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Synthesis of Aliphatic (*S*)-α-Hydroxycarboxylic Amides using a One-Pot Bienzymatic Cascade of Immobilised Oxynitrilase and Nitrile Hydratase

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Abstract: A one-pot bienzymatic cascade combining a hydroxynitrile lyase (*Manihot esculenta*, E.C. 4.1.2.10) and a nitrile hydratase (*Nitriliruptor alkaliphilus*, E.C. 4.2.1.84) for the synthesis of enantiopure aliphatic α-hydroxycarboxylic amides from aldehydes is described. Both enzymes were immobilised as cross-linked enzyme aggregates (CLEAs). Stability tests show that the nitrile hydratase CLEAs are sensitive to water-immiscible organic solvents as well as to aldehydes and hydrogen cyanide (HCN), but are remarkably stable and show useful activity in acidic aqueous environments of pH 4–5. The cascade reactions are consequently carried out by using a portionwise feed of HCN and moderate concentrations of aldehyde in acidic aqueous buffer to suppress the uncatalysed hydrocyanation background reaction. After optimisation, this method was used to synthesise five different kinds of aliphatic α-hydroxycarboxylic amides from the corresponding aldehydes with good yields and with enantiomeric purities comparable to those obtained for the α-hydroxynitriles in the microaqueous hydrocyanation using hydroxynitrile lyase and an excess of HCN.

Keywords: amides; cascade reaction; cross-linked enzyme aggregates; hydrocyanation; hydroxynitrile lyase; nitrile hydratase

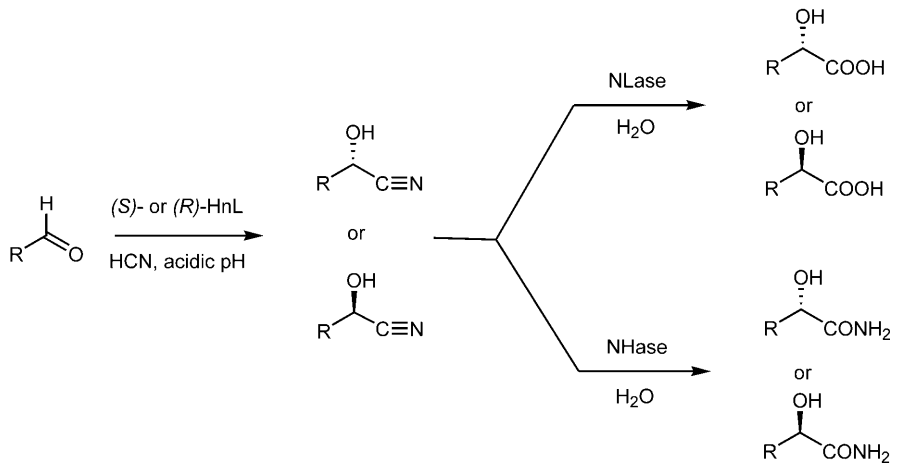
Introduction

Chiral α-hydroxycarboxylic amides, like the corresponding acids, are versatile building blocks in organic synthesis because of the ease of transformation of the functional groups in these molecules. In the last two decades, research was mainly focused on the development of biocatalytic synthesis routes to enantiopure α-hydroxycarboxylic acids.[1] However, a recent brainstorm session and cross company debate of the Pharmaceutical Roundtable identified a definite need for greener and more atom efficient methods of amide formation in the pharmaceutical industry.[2]

Enantiomerically pure *(R)*- and *(S)*-α-hydroxynitriles can be synthesised enzymatically from the corresponding aldehydes using an oxynitrilase (hydroxynitrile lyase, HnL, E.C. 4.1.2.10).[3] Chemical hydration or hydrolysis of these enantiopure nitriles using a strong acid will then give rise to the corresponding enantiopure carboxylic amides or acids. However, chemical hydration or hydrolysis is usually not compatible with sensitive functional groups and generates large amounts of salt waste. A greener route to α-hydroxycarboxylic acids comprises the asymmetric enzymatic hydrolysis of the cyano group using a stereoselective nitrilase (NLase, E.C. 3.5.5.1).[4] The remaining undesired cyanohydrin enantiomer can easily be racemised *in situ* under basic reaction conditions, resulting in a dynamic kinetic resolution (DKR) of the nitrile. The main limitation of this method is that there are only a few nitrilases available that are truly enantioselective and that this enantioselectivity is usually limited to mandelonitrile and its derivatives.

Besides the aforementioned chemoenzymatic routes, a novel method employing a bienzymatic one-pot cascade for the synthesis of (*S*)-α-hydroxycarboxylic acids was recently published. In this method an (*S*)-selective HnL from *Manihot esculenta* is combined with a non-selective NLase from *P. fluorescens* in a combi-CLEA or in the acidotolerant fungal host strain *Pichia pastoris*.[5] This fully enzymatic route to (*S*)-α-hydroxy acids is environmentally benign and compatible with a wide range of hydrolytically sensitive groups because of the mild reaction conditions.

An analogous bienzymatic cascade can be envisaged for the one-pot synthesis of α-hydroxycarboxylic amides by using a nitrile hydratase (NHase, E.C. 4.2.1.84) instead of a NLase in combination with a



Scheme 1. Access to (*R*)- and (*S*)-α-hydroxycarboxylic amides and acids using a one-pot bienzymatic cascade of HnL and NLase or HnL and NHase. The stereogenic centre is set in the molecule by an (*R*)- or (*S*)-selective HnL. Subsequent hydration/hydrolysis is catalysed by an aselective NHase/NLase.

(*R*)- or (*S*)-HnL. Like NLases, NHases are industrially relevant enzymes.[6] Their main drawbacks are low stability in cell-free preparations and low to nonexistent enantioselectivity on most substrates tested, which makes the use of a DKR on α-hydroxynitriles practically impossible. Recently, a new NHase-containing organism from soda lakes was isolated by our group[7] and subsequently the cell-free NHase from this organism was stabilised by the formation of CLEAs.[8] When a bienzymatic cascade of HnL and NHase is used for the production of α-hydroxycarboxylic amides, the low selectivity of the NHase is no longer a drawback since either an (*R*)- or (*S*)-selective HnL will impart its enantioselectivity on the reaction (Scheme 1).

We initially studied the stability of NHase CLEAs under the conditions used for HnL-catalysed hydrocyanation. After establishing the stability, the conditions of the one-pot enzymatic cascade were optimised and consequently α-hydroxycarboxylic amides were produced from several different aliphatic aldehydes.

Results and Discussion

Stability of NHase CLEAs under HnL Hydrocyanation Conditions

HnL-catalysed hydrocyanations are preferably carried out under acidic conditions (pH <5) to suppress the competing unselective uncatalysed hydrocyanation,[9] which will cause erosion of the enantiomeric excess (*ee*) of the α-hydroxynitrile product. Biphasic aqueous-organic as well as micro-aqueous systems can help to further suppress the uncatalysed hydrocyanation.[10] The *Me*HnL CLEA can tolerate these conditions quite well, especially when diisopropyl ether (DIPE) is used as the water-immiscible organic solvent.[11]

For NHases, in contrast, this environment is far from ideal. In order to find out whether NHase CLEAs can still function under HnL hydrocyanation conditions, an assessment was made of their stability in DIPE/buffer mixtures of different composition. *Me*HnL CLEAs tolerate lyophilisation very well and can form a homogeneous suspension in DIPE, which can be kept for weeks in the refrigerator without loss of activity. In contrast, NHase CLEAs suffer from a high loss of activity during freeze drying and are most stable when stored in an aqueous environment.[8] Incubation of the NHase CLEAs in different DIPE/buffer mixtures resulted in an average activity loss of 40% in one hour. In addition to the low stability, it is cumbersome to suspend the NHase CLEAs in these mixtures, since the particles coagulate easily and tend to stick to the walls of the reaction vessel.

Since the use of biphasic as well as micro-aqueous systems was not feasible, the stability of the NHase CLEAs in low pH aqueous buffer systems was determined. Although the optimum pH of the NHase from *Nitriliruptor alkaliphilus* is located around 8 (Figure 1 A), it proved to be surprisingly stable at pH values 2–4 units below the optimum (Figure 1 B) and still had a useful activity under these conditions.

It is well known that NHases are highly sensitive to low to moderate cyanide concentrations.[12] Presumably, the deactivation and/or inhibition of the NHase by cyanide is caused by the strong interaction of cyanide with the cobalt or iron metal centre of this enzyme, which is assumed to be essential for catalysis.[13] NHases that can tolerate moderate to high cyanide concentrations may have interesting applications in the conversion of nitrile compounds that readily re-

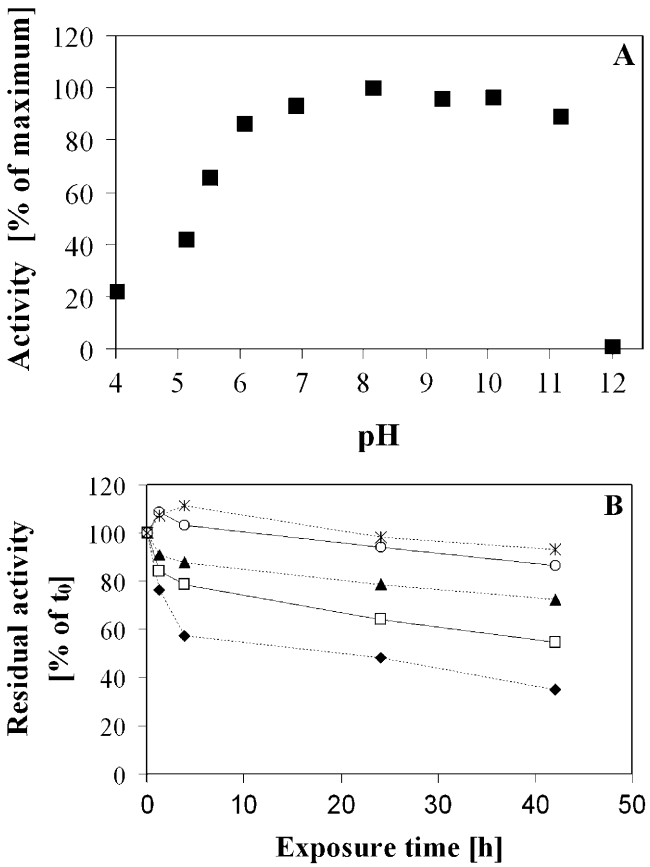


Figure 1. A NHase CLEA pH activity profile.[8] **B** Deactivation of the NHase CLEAs caused by incubation in acidic buffer solutions at 21 °C (pH 4–5.5, 0.01 M citrate buffer; pH 6, MilliQ), pH 4 (◆), pH 4.5 (□), pH 5 (▲), pH 5.5 (○), pH 6 (∗).

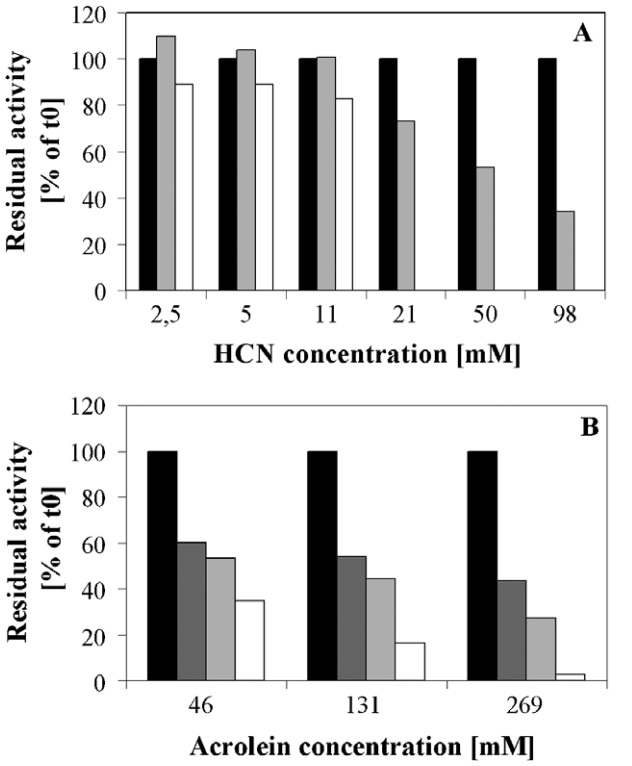


Figure 2. A Deactivation of the NHase CLEAs caused by incubation in the presence of different concentrations of HCN at 21 °C and pH 5.5, incubation of 0 min (■), 30 min (■), 90 min (□). **B** Deactivation of NHase CLEAs caused by incubation in presence of different concentrations of acrolein at 21 °C and pH 5.5, incubation of 0 h (■), 2 h (■), 3.5 h (■), 18.5 h (□).

lease cyanide because of decomposition, like α-hydroxy- or α-aminonitriles.[14] In HnL-catalysed hydrocyanation reactions, HCN is usually added in excess to drive the equilibrium to complete conversion. The stability of the NHase CLEA activity during incubation in the presence of different HCN concentrations is therefore of importance and was investigated (Figure 2 A). The activity was reasonably preserved during incubation in the presence of HCN concentrations $\leq$11 mM. HCN concentrations $\geq$21 mM resulted in severe loss of activity after an incubation of 30 min. These results compare favourably with previously published results.[12] The activity of the CLEAs remained the same after washing them several times with buffer and storage for a week in an open atmosphere, indicating that the loss of activity is permanent. Nonetheless, the relatively high HCN tolerance of the NHase from *Nitriliruptor alkaliphilus* made this enzyme a good candidate for use in the cascade reaction system.

In general, aldehydes have the characteristic to bind to enzymes mostly through non-conjugated Schiff's base formation with the ε-amino groups of free lysine residues but they can also react with thiols, phenols and imidazoles.[15] These interactions with the enzyme can lead to a change in enzyme conformation and subsequent deactivation. In order to investigate the effects of aldehydes on the NHase CLEAs, these were incubated in the presence of different concentrations of acrolein, which was one of the intended substrates of the cascade reaction (Figure 2 B). Deactivation of the NHase CLEAs in the presence of moderate acrolein concentrations was quite severe. When the concentration of acrolein was monitored during experiments without HCN it is clear that the presence of NHase CLEAs is connected to a disappearance of acrolein (Figure 3). No alien peaks were detected during HPLC measurements, which might indicate that a part of the acrolein binds to the cross-linked enzyme aggregates. Apparently the acrolein is able to react either with amine groups that were not occupied during cross-linking of the NHase aggregates or with other essential functional groups. In contrast, propionaldehyde did not disappear during incubation with

the NHase CLEAs, which indicates that the enzyme reacts with the C=C double bond of acrolein in a Michael-type addition. Nevertheless, the enzyme was deactivated by moderate concentrations of propionaldehyde.

During the preparation of NHase CLEAs with glutaraldehyde no reduction step was carried out since no leakage of free enzyme from the CLEA matrix was detected. No noticeable effect on CLEA stability in organic solvent or in presence of acrolein was observed after reduction of the CLEAs using $NaBH_4$. The Schiff's bases formed by glutaraldehyde with the free amino groups of the enzyme are apparently very stable or cross-linking of the NHase CLEAs also takes place through the formation of other (stable) connections.[15]

In view of the results discussed above, carrying out a one-pot bienzymatic cascade using HnL and NHase to synthesise α-hydroxyamides from aldehydes and HCN is affected by several severe limitations. Because of the bad performance of the NHase CLEAs in DIPE mixtures, the reactions have to be carried out in an aqueous environment and, hence, the uncatalysed hydrocyanation has to be suppressed by lowering the pH of the reaction as much as possible. Because of the low HCN tolerance of the metalloenzyme, the HCN concentration in the reaction should be kept low. A low HCN concentration will also have the benefit of reducing the uncatalysed hydrocyanation reaction rate and since the NHase will convert the formed α-hydroxynitrile to the stable amide, the equilibrium will shift to the right without the need of an HCN excess. The preferred mode of carrying out the cascade reactions was therefore the fed-batch addition of portions of HCN to modest aldehyde concentrations in acidic aqueous environment.

HnL-NHase One-Pot Bienzymatic Cascade

Because of the unfavourable reaction equilibrium and the low *ee*s obtained in the *Me*HnL-catalysed hydrocyanation of acrolein,[11] this challenging aldehyde was chosen as the substrate for optimising the cascade reaction conditions, since changes in reaction conditions together with enzyme ratio were expected to have a more profound impact on the *ee* of the amide product.

As was previously mentioned, the uncatalysed background hydrocyanation reaction should be suppressed as much as possible, because it is unselective and will deteriorate the *ee* of the α-hydroxynitrile intermediate and consequently of the final amide product. In order to investigate the effect of the pH on the background reaction in more detail, the latter was monitored for acrolein under cascade reaction conditions (5 mM HCN, 45 mM acrolein, 21 °C) at different

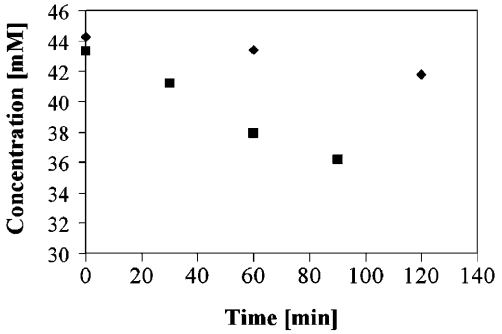


Figure 3. Disappearance of acrolein during incubation in buffer (0.01 M citrate buffer, pH 5.5) with and without the presence of NHase CLEAs, acrolein in buffer (◆), acrolein in buffer + 3.7 mg NHase CLEA (■).

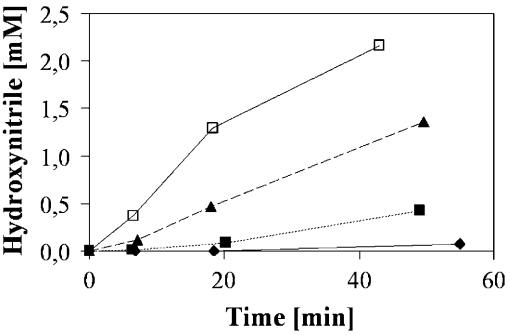
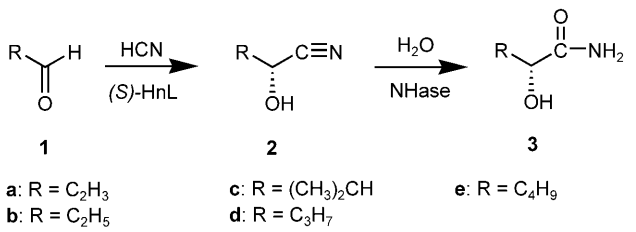


Figure 4. Rate of the uncatalysed hydrocyanation of acrolein at different pH under cascade reaction conditions, pH 4 (◆), pH 4.5 (■), pH 5 (▲), pH 5.5 (□).

pH values (Figure 4). As expected, lowering the pH of the cascade reaction decreased the rate of the uncatalysed hydrocyanation.

The relative contribution of the background reaction is also dependent on the rate of enzymatic hydrocyanation. An increase of HnL activity in the cascade reaction would decrease the effect of the background reaction on the *ee* of the intermediate nitrile product. However, there is a limit to the amount of HnL that can be added to the cascade reaction, since its cascade partner should still be able to keep the intermediate nitrile concentration low to prevent chemical and/or enzymatic racemisation. The activity ratio of the two enzymes in this cascade is therefore an important variable.

During the cascade reaction studies (Scheme 2), the HnL activity added to the reaction mixture was fixed and the NHase activity was varied. The activity of the HnL CLEA for acrolein under cascade reaction conditions at pH 4 was 0.98 μmol $min^{-1} mg^{-1}$. During the cascade reactions a fixed amount of 1.8–1.9 $mg mL^{-1}$ of HnL CLEA was used, which enabled reasonably fast reactions without building up high concentrations of intermediate nitrile. Under these conditions the



Scheme 2. Synthesis of aliphatic (*S*)-α-hydroxyamides using a bienzymatic one-pot cascade of the (*S*)-HnL from *Manihot esculenta* and the NHase from *Nitriliruptor alkaliphilus.*

Table 1. HnL-NHase one-pot bienzymatic cascade reactions with **1a** (45–46 mM) at different pH, 21 °C, and a maximum HCN concentration of 5 mM.

pH	Background [%][a]	Factor[b]	**3a** produced [mM]	*ee* [%] (*S*)
5.5[c]	3.1	12	24.4	35
5.5[c]	3.1	24	26.6	35
5[c]	1.6	24	24.8	39
4.5[d]	0.5	24	20.0	43
4.5[e]	0.5	24	35.5	43
4[c]	0.1	24	28.1	39
4[e]	0.1	48	40.0	48

[a] Relative background reaction when using 1.8–1.9 mg HnL CLEA ($T = 21$ °C).
[b] Ratio of NHase activity to HnL activity. NHase activity was measured for the hydration of **2a** at pH 6 and HnL activity was measured for the production of **2a** under cascade reaction conditions at pH 4.
[c] Reaction was stopped after spiking 5 times with 5 mM HCN.
[d] Reaction was stopped after spiking 4 times with 5 mM HCN.
[e] Reaction was continued to the point were **1a** was completely converted.

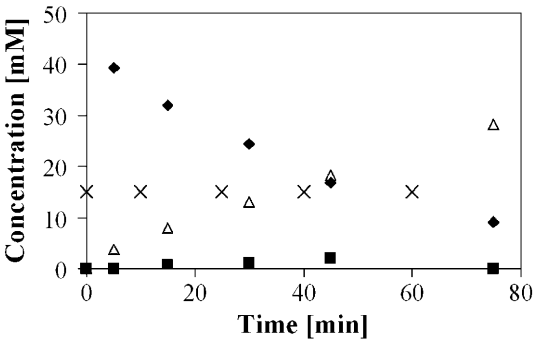


Figure 5. HnL-NHase one-pot bienzymatic cascade conversion of acrolein at pH 4, **1a** (◆), **2a** (■), **3a** (△), HCN spike (×).

relative background reaction during the cascade reaction of acrolein (**1a**) was 3.1% or less (Table 1).

The pH of the cascade reaction clearly had an effect on the final *ee* of **3a**. This is to be expected, since the competing unselective background hydrocyanation rate increases at a higher pH. The drop in *ee* between pH 4.5 and the first experiment at pH 4 is most probably the result of enzymatic racemisation of **2a**, since moderate concentrations of **2a** were detected by HPLC at this pH (Figure 5).[11] Apparently, it was not possible for the NHase to keep up with the HnL in this situation. When the NHase activity at pH 4 was doubled, the nitrile intermediate was no longer detected and the enantiomeric purity of the amide improved. A two-fold increase of the relative NHase activity in the cascade reaction at pH 5.5, in contrast to pH 4, did not lead to an improvement in product *ee*, indicating that the conversion of the α-hydroxynitrile intermediate by the NHase CLEA is fast enough to prevent enzymatic racemisation of **2a** at this pH.

Complete conversion of **1a** at pH 4.5 did not result in a lower *ee* of **3a** compared to the reaction where **1a** was not fully converted. This indicates that stopping the reaction prematurely to maintain a favourable equilibrium and to prevent enzymatic racemisation was not necessary. The yield on **1a** was between 77 and 89%, depending on the conditions and time course of the reaction (Figure 5). The loss of **1a** in the reaction is probably caused by attachment of the substrate to the enzyme, as was demonstrated before (Figure 3). In these reactions, the formation of α-hydroxy-3-butenoic acid was not observed by HPLC, GC, and NMR.

The *Manihot esculenta* HnL is known to have a rather low selectivity in converting acrolein (47–56%).[11,16] In order to attempt the production of α-hydroxycarboxylic amides with higher enantiomeric purity, the same cascade reaction was used for the conversion of aliphatic aldehydes for which the HnL

Table 2. HnL-NHase one-pot bienzymatic cascade reactions of **1b–1e** (35–45 mM) at pH 4.5, 21 °C, and a maximum HCN concentration of 5 mM. Unless stated otherwise, reactions were stopped after full conversion of the aldehyde.

Compound	**3** produced [mM]	Factor[a]	*ee* [%] (*S*)	*ee* [%] (*S*)-**2**[16,17]
b	44	24	86	91
c[b]	28	24	84	95
c	35	48	88	95
d	41	24	88	88
e	43	48	90	91

[a] Ratio of NHase activity to HnL activity. NHase activity is measured for the hydration of **2a** at pH 6 and HnL activity is measured for the production of **2a** under cascade reaction conditions at pH 4.
[b] Full conversion was not achieved with this amount of NHase.

from *Manihot esculenta* is known to have a higher selectivity (Table 2).

The *ee* of the amide products significantly improved when aldehydes were used for which the *Me*HnL is known to show a higher selectivity. In all cases, except for **d** and **e**, the *ee* of the amide products is lower than the *ee* of the nitriles as reported in literature for *Me*HnL catalysed hydrocyanations in microaqueous systems using an excess of HCN. The higher *ee*s obtained for compounds **3d** and **3e** could be the result of a low preference for the corresponding (*S*)-α-hydroxy nitriles by the NHase. The enantioselective properties of the NHase from *Nitriliruptor alkaliphilus* are currently under investigation.

The yields of the α-hydroxy amide on the aldehyde are >95%, which contrasts with the lower yields obtained when acrolein is used as the substrate. Unlike acrolein, the aliphatic aldehydes did not disappear during the reaction because of possible attachment to the enzyme. No traces of the corresponding α-hydroxycarboxylic acids were detected during these experiments on HPLC and GC.

Conclusions

A bienzymatic one-pot cascade reaction to synthesise aliphatic α-hydroxycarboxylic amides using *Me*HnL and NHase CLEAs was made possible by using aqueous acidic citrate buffer of pH 4–pH 5.5 as reaction medium containing moderate concentrations of aldehyde and the subsequent portionwise feed of HCN. After optimisation of the pH together with the HnL:NHase activity ratio in the reaction, five aliphatic aldehydes were successfully converted into the α-hydroxycarboxylic amides. Except for acrolein, the aldehydes were converted into the stable α-hydroxycarboxylic amides with good yield and high *ee* without the need for organic solvent and an excess of HCN.

The *ee* of the α-hydroxycarboxylic amides as well as the stability of the immobilised nitrile hydrating biocatalyst can probably be further improved by the controlled continuous addition of low concentrations of aldehyde and pure HCN and by co-immobilisation of the two biocatalysts in the form of a combi-CLEA. An NHase with an inherent or designed increased stability in microaqueous systems could further increase the performance of this system and the substrate scope of the cascade reaction system could possibly be broadened to aromatic aldehydes by using an NHase with a higher activity for aromatic nitriles.

Experimental Section

General Comments

Reactions and stability tests were carried out in Eppendorf tubes using a ThermoTWISTER comfort shaker of QUANTIFOIL Instruments. Freeze drying was performed by a Christ Alpha 2–4 freeze dryer at a reduced pressure of 0.1 mbar and a temperature of −80 °C, after freezing the samples with liquid N_2. An Eppendorf 5415R centrifuge was used for centrifugation at 13200 rpm. The temperature of the centrifuge was adjusted according to the needs of the experiment.

Materials

Semi-purified (*S*)-hydroxynitrile lyase from *Manihot esculenta* was obtained from Jülich Fine Chemicals (now Codexis). The NHase was produced by cultivation and induction of the haloalkaliphilic actinobacterium *Nitriliruptor alkaliphilus*.[7] NHase and *Me*HnL CLEAs were prepared as described previously.[8,11] NHase CLEAs were stored at +4 °C in Tris-HCl buffer (0.01 M, pH 8), while the *Me*HnL CLEAs were stored at −20 °C in lyophilised form. Hexanenitrile (98%, Aldrich), hexanamide (98%, Aldrich), sodium borohydride (>96%, Fluka), diisopropyl ether (DIPE, >99%, Fluka), acrolein (>95%, Fluka), propionaldehyde (99+%, Janssen), isobutyraldehyde (99+%, Acros), butyraldehyde (>99.5%, Aldrich), valeraldehyde (>97%, Fluka), α-hydroxybutyronitrile (Fluka, purum), α-hydroxybutyric acid (Fluka, >97%), and α-hydroxycaproic acid (Aldrich, 98%) were used in the experiments as received without additional purification. A 2 M solution of hydrogen cyanide in DIPE was prepared from sodium cyanide (98+%, Acros) as described previously.[18]

Warning: both sodium cyanide and HCN are highly poisonous. They should always be handled in a fume cupboard with a good draught. It is strongly advised to use a well-calibrated HCN detector during HCN work.

Analytical Procedures

HPLC analysis: The progress of the reactions was monitored by HPLC using one or three 4.6×50 Merck Chromolith SpeedROD RP-18e columns, depending on the polarity of the compounds analysed. The eluent (MilliQ with 0.1 v% TFA) was pumped through the column(s) at a flow rate of 1 mL min^{-1} using a Waters 590 HPLC pump. The HPLC analyses were carried out at room temperature. All compounds were detected using a Shimadzu RID 10 A refractive

Table 3. Retention times on HPLC of the different compounds in the cascade reaction.

Compound	Column #	Retention time [min]		
		1	**2**	**3**
a	3	5.2	4.4	3.2
b	3	4.8	4.6	3.5
c	3	9.4	12.2	5.3
d	3	8.9	10.9	5
e	1	7.6	11.6	4

Table 4. Retention times and temperature programs used for separation of the (*S*)- and (*R*)-enantiomers of the formed aliphatic α-hydroxycarboxylic amides.

Compound	T program [°C]	R_t (*R*) [min][a]	R_t (*S*) [min][a]
3a	100	34	36
3b	100	37	39
3c	105	43	46
3d	105	21[b]	25[b]
3e	115	39[c]	36[c]

[a] Since the *Me*HnL is (*S*)-selective, the peak with the largest area was designated as the (*S*)-enantiomer.
[b] This α-hydroxycarboxylic amide was first derivatised by a one hour incubation in a mixture of acetic anhydride (0.8 mL) and pyridine (0.8 mL) in dichloromethane (10 mL).
[c] The elution of the (*S*)- and (*R*)-enantiomers on this column are in reversed order.

index detector and/or a Shimadzu SPD-10 A VP UV-VIS detector at a wavelength of 210 nm (Table 3).

GC analysis: Enantiomeric excess (*ee*) of the formed aliphatic α-hydroxycarboxylic amides **3a–d** was determined using a 25 m Chirasil dex CB column with i.d. 0.32 mm and d_f 0.25 μm on a Shimadzu GC-17 A gas chromatograph equipped with a FID detector ($T_{injector}$ = 200 °C, $T_{detector}$ = 200 °C). The carrier gas used was N_2 at a column flow rate of 5.92 mL min^{-1}. The (*S*)- and (*R*)-enantiomers were separated using the temperature programmes in Table 4. The enantiomers of compound **3e** were separated using a 50 m Chiradex GTA column with i.d. 0.25 mm and d_f 0.12 μm on a Shimadzu GC-2010 gas chromatograph equipped with an FID detector ($T_{injector}$ = 200 °C, $T_{detector}$ = 200 °C). The carrier gas used was N_2 at a column flow rate of 5.00 mL min^{-1}.

Cyanide concentration: Cyanide concentrations in the reactions and stability tests were determined by using the Merck Spectroquant® cyanide test kit for the determination of free and readily liberated cyanide in water according to the manual of the manufacturer. Cyanide stock solutions for calibration were prepared by dissolving potassium cyanide in a KOH solution (35 mM).

Standard Activity Assay

NHase CLEAs were suspended in buffer (1000 μL, 0.01 M Tris-HCl of pH 8, unless stated otherwise) in an Eppendorf tube and subsequently hexanenitrile was added (15 μL, ~120 mM). The reaction was allowed to proceed for 5 min after which the CLEAs were centrifuged off in 10–20 seconds. A sample (200 μL) was withdrawn from the supernatant and mixed with the same amount of HCl (1 M) to make sure that any accidentally transferred CLEA was deactivated. After centrifugation the supernatant was directly injected on HPLC.

NHase CLEA Stability Tests

Two-phase DIPE-buffer system: NHase CLEAs were incubated in mixtures of DIPE (50 v%, 90 v% and >99 v%) and Tris-HCl buffer (0.01 M, pH 8). After an incubation time of one hour (900 rpm, 21 °C), the CLEAs were washed 3 times with Tris-HCl buffer (0.01 M, pH 8) to remove all traces of DIPE. The residual activities were determined by the standard activity assay.

Acidic pH: NHase CLEAs were incubated in buffers with different acidic pH values (pH 4–5.5: 0.01 M citrate buffer) and in MilliQ (pH ~6) at 21 °C. The residual activities after different incubation times were determined by the standard activity assay. Changing buffers for incubation was carried out by washing the CLEAs 3 times with the corresponding buffer.

HCN: NHase CLEAs were washed 3 times with citrate buffer (0.01 M, pH 5.5). To a mixture of this buffer (1000 μL) and the CLEAs, HCN was added (2.5, 5, 11, 21, 50, and 98 mM). These mixtures were then shaken at 900 rpm and 21 °C. After 30 and/or 90 min of incubation the incubation mixture was centrifuged and the CLEAs washed 3 times with citrate buffer. The residual activity was determined by the standard activity test at pH 5.5. After the activity test the CLEAs were washed 3 times with Tris-HCl buffer (0.01 M, pH 8) and stored at +4 °C in an open atmosphere. Activity of the CLEAs was reassessed after one week of storage.

Acrolein and propionaldehyde: NHase CLEAs were washed 3 times with citrate buffer (0.01 M, pH 5.5). To a mixture of this buffer (1000 μL) and the CLEAs, acrolein or propionaldehyde were added (46, 131, and 269 mM). After 2, 3.5 and 18.5 h of incubation (900 rpm, 21 °C), the incubation mixture was centrifuged and the CLEAs washed 3 times with citrate buffer. The residual activities were determined by the standard activity test at pH 5.5.

Preparation of the Racemic Amides

Racemic **2a** was prepared according to a literature procedure.[19] Racemic **2b** (purum) was purchased at Fluka. Racemic **3a** and **3b** were prepared by adding racemic **2a** and **2b** in fed-batch fashion to a suspension of NHase CLEAs in MilliQ (21 °C, 900 rpm). After the hydration reaction was finished the CLEA particles were spun down by centrifugation and the supernatant was removed. Freeze drying of the obtained supernatant resulted in fluffy white amide crystals of high purity. Racemic **2c–2e** were produced by adding equimolar amounts of **1c–1e** and HCN to a suspension of HnL CLEAs in citrate buffer (pH 4.5, 0.01 M), after which the reaction was allowed to proceed overnight. Racemic **3c–3e** were then prepared by the removal of the HnL CLEAs by centrifugation, after which NHase CLEAs were added to the supernatant of the enzymatic hydrocyanation reaction. After the hydration reaction was completed, the NHase CLEAs were removed by centrifugation and the supernatant was subsequently freeze dried to obtain the racemic amide crystals.

HnL-NHase Cascade

In order to obtain better suspension behaviour, HnL CLEAs were incubated in citrate buffer (0.01 M) under reduced pressure to remove air from the very fluffy lyophilised particles before the reaction. To a suspension of NHase and HnL CLEAs with a certain activity ratio in citrate buffer (0.01 M, pH 4–6), aldehydes were added (30–50 mM). At these concentrations the aldehydes were completely soluble in citrate buffer. After sampling for determi-

nation of the exact aldehyde starting concentration, the cascade reaction was initiated by the addition of HCN (5 mM). The reaction was spiked with portions of HCN (5 mM) several times. The time intervals between the HCN spikes depended on the speed of hydration of the α-hydroxynitrile intermediate. Sampling was carried out by stopping the reaction with a 30 second centrifugation after which a sample (50 μL) was withdrawn. The sample was directly injected on the HPLC and the excess sample liquid was returned to the reaction. At the end of the reaction the NHase and HnL CLEAs were spun down by a 10 min centrifugation and subsequently all supernatant was removed. After removing excess HCN under vacuum, the supernatant was freeze dried, after which white fluffy crystals were obtained. Citrate, which is an impurity in the crystalline product, can be removed by the addition of methanol in which the buffer does not dissolve. After removing insoluble citrate by filtration, the enantiomeric excess of the final product was determined by chiral GC as discussed previously.

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