mixture. As shown in Figure 6, benzoic acid exerts a strong negative effect on the hydrocyanation rate of *MeHNL*. Thus, in order to achieve a cascade synthesis of **3** with high yield and selectivity, the *CALA*-catalyzed hydrolysis of phenyl benzoate and **3** should be minimized as much as possible. This observation is not surprising, since benzoic acid has previously been reported as a competitive inhibitor for other hydroxynitrile lyases [49–51]. Based on these results, the observed negative effect of benzoic acid on *CALA* is minor when compared to its effect on *MeHNL*.

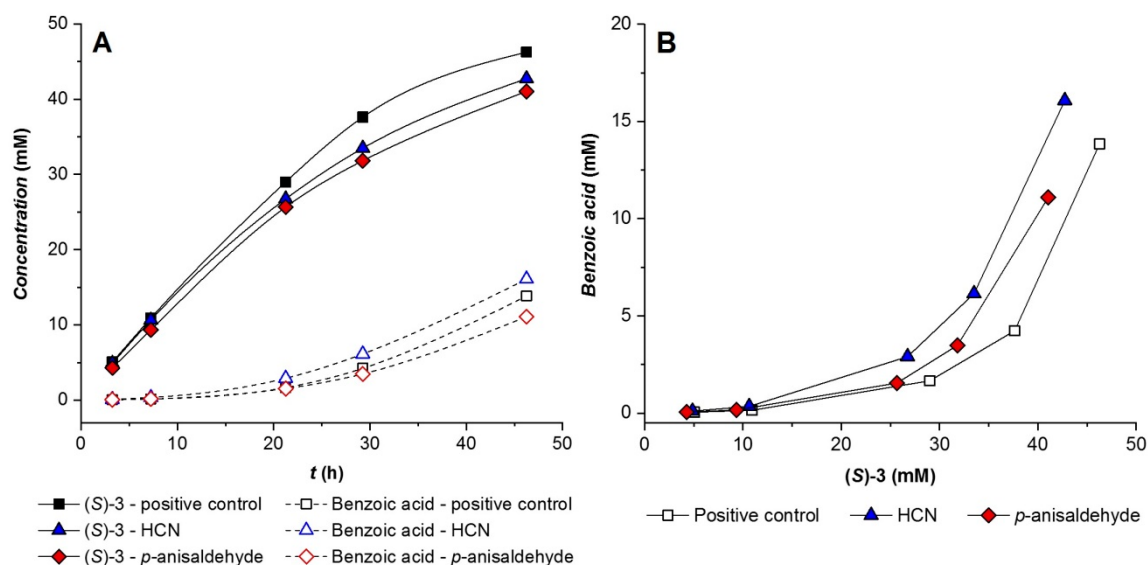


Figure 5. Effect of 60 mM 4-anisaldehyde and 209 mM HCN on the transesterification and hydrolysis rate of CALA (A) as well as its selectivity towards transesterification over hydrolysis (B) using 65 mM ($\pm$)-4-methoxymandelonitrile and 200 mM phenyl benzoate.

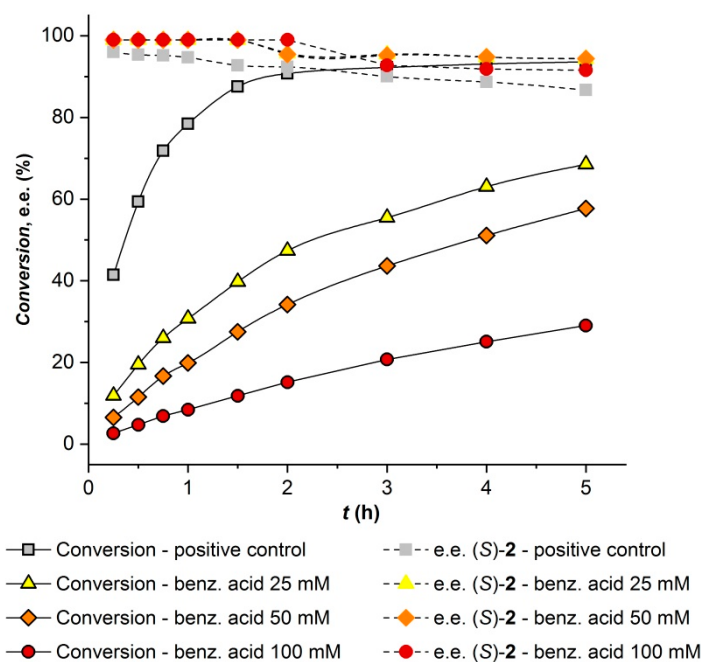


Figure 6. Effect of 25 mM, 50 mM, or 100 mM benzoic acid on MeHNL-catalyzed hydrocyanation of 100 mM 4-anisaldehyde using 6.5 equivalents of HCN. With exception of the positive control, the enantiomeric excess values overlap at low reaction times.

2.5. Cascade Synthesis of (S)-4-Methoxymandelonitrile Benzoate

For the first cascade synthesis test (see Table 1, entry 1), the used reaction conditions in terms of enzyme amount, temperature, water buffering salts and the time point of CALA addition were selected based on the results obtained before. The reaction temperature was set at 10 °C, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}/\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ was added to set the water activity to $a_w = 0.57$, and the lipase was added after 16 h of hydrocyanation reaction with the idea to minimize the exposure of MeHNL to benzoic acid. In this first reaction, a low reaction rate of the overall process was observed, resulting in only 10.5% conversion to the corresponding ester with a fair e.e. of 89% after 47 h reaction time.

The results, however, showed that both enzymes were simultaneously active after adding the lipase to the reaction mixture, since the 4-anisaldehyde was further converted and (S)-4-methoxymandelonitrile benzoate was also formed. The same process was repeated at 25 °C (Table 1, entry 2) resulting in a significantly improved reaction rate, while maintaining the enantiomeric excess of the ester. Based on these observations, all further reactions were performed at room temperature (20 °C).

For comparison, in experiment 3 (Table 1, entry 3) a concurrent approach, where both enzymes were added from the beginning, was tested. Due to the inhibitory effect of 4-anisaldehyde and HCN on the acyltransferase activity of CALA, a higher amount of lipase was added. This approach significantly improved the enantiopurity of the final ester (97% e.e.), while maintaining a good reaction rate. After 30.5 h of reaction, 10 mM cyanohydrin was still present in the reaction mixture. This suggested that the transesterification rate was not too high in comparison to the *MeHNL*-catalyzed hydrocyanation. Therefore, it was decided to maintain the CALA/*MeHNL* ratio for further reactions. Strikingly, the results could be further improved by omitting the water buffering salts from the system (Table 1, entry 4). Elimination of these salts provided overall higher yield and selectivity, which may be explained by the previously identified negative effect of low water activity on the enzymatic hydrocyanation reaction. Moreover, comparison of the benzoic acid concentration in experiments 3 and 4 indicated that in the former cascade (including the salts) the hydrolysis reaction was not suppressed to a higher extent than in the latter (without salts). Thus, the use of the salt hydrate pair did not further reduce the hydrolysis reaction in the studied system.

Salt-free experiments with varying initial amounts of benzoyl donor (Table 1, entries 4–6) showed a detrimental effect of higher donor concentrations on the hydrocyanation reaction, while the amount of hydrolysis side product, benzoic acid, increased substantially. In all cases, however, excellent enantiopurity of (S)-4-methoxymandelonitrile benzoate (99% e.e.) was maintained throughout the process. These results prompted us to test gradual addition of phenyl benzoate to the process, which was found to minimize the hydrolytic side reaction at the expense of some loss in reaction rate (see Table 1, entry 7), leading to 64% yield of ester 3 (98% e.e.) in 49 h, with 50% less benzoic acid produced compared to Table 1, entry 5. Finally, we hypothesized that a reduction of the lipase units could further decrease the amount of formed benzoic acid. Using 0.5 U/ml CALA afforded ester 3 (98% e.e.) with 81% yield in 122 h, with a total conversion of 4-anisaldehyde of 95% (see Table 1, entry 8 and Figure S3). The reaction time required for high conversion values could be reduced by approximately one day when the reaction temperature was increased from 20 to 25 °C (see Table 1, entry 10).

Furthermore, we also tested the possibility of decreasing the amount of HCN used in the cascade. However, when performing the cascade using 5 instead of 6.5 equivalents of HCN (using the same conditions detailed in Table 1, entry 7), after 122 h only 60 mM (S)-3 was produced with a poor enantiomeric excess of 87%. Similarly, a decrease in yield and enantiomeric excess of (S)-2 was observed in the immobilized *MeHNL*-catalyzed hydrocyanation of 4-anisaldehyde when decreasing the HCN concentration (see Figure S4).

Overall, our results demonstrate that the concurrent combination of a *MeHNL*-catalyzed hydrocyanation with a CALA-catalyzed transesterification is possible after careful investigation of the individual reactions in search for compatible conditions. However, further optimization is required to improve the cascade, since 5% residual 4-anisaldehyde could not be converted by *MeHNL* (see Table 1, entry 8 and Figure S4A). Presumably, this is due to an observed increase in benzoic acid concentration as the cascade proceeds. Moreover, roughly 11 mM cyanohydrin 2 remained in the reaction media, which could not be further converted to 3 by CALA. This indicates a rather low affinity of the lipase for 4-methoxymandelonitrile, which, at low concentrations, cannot compete with the water present in the reaction medium for the acyl-enzyme complex. Hence, hydrolysis is the main reaction taking place in the end, further increasing the amount of benzoic acid.

The use of an engineered CALA with enhanced selectivity towards transesterification reactions versus hydrolysis could significantly improve the performance of our designed cascade [29].

Table 1. Cascade synthesis of (S)-4-methoxymandelonitrile benzoate catalyzed by immobilized MeHNL and CALA starting from 100 mM 4-anisaldehyde and 650 mM HCN using 200–400 mM phenyl benzoate. Reactions were performed in duplicate and relative standard deviations were generally below 2%.

Exp.	T (°C)	Equiv. Phenyl Benzoate	MeHNL (U/mL) ¹	CALA (U/mL) ²	Time (h)	[1] (mM)	[2] (mM)	e.e. (S)-2 (%)	[3] (mM)	e.e. (S)-3 (%)	[benzoic acid] (mM)
1 ³	10	3	0.21	0.50 ⁴	16 47	90 82	10 7	58 12	- 11	- 89	- ND
2 ³	25	3	0.21	0.50 ⁵	15 24	75 59	25 11	63 15	- 30	- 90	- ND
3 ³	20	2	0.26	2.20	31	47	10	70	43	97	64
4	20	2	0.26	2.20	31 73	23 15	21 23	92 86	56 62	99 98	57 111
5	20	3	0.26	2.20	31	33	8	76	59	99	109
6	20	4	0.26	2.20	31	45	4	68	51	99	140
7	20	3 ⁶	0.26	2.20	49 122	18 15	19 13	74 79	64 72	98 93	56 210
8	20	3 ⁶	0.26	0.50	122 164	5 4	14 11	60 61	81 85	98 96	50 94
9	20	0	0.26	0	24	15	85	91	-	-	-
10	25	3 ³	0.26	0.50	94	7	13	46	80	97	39

¹ Activity of immobilized MeHNL: 0.03 U/mg. ² Activity of immobilized CALA: 0.11 U/mg. ³ Na₂HPO₄·2H₂O/Na₂HPO₄·7H₂O was added (0.34 mmol per mL of reaction). ⁴ CALA was added after 16 h. ⁵ CALA was added after 15 h. ⁶ Gradual addition: Each 1 equivalent of phenyl benzoate was added at the beginning, after 24 h, and after 48 h.

3. Materials and Methods

3.1. Enzymes

Hydroxynitrile lyase from *Manihot esculenta* was heterologously expressed in *E. coli* K12 Top 10F' harboring the pSE420-MeHNL plasmid, which was kindly provided by Dr. Kerstin Steiner from the Austrian Centre of Industrial Biotechnology (ACIB). 2 × YT medium (100 mL, containing 100 µg/mL ampicillin) was inoculated from a glycerol stock (10 µL) and the culture was incubated overnight at 37 °C, 200 rpm. A 5 mL aliquot of this preculture (OD₆₀₀ = 4.4) was used to inoculate 2 × YT medium (500 mL, containing 100 µg/mL ampicillin), and the new culture was grown at 37 °C, 200 rpm. At an optical density at 600 nm (OD₆₀₀) of 0.8, IPTG was added to a final concentration of 0.1 mM and the culture was further incubated at 18 °C, 160 rpm for 48 h. Cells were harvested by centrifugation at 4000 g, 4 °C for 10 min, washed with 25 mM sodium acetate buffer, pH 5.8, and centrifuged again (4000 g, 4 °C for 30 min). Cell pellets were resuspended in 25 mM sodium acetate, pH 5.8 (4 mL per g of wet cells), and 10 mL aliquots were sonicated at 4 °C using a Fisher Scientific Model 120 Sonic Dismembrator at 60% amplitude, 2 s pulse, 5 s pause for 7 min. The resulting crude extract was centrifuged to remove cell debris (18,500 g, 4 °C for 45 min) and the obtained cell free extract was stored at −20 °C. Concentration of cell free extract was performed in an Amicon Ultra Filter Device with 10 kDa cut-off at 4 °C.

Candida antarctica lipase A (Novozym®CALA L, LDN 00025) was purchased from Novozymes.

3.1.1. Enzyme Activity Measurements

The enzymatic activity of free MeHNL was measured following the cleavage of racemic mandelonitrile into benzaldehyde and HCN, according to a literature procedure [52]. Samples were prepared by diluting the MeHNL cell free extract in 5 mM phosphate buffer, pH 6.5. The enzymatic activity of immobilized MeHNL was determined following the hydrocyanation of 4-anisaldehyde (100 mM) using 6.5 equivalents of HCN in isopropyl ether at 20 °C, 800 rpm. A negative control was performed using the enzyme support instead. Samples were analyzed by chiral HPLC.

The enzymatic activity of CALA was measured according to a modified literature procedure following the hydrolysis of tributyrin in phosphate buffer at pH 7 [53]. The resulting butyric acid was titrated with 0.1 M sodium hydroxide, and the consumption of the latter was recorded as a function of time. The enzymatic activity of immobilized CALA was determined following the hydrolysis of 4-nitrophenyl butyrate (1.67 mM) in acetonitrile/50 mM phosphate pH 7.4 (1/5). A calibration curve of the product 4-nitrophenol was used to calculate the reaction rate based on the absorbance of the reaction mixture at 410 nm.

3.1.2. Enzyme Immobilization

General procedure for MeHNL: In a 5 mL glass vial containing a magnetic stirring bar and the carrier (100 mg), MeHNL-containing cell-free extract (40 µL) was added dropwise while stirring. The mixture was stirred for 30 min and was then dried at room temperature under high vacuum for 48 h.

Immobilization of CALA: CALA (30 g), Relizyme EXE309 (30 g), and demineralized water (30 mL) were mixed in a 100 mL bottle. The pH was adjusted to 6 with a solution of 2 M sodium hydroxide and the mixture was incubated at room temperature in an orbital shaker overnight. After filtering the solution, the activity of the supernatant was measured and compared to the initial activity of the enzyme solution before immobilization, giving an immobilization yield of 84%. The immobilized enzyme was washed several times with demineralized water and dried at room temperature under high vacuum for 48 h.

3.1.3. Evaluation of the MeHNL Immobilisates

To 1.5 mL glass vials containing different MeHNL immobilisates (15 mg) and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (30 mg, 0.17 mmol), each a solution of 100 mM 4-anisaldehyde and 650 mM HCN in isopropyl ether (500 μL) was added. Duplicate reactions per immobilisate were incubated at 20 °C in an orbital shaker at 800 rpm. Samples (20 μL) were analyzed by HPLC.

3.2. Chemicals

Unless otherwise indicated, reagents and organic solvents were purchased from Fisher Scientific, Sigma-Aldrich, TCI Chemicals and Acros Organics, and were of the highest available purity.

Celite R633 was a gift from Imerys S.A., Relizyme EXE309 was kindly provided by Resindion S.r.l., and SYLOID silicas were a gift from Grace Davison, Inc. ($\pm$)-4-Methoxymandelonitrile was synthesized via chemical hydrocyanation of 4-anisaldehyde following a literature procedure [22].

For the preparation of hydrogen cyanide solution, potassium cyanide (26 g, 0.4 mol) was dissolved in a stirred mixture of distilled water (200 mL) and diisopropyl ether ($^i\text{Pr}_2\text{O}$) (100 mL). The solution was cooled in an ice-water bath and 1.33 M citric acid (100 mL, 0.13 mol, 1 equivalent) was slowly added. The aqueous layer was extracted twice with 50 mL of $^i\text{Pr}_2\text{O}$. The combined organic phases were stored in a dark bottle containing 1 M citrate buffer pH 5.5 (10 mL). The HCN concentration was determined by measuring the absorption of the $[\text{Ni}(\text{CN})_4]^{2-}$ complex ion at 267 nm. HCN solution (20 μL) was added to 3.5 mM $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ in 1 M NH_3 (4 mL) [54]. After vigorous mixing, the solution was further diluted 50 times with 1 M NH_3 in a cuvette. The absorption was measured and the resulting HCN concentration was calculated based on a calibration curve.

Synthesis of ($\pm$)-4-Methoxymandelonitrile (2)

In a 100 mL round-bottom flask, NaCN (4.4 g, 90 mmol) was dissolved in water (30 mL) and NaHSO_3 (9.4 g, 90 mmol) was slowly added. 4-Anisaldehyde (2.4 g, 18 mmol) was dissolved in ethyl acetate (20 mL) and added to the reaction mixture. After 1 h of vigorous stirring, the flask was introduced in an ice-water bath and stirred for another 6 h. The reaction was followed by HPLC until it reached plateau after 90% conversion. Distilled water (30 mL) was added to dissolve the salts and the aqueous phase was extracted ethyl acetate (2×10 mL). The combined organic phase was washed with brine, dried over anhydrous MgSO_4 , and concentrated on a rotary evaporator using a 10 °C bath, whereupon crystallization occurred. The crystals were washed with cold heptane/ethyl acetate (2:1) and stored at 5 °C. HPLC analysis showed that the ($\pm$)-4-methoxymandelonitrile crystals contained 3% 4-anisaldehyde impurity.

3.3. Bioconversions

All bioconversions were performed in 1.5 mL glass vials in an orbital shaker at 800 rpm. Unless otherwise stated, the reaction temperature was set at 20 °C.

3.3.1. Benzoyl Donor Screening

To a vial containing immobilized CALA (4.2 mg, 0.46 U), racemic 4-methoxymandelonitrile (200 mM) in isopropyl ether (250 μL) and benzoyl donor (300 mM) in isopropyl ether (500 μL) was added. Reactions were performed in duplicate and were incubated at 25 °C. Samples (20 μL) were taken after 4, 20, and 27.5 h and analyzed by HPLC. The enantioselectivity was calculated according to the method of Chen et al. for conversion values between 5 and 20% [55].

3.3.2. Temperature Effect on MeHNL

4-Anisaldehyde (530 mM) in isopropyl ether (188 μL), HCN (800 mM) in isopropyl ether (812 μL), citrate buffer pH 5 (500 mM, 125 μL), and MeHNL-containing cell-free extract (33.2 μL , 5 U) were incubated at 5, 10, and 20 °C. Reactions were performed in duplicate and a negative control reaction

was performed for each temperature value, where no enzyme was added. Samples (20 μ L) were taken from the upper organic phase after 6, 22, and 75 h and analyzed by HPLC.

3.3.3. Temperature Effect on CALA

To a vial containing CALA (2 mg, 0.22 U), ($\pm$)-4-methoxymandelonitrile (195 mM) in isopropyl ether (125 μ L) and phenyl benzoate (300 mM) in isopropyl ether (250 μ L) was added. The mixture was incubated at 5, 10, and 25 $^{\circ}$ C. Reactions were performed in duplicate. Samples (20 μ L) were taken after 3, 7, 21, and 46 h and analyzed by HPLC.

3.3.4. Effect of Na_2HPO_4 Salt Hydrates on Chemical Hydrocyanation

To a solution of 4-anisaldehyde (100 mM) and HCN (650 mM) in isopropyl ether (1 mL), anhydrous Na_2HPO_4 (70 mg, 0.5 mmol), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (85 mg, 0.5 mmol), or $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (134 mg, 0.5 mmol) was added. Reactions were performed in duplicate and a control reaction without salt was run in parallel. The amount of salt added is based on the maximum solubility of water in isopropyl ether (0.55 wt%) [56] and the water capacity of the $\text{Na}_2\text{HPO}_4/\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ salt pair (11.2 mmol/g) [57]. Reactions were stopped after 24 h and analyzed by HPLC.

3.3.5. Effect of a_w on MeHNL

To a vial containing MeHNL immobilized on Celite R633 (12 mg, 0.36 U), a solution of 4-anisaldehyde (90 mM) and HCN (590 mM) in isopropyl ether (550 μ L) was added, together with either anhydrous Na_2HPO_4 (35 mg, 0.25 mmol), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (42 mg, 0.25 mmol) or $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (67 mg, 0.25 mmol). Reactions were performed in duplicate. Samples (20 μ L) were taken after 5 and 24 h and analyzed by HPLC.

3.3.6. Effect of a_w on CALA

To a vial containing CALA immobilized on Relizyme EXE309 (4 mg, 0.44 U), a solution of ($\pm$)-4-methoxymandelonitrile (100 mM) and vinyl benzoate (300 mM) in isopropyl ether (500 μ L) was added, together with either anhydrous Na_2HPO_4 (35 mg, 0.25 mmol), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (42 mg, 0.25 mmol), or $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (67 mg, 0.25 mmol). Samples (20 μ L) were taken after 6, 22, and 30 h and analyzed by HPLC.

3.3.7. Effect of Phenol and Phenyl Benzoate on MeHNL

To a solution of 4-anisaldehyde (100 mM) and HCN (600 mM) in isopropyl ether (670 μ L), MeHNL on Celite R633 (8 mg, 0.24 U) and either phenol (18.8 mg, 200 μ mol) or phenyl benzoate (39.6 mg, 200 μ mol) were added. A control was performed without addition of phenol or phenyl benzoate. Samples (20 μ L) were taken after 17 and 22 h and analyzed by HPLC.

3.3.8. Effect of 4-Anisaldehyde and HCN on CALA

To a solution of ($\pm$)-4-methoxymandelonitrile (65 mM) and phenyl benzoate (200 mM) in isopropyl ether (375 μ L), CALA on Relizyme EXE309 (2 mg, 0.22 U) and either 4-anisaldehyde (200 mM) in isopropyl ether (160 μ L) or HCN (700 mM) in isopropyl ether (160 μ L) were added. Reactions were performed in duplicate and a control was performed adding isopropyl ether (160 μ L) instead of the hydrocyanation reagents. The reactions were incubated at 25 $^{\circ}$ C. Samples (20 μ L) were taken after 3, 7, 21, 29, and 46 h and analyzed by HPLC.

3.3.9. Effect of Benzoic Acid on MeHNL

To a vial containing MeHNL on Celite R633 (15 mg, 0.45 U) and benzoic acid (1.5 mg, 12 μ mol; 3 mg, 25 μ mol; or 6 mg, 50 μ mol), a solution of 4-anisaldehyde (100 mM), HCN (650 mM), and mesitylene

(100 mM) (internal standard) in isopropyl ether (500 μ L) was added. A control was performed without addition of benzoic acid. Samples (20 μ L) were analyzed by HPLC.

3.3.10. Effect of Benzoic Acid on CALA

To a solution of ($\pm$)-4-methoxymandelonitrile (100 mM), phenyl benzoate (300 mM) and mesitylene (100 mM) (internal standard) in isopropyl ether (350 μ L), CALA on Relizyme EXE309 (2 mg, 0.22 U), and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (30 mg, 0.17 mmol), benzoic acid (70 mM) in isopropyl ether (150 μ L, 1 equivalent) were added. A control was performed adding isopropyl ether (150 μ L) instead of benzoic acid solution. Samples (20 μ L) were analyzed by HPLC.

3.3.11. General Procedure for the Cascade Synthesis of (S)-4-Methoxymandelonitrile Benzoate

To a vial containing immobilized MeHNL and CALA, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (30 mg, for experiments 1-3, Table 1) was added. A solution of 4-anisaldehyde (360 mM) and mesitylene (360 mM) (internal standard) in isopropyl ether (140 μ L) and HCN (900 mM) in isopropyl ether (360 μ L) was subsequently added. To this mixture, phenyl benzoate (2-4 equivalents) was added (in case of experiments 7 and 8 from Table 1, 1 equivalent was added each time at $t = 0$, after 24 h and after 46 h) and the reactions were incubated at 10, 20, or 25 $^\circ\text{C}$. Negative control reactions without enzyme addition were performed in parallel. Reactions were performed in duplicate and 20 μ L samples were analyzed by HPLC.

3.4. HPLC Analysis

The quantification of 4-anisaldehyde, (S)- and (R)-4-methoxymandelonitrile and (S)- and (R)-4-methoxymandelonitrile benzoate was achieved using chiral normal phase HPLC. Samples from reaction mixtures were diluted 50 $\times$ with n-heptane/isopropanol 8:2 v/v and dried over anhydrous magnesium sulfate. Analysis was performed using a Hitachi Elite LaChrom HPLC system, consisting of a VWR Hitachi L-2130 pump, L-2200 auto-sampler, L-2350 column oven, and L-2400 UV detector using a D₂ lamp coupled to a Hitachi organizer module. Separation was achieved on a Chiralpak AD-H column (250 mm $\times$ 4.6 mm $\times$ 5 μ m; Daicel, Japan), using n-heptane/isopropanol (82/18) at a flow rate of 0.65 mL/min. The column oven temperature was set at 35 $^\circ\text{C}$ and a detection wavelength of 225 nm was chosen. Under these conditions, the compounds were separated with the following retention times; mesitylene (internal standard) 5.0 min, benzoic acid 6.7 min, phenol 7.0 min, phenyl benzoate 7.3 min, 4-anisaldehyde 8.3 min, (S)-4-methoxymandelonitrile 9.6 min, (R)-4-methoxymandelonitrile 10.5 min, (S)-4-methoxymandelonitrile benzoate 13.1 min, and (R)-4-methoxymandelonitrile benzoate 13.9 min.

Reverse-phase HPLC was used for the quantification of benzoic acid. Samples from reaction mixtures were diluted 50 $\times$ with acetonitrile/water 1:1 v/v. Analysis was performed using a Shimadzu Nexera XR HPLC system equipped with a DGU-20A5R degassing unit, coupled to two LC-20AD solvent delivery units, a SIL-20AC Autosampler, a CTO-20A column oven, an SPD-20A UV/VIS detector using a D₂ lamp, and a CBM-20A system controller. Separation was achieved on a Nucleoshell RP 18 column (150 mm $\times$ 3 mm $\times$ 2.7 μ m; Macherey-Nagel, Germany) at 30 $^\circ\text{C}$, 0.7 mL/min flow rate using MilliQ® water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B) as mobile phase and UV detection at 225 nm. The following eluent program was used; 10% solvent B for 0.5 min, followed by a linear gradient to 45% solvent B in 5.5 min, 45% solvent B for 10 min, another linear gradient to 100% solvent B in 5 min, 100% solvent B for 3 min, and finally a linear gradient to 10% solvent B in 3 min followed by 10% solvent B for 3 min. Under these conditions, the compounds were separated with the following retention times: phenol 4.3 min, benzoic acid 4.9 min, ($\pm$)-4-methoxymandelonitrile 5.2 min, 4-anisaldehyde 5.9 min, phenyl benzoate 14.8 min, ($\pm$)-4-methoxymandelonitrile benzoate 16.1 min, and mesitylene (internal standard) 19.3 min.

Calibration curves were prepared using commercial 4-anisaldehyde (>99.0%), (R)-4-methoxymandelonitrile (98.0%) and benzoic acid (>99.5%) as well as chemically synthesized ($\pm$)-4-methoxymandelonitrile benzoate.

4. Conclusions

In the present work, the successful concurrent cascade synthesis of (S)-4-methoxymandelonitrile benzoate starting from 4-anisaldehyde and catalyzed by MeHNL and CALA was accomplished. Systematically studying each enzymatic reaction first was key for their effective combination. When developing the one-pot cascade, however, further fine-tuning of reaction conditions was required to achieve optimal performance. This way, 95% conversion of **1** with 81% yield of the final ester (S)-**3** and excellent enantiopurity of 98% e.e. was achieved. Compared to the enzymatic hydrocyanation performed under the same conditions, which yielded **2** with 85% conversion and 91% e.e. (see Table 1, entry 9, and Figure S4B), this result proves that the CALA-catalyzed benzoylation of (S)-**2** coupled to the MeHNL-catalyzed hydrocyanation of **1** can shift the equilibrium of the latter by removal of the unstable intermediate. Nevertheless, there is still room for further improvement, since the low affinity of CALA for cyanohydrin **2** has been identified as the main bottleneck that prevents the cascade from reaching full conversion.

In principle, our approach reported herein can be transferred to the synthesis of other acylated cyanohydrins. In any case, a key requirement for success will be the application of a lipase with high selectivity for transesterification over hydrolysis in microaqueous media.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4344/9/6/522/s1>, Figure S1: Conversion and enantiomeric excess (e.e.) of the remaining 4-methoxymandelonitrile benzoate after 18 h of hydrolysis reaction, Figure S2: Conversion and enantiomeric excess (e.e.) values obtained for the CALA-catalyzed benzoylation of 67 mM ($\pm$)-4-methoxymandelonitrile using 200 mM vinyl benzoate (VB), acetoxime benzoate (AB) or phenyl benzoate (PB), Figure S3: Comparison of the cascade synthesis of (S)- 4-methoxymandelonitrile benzoate catalyzed by MeHNL and CALA starting from 100 mM **1** (A, Table S2, entry 8) and the hydrocyanation of 100 mM **1** catalyzed by MeHNL under the same conditions (B, Table S2, entry 9), Figure S4: Hydrocyanation of 100 mM 4-anisaldehyde catalyzed by immobilized MeHNL using 3–6.5 equivalents of HCN at 20 °C. Table S1: Assigned number and specifications of the lipases screened in the hydrolysis of ($\pm$)-4-methoxymandelonitrile benzoate, Table S2: Results of hydrocyanation of 100 mM 4-anisaldehyde with 6.5 equivalents of HCN catalyzed by immobilized MeHNL in isopropyl ether with 0.34 mmol Na₂HPO₄·2H₂O/Na₂HPO₄·7H₂O per mL (water activity of 0.57). Detailed information and additional data on the screening of hydrolases for the selective hydrolysis of ($\pm$)-4-methoxymandelonitrile benzoate, the screening of benzoyl donors, the immobilization of MeHNL, the effect of HCN concentration on MeHNL-catalyzed hydrocyanation, the optimized cascade, and the chemical synthesis of ($\pm$)-4-methoxymandelonitrile benzoate.

Author Contributions: Conceptualization, L.v.L. and F.H.; methodology, A.S.; validation, L.L., A.S., and L.v.L.; formal analysis, L.L.; investigation, L.L.; resources, A.S., L.v.L. and F.H.; data curation, A.S. and L.v.L.; writing—original draft preparation, L.L. and L.v.L.; writing—review and editing, A.S. and F.H.; visualization, L.L.; supervision, A.S., L.v.L., and F.H.; project administration, A.S. and L.v.L.; funding acquisition, L.v.L., F.H., and A.S.

Funding: This project was funded by the European Union's Horizon 2020 MSCA ITN-EID program under grant agreement No 634200 (BIOCASCADES). This communication reflects only the beneficiary's view and the European Commission is not responsible for any use that may be made of the information it contains.

Acknowledgments: We thank Antje Spieß (Institute of Biochemical Engineering, Technische Universität Braunschweig, Germany) for fruitful discussion and for providing an HPLC system for chiral analysis. L. Leemans also thanks Yvonne Goecke (Institute of Biochemical Engineering, Technische Universität Braunschweig, Germany) for technical support with the HPLC equipment. Further, Kerstin Steiner (ACIB, Graz, Austria) is acknowledged for the generous gift of *E. coli* K12 Top 10F' clone harboring the pSE420-MeHNL plasmid. Imerys S.A. and Resindion S.r.l. are acknowledged for providing Celite R633 and supplying Relizyme EXE309, respectively.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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