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

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Screening of hydrolases for the selective hydrolysis of (±)-4-methoxymandelonitrile benzoate

A total of 41 hydrolase preparations were evaluated in a hydrolysis test. In a 10 mL glass vial containing 1 mL 20 mM (±)-4-methoxymandelonitrile benzoate in isopropyl ether and 1 mL 100 mM phosphate pH 7, 50 mg or 50 µL of enzyme was added. The reaction mixture was incubated at room temperature, 800 rpm for 18 h. 20 µL samples were diluted with 980 µL *n*-heptane/2-propanol 8:2, dried over anhydrous $MgSO_4$ and analyzed in chiral HPLC.

Table S1: Assigned number and specifications of the lipases screened in the hydrolysis of (±)-4-methoxymandelonitrile benzoate.

Exp. n°	Name	Provider	Enzyme Type	Origin
1	SPRIN imibond THERMOLYSIN	Sprin Technologies	protease	*Geobacillus* sp.
2	SPRIN epobond THERMOLYSIN	Sprin Technologies	protease	*Geobacillus* sp.
3	Protease from Bacillus Licheniformis	Sigma Aldrich	protease	*Bacillus licheniformis*
4	CALA L	Novozymes	lipase	*Candida antarctica*
5	Porcine PEM	Novozymes	protease mixture	*Sus domesticus*
6	Novozym 388	Novozymes	lipase	*Rhizomucor miehei*
7	Lipex 100L	Novozymes	lipase	*Aspergillus oryzae*
8	Acylase "Amano"	Amano Enzyme Inc.	acylase	*Aspergillus melleus*
9	Esperase 8.0 L	Novozymes	protease	*Bacillus lentus*
10	Lipase AK "Amano" 20	Amano Enzyme Inc.	lipase	*Pseudomonas fluorescens*
11	Protease N "Amano"	Amano Enzyme Inc.	protease	*Bacillus subtilis*
12	Lipase AYS "Amano"	Amano Enzyme Inc.	lipase	*Sus domesticus*
13	Protease S "Amano"	Amano Enzyme Inc.	protease	*Bacillus* sp.
14	Lipase AS "Amano"	Amano Enzyme Inc.	lipase	*Aspergillus niger*
15	Lipase PS "Amano" SD	Amano Enzyme Inc.	lipase	*Bulkholderia cepacia*
16	Neutrase 0.8 L	Novozymes	metalloprotease	*Bacillus amyloliquefaciens*
17	Savinase 16L, Type EX	Novozymes	protease	*Bacillus lentus*
18	Lipase from porcine pancreas, Type II, L3126-100G	Sigma Aldrich	lipase	*Sus domesticus*

19	ASSEMBLASE liquid	DSM Anti-Infectives	amidase	*Escherichia coli*
20	BIOCATALYST CALB 10L	Fermenta Biotech Ltd	lipase	*Candida antarctica*
21	Lipase R "Amano"	Amano Enzyme Inc.	lipase	*Penicillium roqueforti*
22	Lipase AY "Amano" 30SD-K	Amano Enzyme Inc.	lipase	*Candida rugosa*
23	Lipase MH "Amano" 10SD	Amano Enzyme Inc.	lipase	*Mucor javanicus*
24	Lipase A "Amano" 12-K	Amano Enzyme Inc.	lipase	*Aspergillus niger*
25	Lipase DF "Amano" 15-K	Amano Enzyme Inc.	lipase	*Rhizopus oryzae*
26	Lipase R "Amano"-K	Amano Enzyme Inc.	lipase	*Penicillium roqueforti*
27	Papain from papaya latex, P3375-25G	Sigma Aldrich	protease	*Carica papaya*
28	Alcalase	Clea Technologies	protease	*Bacillus licheniformis*
29	ECS-PLE06	Enzymicals	esterase	*Sus domesticus*
30	Trypsin from bovine pancreas cat: 93610	Fluka, Bio Chemika	protease	*Bos taurus*
31	Esterase, from porcine liver, crude.	Sigma Aldrich	esterase	*Sus domesticus*
32	CES P-1	Amano Enzyme Inc.	protease	-
33	CES P-2	Amano Enzyme Inc.	protease	-
34	CES P-3	Amano Enzyme Inc.	protease	-
35	CES L-1	Amano Enzyme Inc.	lipase	-
36	CES L-2	Amano Enzyme Inc.	lipase	-
37	CES L-3	Amano Enzyme Inc.	lipase	-
38	CES L-4	Amano Enzyme Inc.	lipase	-
39	CES L-5	Amano Enzyme Inc.	esterase	-
40	CES E-1	Amano Enzyme Inc.	esterase	-
41	CES E-2	Amano Enzyme Inc.	esterase	*Aspergillus melleus*

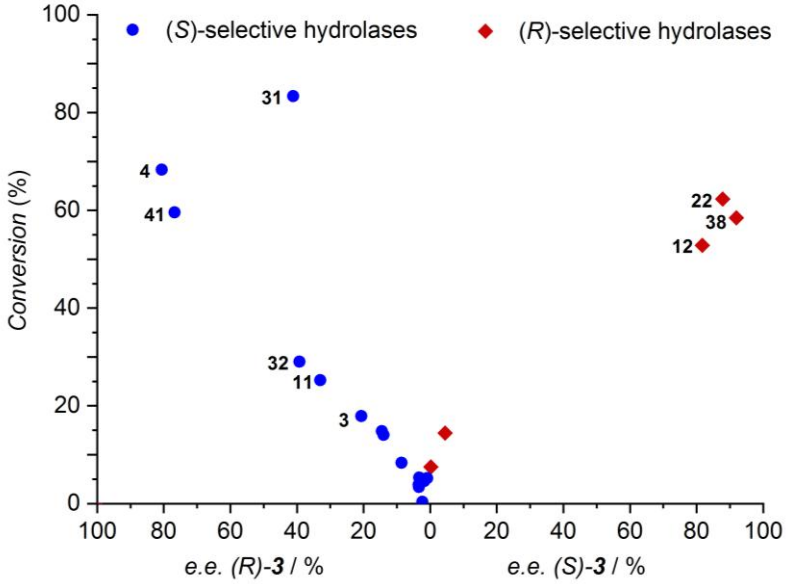

Figure S1: Conversion and enantiomeric excess (e.e.) of the remaining 4-methoxymandelonitrile benzoate after 18 hours of hydrolysis reaction. The number next to the data points indicate the hydrolase to which they correspond (see table S1). Hydrolases 5, 7, 8, 9, 14, 17, 18, 24, 28, 29 and 33 showed low activity and/or selectivity and their numbers are therefore not specified on the graph. Hydrolases with no activity are not shown.

Screening of benzoyl donors

For the CALA-catalyzed transesterification reaction to take place, an activated benzoyl donor was required, as simple esters of benzoic acid such as methyl benzoate did not afford the benzoylation of 4-methoxymandelonitrile (data not shown). Enol esters are highly activated acyl donors and have been extensively used in lipase-catalyzed transesterifications [1,2]. As an alternative to enol esters, several studies have proposed oxime esters, which are generally considered quasi-irreversible acyl donors [1,3–6]. Another activated ester, reported by Sakulsombat *et al*. on the successful acetylation of cyanohydrins catalyzed by *Burkholderia cepacia* lipase, is phenyl acetate [7]. Based on this information, vinyl benzoate, acetoxime benzoate and phenyl benzoate were studied in the transesterification reaction of 4-methoxymandelonitrile.

As shown in Figure S2, all three activated benzoyl donors are accepted by CALA in the benzoylation of 4-methoxymandelonitrile. Phenyl benzoate afforded the highest reaction rate, followed by vinyl benzoate and, finally, acetoxime benzoate. The enantioselectivity of the reaction was not significantly affected by the donors, resulting in an enantiomeric ratio [8] (E value) of 8 when vinyl benzoate was used and 6 when acetoxime benzoate or phenyl benzoate were used.

Among the three tested donors, vinyl benzoate is the only one that can yield an irreversible benzoylation of 4-menthoxymandelonitrile, due to the tautomerization of the byproduct to acetaldehyde. This constitutes an advantage for the transesterification reaction, but acetaldehyde might compete with 4-anisaldehyde as a substrate for *Me*HNL. After confirming that *Me*HNL could catalyze the hydrocyanation of acetaldehyde to yield lactonitrile (data not shown), vinyl benzoate was ruled out as suitable benzoyl donor for the cascade approach.

Phenyl benzoate and acetoxime benzoate are both activated donors due to the poor nucleophilicity of the byproducts (phenol and acetone oxime), although they do not yield irreversible transesterifications. Given that phenyl benzoate afforded considerably higher transesterification rates than acetoxime benzoate and after confirming that, as well as the byproduct phenol, it did not exert a negative effect on the hydrocyanation reaction, it was selected for the cascade synthesis of (*S*)-4-methoxymandelonitrile benzoate.

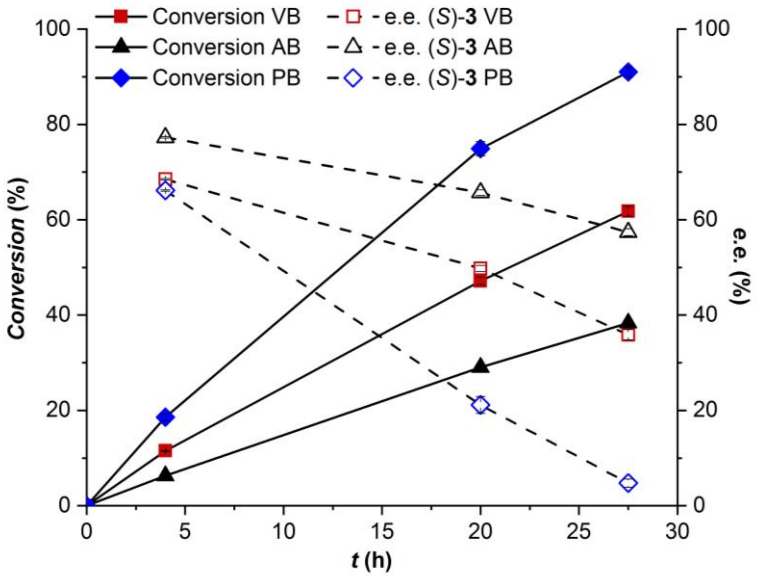

Figure S2: Conversion and enantiomeric excess (e.e.) values obtained for the CALA-catalyzed benzoylation of 67 mM (±)-4-methoxymandelonitrile using 200 mM vinyl benzoate (VB), acetoxime benzoate (AB) or phenyl benzoate (PB).

Immobilization of *Me*HNL

A simple and generally effective immobilization method when working in organic solvents is physical adsorption. Crystalline cellulose and Celite have been widely used for HNL immobilization, affording high activities and selectivities in organic solvents [9–14]. Furthermore, Celite has been used to control water activity in organic media [15]. In a recent study of various supports for the immobilization of *Me*HNL, silica gel exhibited very high activity recovery [16]. Based on this information, microcrystalline cellulose, Celite R-633 (which has been used for immobilization of AtHNL and HbHNL) [10,12] and several silica supports were used as carriers for *Me*HNL. The resulting immobilisates were tested in the hydrocyanation of 4-anisaldehyde in a controlled low water medium (see Table S2).

Under the tested conditions, most *Me*HNL formulations performed poorly, compared with the excellent yield and selectivity values obtained for the hydrocyanation of 4-anisaldehyde in a biphasic medium using free *Me*HNL (see Figure 1). This observation could be partially attributed to the reported lower activity and selectivity of *Me*HNL when working at reduced water content [9,11]. Furthermore, it was observed that Celite R633 and the silica carriers catalyzed the unselective hydrocyanation, thus contributing to formation of the unwanted enantiomer. Nevertheless, the screening clearly identified Celite RR633 as the best carrier for *Me*HNL, with the highest activity and selectivity of the resulting biocatalyst (enzyme loading = 0.41 U/mg), which afforded a conversion of 82 % and an e.e. value of 93 % after 2 h under the studied conditions. However, the hydrocyanation reaction reached plateau shortly after 2 hours and, as the reaction continued, the e.e. dropped to 69 %, with 86 % conversion after 25 hours. This decrease in e.e. value after long reaction times is due to the mentioned chemical background reaction catalyzed by the carrier.

Table S2: Results of hydrocyanation of 100 mM 4-anisaldehyde with 6.5 equivalents of HCN catalyzed by immobilized *Me*HNL in isopropyl ether with 0.34 mmol $Na_2HPO_4 \cdot 2H_2O/Na_2HPO_4 \cdot 7H_2O$ per mL (water activity of 0.57).

Support	Enzyme Loading (U Free Enzyme/mg support)	mg Immobilizate/mL of Reaction	Reaction Time (h)	Conversion (%)	e.e. (S)-2 (%)
Microcrystalline	0.36	14	23	18	48

cellulose					
Celite R633	0.22	23	23	63	60
Celite R633	0.41	30	2	82	93
			25	86	69
SP53D-11785	0.41	30	2	12	76
			25	54	60
SP 540-10297	0.41	30	2	14	62
			25	70	35
SYLOID 244 FP	0.41	30	2	7	42
			25	48	26
SYLOID AL1-FP	0.41	30	2	10	47
			25	78	24
Perkasil SM 660	0.41	30	2	3	99
			25	22	48

Optimized cascade

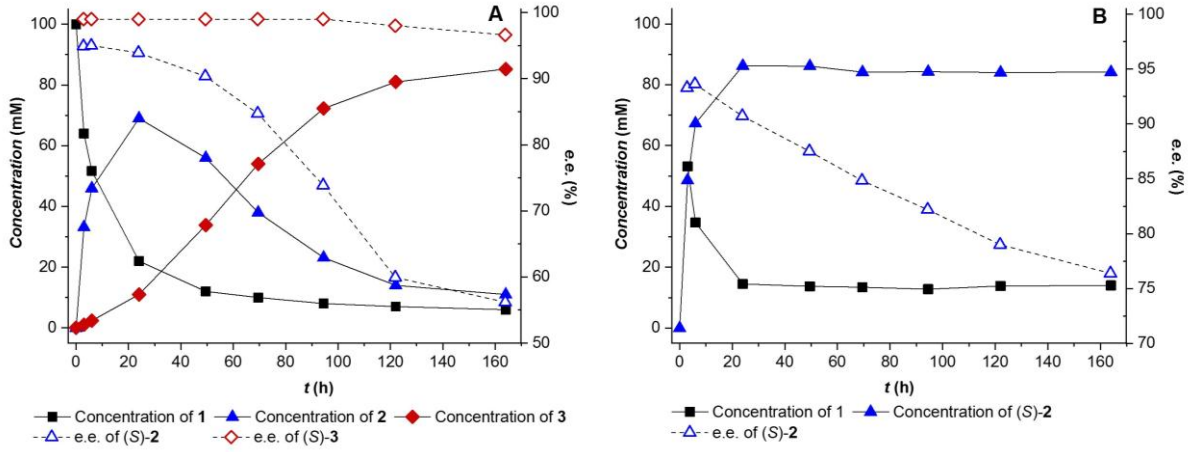

Figure S3: Comparison of the cascade synthesis of (*S*)-4-methoxymandelonitrile benzoate catalyzed by *Me*HNL and CALA starting from 100 mM **1** (A, Table 1, entry 8) and the hydrocyanation of 100 mM **1** catalyzed by *Me*HNL under the same conditions (B, Table 1, entry 9).

Effect of HCN equivalents on hydrocyanation catalyzed by immobilized *Me*HNL

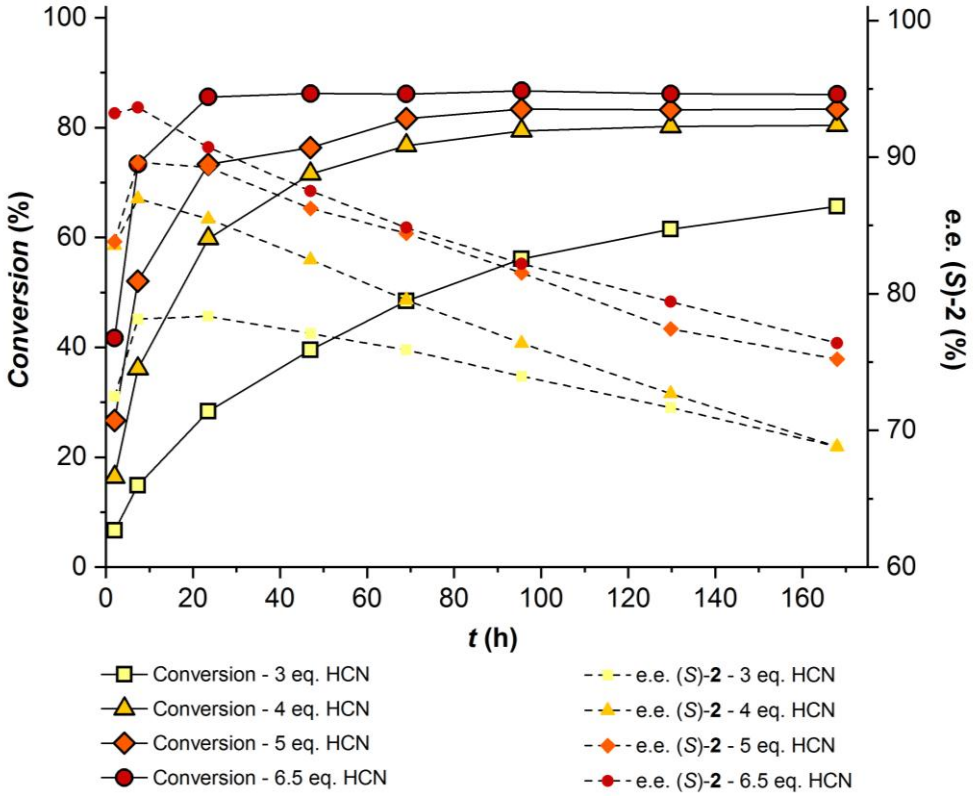

Figure S4: Hydrocyanation of 100 mM 4-anisaldehyde catalyzed by immobilized *Me*HNL using 3-6.5 equivalents of HCN at 20 °C.

Synthesis of (±)-4-methoxymandelonitrile benzoate

The racemic cyanohydrin ester was synthesized *via* chemical benzoylation of (±)-4-methoxymandelonitrile. A sealed 50 mL round-bottom flask containing 1.96 g (±)-4-methoxymandelonitrile (12 mmol) and 1.5 mL benzoyl chloride (13.2 mmol) in 20 mL dry dichloromethane under N_2 atmosphere, was introduced in an ice-water bath and 10.7 mL anhydrous pyridine (13.2 mmol) was added dropwise with a syringe. The reaction mixture was vigorously stirred for 2 hours. The mixture was then transferred to a 100 mL separatory funnel and washed twice with 20 mL demineralized water, twice with 20 mL citrate 0.2 M pH 4 and twice with 20 mL phosphate 0.2 M pH 8. The organic phase was then dried over anhydrous magnesium sulphate and concentrated in a rotary evaporator. Recrystallization (dichloromethane-diethyl ether = 1/4) afforded 1.9 g (58 %) of (±)-4-methoxymandelonitrile benzoate.

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