Hydroxynitrile Lyase-Catalyzed Enantioselective Conversion of Ketones into Cyanohydrins

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To Nana, for inspiring my Education

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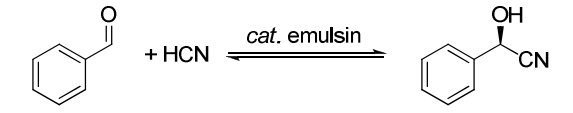
CHAPTER 1

Introduction

A Brief History of Hydroxynitrile Lyases

A century ago, in 1908, Rosenthaler reported the preparation of (*R*)-mandelonitrile from benzaldehyde and hydrogen cyanide using emulsin as catalyst[1] (Scheme 1). This publication, arguably, marks the birth of asymmetric biocatalysis.

Scheme 1. The preparation of (*R*)-mandelonitrile catalyzed by emulsin.



Benzaldehyde (*R*)-Mandelonitrile

The reaction was later shown to occur in the presence of crude extracts from various plants.[2,3]. However, the enzymatic nature of the catalytic process remained challenged by other research groups who argued in favour of chiral impurities in the preparation acting as asymmetric catalyst.[4,5] Eventually, enzymatic activity was demonstrated with crude enzyme extract from

almonds.[6] Subsequent enzyme characterization after partial purification of these extracts[7] demonstrated that Hydroxynitrile Lyases (HNLs or Oxynitrilases) were indeed asymmetric biocatalysts. However, the potential of these enzymes for the preparation of cyanohydrins only gained interest in the 1960s. Investigations on the substrate range showed that the (*R*)-selective HNL from *Prunus amygdalus* (*Pa*HNL) accepted a wide range of carbonyl compounds.[8] In parallel, the discovery of the HNL from *Sorghum vulgare*[9] revealed fundamental differences in this enzyme when compared to the HNL from almond. Both enzymes vary in terms of natural substrate, molecular weight, prosthetic group (FAD is required for *Pa*HNL while no cofactor is required for the *sorghum* enzymes), and activity on a non-natural substrate.[10] More than 30 years later, the HNL from *Sorghum bicolor* (*Sb*HNL, homologous to the enzyme from *Sorghum vulgare*) was found to be (*S*)-selective.[11] The substrate range for this enzyme was nonetheless disappointingly limited since it did not accept ketones and aliphatic aldehydes.[11-13] At this point in time, the perspective of discovering a (*S*)-selective HNL with the application potential of the (*R*)-selective *Pa*HNL prompted investigations on the selectivity and substrate range of a number of known HNLs. Isolation and characterization of the HNLs from *Hevea brasiliensis*: *Hb*HNL,[14-16] *Manihot esculenta*: *Me*HNL,[17-19] and *Linum usitatissimum*: *Lu*HNL[20,21] had been reported earlier. (*S*)-*Me*HNL[22] and (*S*)-*Hb*HNL[23] were found to have a wide substrate range, shared several structural and catalytic similarities and were serologically related.[24] However, *Lu*HNL was inactive on aromatic aldehydes, (*R*)-selective on aliphatic aldehydes,[25] and (*S*)-selective on phenylacetone and benzylacetone derivatives.[26] This feature was found to be specific to the sole representative of HNLs evolved from the Zinc-dependent alcohol dehydrogenase family: *Lu*HNL.[27]

The production of substantial amounts of *Pa*HNL by isolation from almonds had made this enzyme inexpensive and readily available for synthetic

application as early as the 1960s. Difficult and low yielding purification of enzymes from natural sources, which had limited the practicality of *Sb*HNL,[28] was no longer an obstacle when it came to prepare bulk quantities of the HNLs discovered in the 1990s. The production of *Me*HNL,[29,30] *Hb*HNL,[16,31] and *Lu*HNL[25,27] by cloning and over-expression was indeed described shortly after their potential as biocatalysts for the preparation of cyanohydrins was recognized.

Five HNLs have been commercialized to date (Table 1). These enzymes are relatively inexpensive asymmetric catalysts with a wide substrate range but most importantly they catalyze the formation of very useful chiral intermediates: cyanohydrins.

Table 1. HNLs commercialized.

	Source	**Selectivity**	**Substrate Range**	**Price**[a]
*Pa*HNL	Almond	(*R*)	Aliphatic/Aromatic carbonyls	€ 12.5 /kU
*Sb*HNL	Millet	(*S*)	Aromatic aldehydes	*Discontinued*[c]
*Lu*HNL	Flax	(*R*)/(*S*)[b]	Aliphatic carbonyls, phenyl/benzylacetones	€ 850 /kU
*Me*HNL	Maniok	(*S*)	Aliphatic/Aromatic carbonyls	€ 8.5 /kU
*Hb*HNL	Rubber tree	(*S*)	Aliphatic/Aromatic carbonyls	*Industrial use*[d]

[a] Price according to Jülich Chiral Solutions (2007 Catalogue). Unit definition according to the corresponding standard activity assay. [b] The stereoselectivity of *Lu*HNL is substrate dependant. [c] *Sb*HNL is apparently no longer commercialized. [d] *Hb*HNL is currently under exclusive licensing and not commercially available for research purposes.

HNL–Catalyzed Preparation of Cyanohydrins

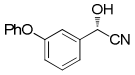
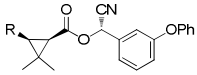
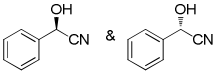
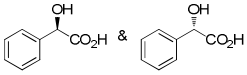
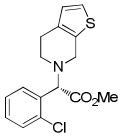
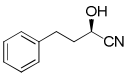
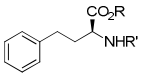
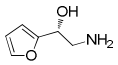
Cyanohydrins are remarkably versatile building blocks for the fine chemical and pharmaceutical industries. The derivatization potential of the combined α-hydroxyl and nitrile functionalities, that defines a cyanohydrin, offers straightforward synthetic routes towards a range of intermediates and active pharmaceutical ingredients (APIs) such as α-hydroxyacids, β-hydroxyamines, α-hydroxyketones.

The main contributor to a cyanohydrin as the intermediate of choice when compared to alternative synthetic routes is cost. Cyanohydrins can indeed be readily prepared from inexpensive hydrogen cyanide (HCN) and the corresponding carbonyl compounds. As a result, synthetic mandelonitrile was first reported by Winkler as early as 1832, only 21 years after Gay-Lussac prepared anhydrous HCN and characterized it.[32] Historically, the preparation of synthetic cyanohydrins even predates that of other nitrogen-containing substances such as amines (discovered by Wurtz in 1849). The toxicity of cyanohydrins, and the potential release of HCN upon decomposition in particular, explain the reluctance to produce these chemicals industrially in their early days. As a result, the production of cyanohydrins industrially started in the 1930s when a better understanding of their toxicity was available and could be controlled on large scale.[32] Still today cyanohydrins are in general considered as in-house industrial intermediates towards more stable and marketable compounds.

The base-catalyzed C-C bond forming reaction of a cyanohydrin from HCN and aldehydes or ketones remains nonetheless a remarkable tool in organic synthesis. Furthermore, the discovery of HNLs as asymmetric biocatalysts in the first half to the 20th century led to the preparation of enantioenriched mixtures of cyanohydrins from prochiral aldehydes and ketones. Over the

years, the growing interest in chiral intermediates for the fine chemical and pharmaceutical industries contributed to the development of robust and cost effective large scale processes for the HNL-catalyzed preparation of chiral cyanohydrins (Figure 1)

Table 2. Cyanohydrins produced industrially (HNL-catalyzed) and the corresponding target compound.[33]

Cyanohydrin	Catalyst and Scale	Target Product	Name (application)
	cat. *Hb*HNL > 50 tons/year		Pyrethroids (Insectisides)
	cat. *Pa*HNL & *Hb*HNL Ton scale		Mandelic Acid (Kinetic resolution reagent)
	cat. *Pa*HNL 10 tons/year		Clopidogrel (Plavix) (Antiplatelet Agent)
	cat. *Pa*HNL 1 ton/year		"Prils" (example: benazepril) (ACE Inhibitors)
	cat. *Hb*HNL kg scale (Process Dev.)		Hydroxy-amine (Chiral intermediate)

The HNL-catalyzed preparation of cyanohydrins has been reviewed extensively[33-37] and a number of challenges have been highlighted together with the various approaches designed to answer them. For instance, the non-catalyzed addition of HCN to the carbonyl carbon is a relatively fast process that had enabled the preparation and isolation of synthetic mandelonitrile as early as 1832. In the HNL-catalyzed system, the rate of the uncatalyzed reaction becomes a major obstacle since it reduces the practical enantiopurity of the cyanohydrin. In order to minimize the effect of the competing non-catalyzed reaction, biphasic systems constituted of an aqueous buffer and first ethyl acetate,[38-40] then ether type solvents such as diethyl ether[41] and

diisopropyl ether[42] proved to be successful. Besides reducing the rate of the non-catalyzed reaction, the extent of cyanohydrin racemization was also decreased significantly in biphasic systems. Miscible co-solvents like ethanol were also evalauted[43,44] but little improvement was observed when compared to an aqueous medium. Decreasing the pH of the aqueous buffer proved to be successful in limiting the rate of decomposition of the cyanohydrin and the competing non-catalyzed addition of HCN[45-49] since the reaction is base-catalyzed with linear dependence on the CN^- concentration in the media. Unfortunately, a low pH is not compatible with the optimum pH range of most HNLs[50,51] and a significant drop in enzymatic activity was observed. Similarly, biphasic systems were often deleterious to enzymatic activity and high catalyst loadings were required for the rate of the reaction to be practical.

Immobilized HNLs

In order to enable enzyme catalysis in biphasic systems, improved operational stability was needed. As a result, the immobilization of HNLs has attracted significant interest. Immobilization of HNLs on supports such as celite,[52] cellulose,[52-55] nitrocellulose,[54,55] poly(vinyl alcohol) hydrogels (Lentikats®),[56] and aqua-gels[57] has been reported to improve the enzyme robustness in biphasic media. Furthermore, immobilization allowed for the recycling of the biocatalyst thereby reducing the cost contribution of the enzyme to the overall process. Immobilization offers a wide range of benefits but no specific method suits all enzymes even within the same class. Consequently, a range of immobilization strategies is typically attempted until the optimized conditions are determined for a specific enzyme.[58] The main drawback of the supported catalyst approach is the support itself. The carrier typically accounts for the largest portion of the catalyst weight. The low

protein content in the biocatalyst translates into poor volumetric activity and lengthy separation from the reaction medium after reaction.[59]

For HNLs, attempts to answer these limitations were first reported with the development of cross-linked-enzyme-crystals (CLECs).[60] Although this immobilization strategy allowed for the stabilization of the enzyme in the absence of a carrier, a number of challenges – inherent to the scale up of protein crystallization[61] – have limited the applications of CLECs.[62] The cross linking of aggregates instead of crystals of the protein circumvented the major drawbacks of the CLEC approach and resulted in the development of a new generation of biocatalysts: Cross-Linked Enzyme Aggregates (CLEA®), where the benefits of a carrier-free immobilized enzyme are retained in a cost effective manner.[63-66]

The development of strategies for suppressing the non-catalyzed (racemic) addition of HCN to the carbonyl compound and the cyanohydrin decomposition is a very good example of biocatalyst development prompted by reaction engineering prerequisites. Nevertheless some aspects of the HNL-catalyzed enantioselective preparation of cyanohydrins remain major challenges to this day. Among them, one has attracted a significant amount of attention in academia: the use of ketones as substrates for HNLs.

The Ketone Case

A rapid review of the carbonyl compounds recognized by HNLs reveals that less than 50 ketones have been described. In comparison, more than 120 aldehydes have been reported. This disparity is all the more surprising since three out of the five HNLs presented in Table 1 have ketones as natural substrates. Furthermore, the conversion obtained in the HNL-catalyzed

preparation of ketone cyanohydrins is acceptable only for alkyl methyl ketones.[34] Although the enantioselectivity of the enzyme is usually high, low conversion is typically obtained for ethyl ketones[47] or methyl aryl ketones.[26,49] The equilibrium position in these substrate/product systems is typically shifted toward the starting material.[32,67]

The preparation of a chiral quaternary centre via enantioselective hydrocyanation of ketones is nonetheless a valuable reaction in the organic chemist's toolbox. Although the thermodynamics of the system are in general not favoured for ketones as substrates a number of interesting results were obtained with carefully selected substrates. For instance the preparation of cyanohydrins derived from substituted cyclopentanones[68] and cyclohexanones[69-72] could be achieved in high yields and stereospecificity. In particular results obtained for 4-substituted cyclohexanone derivatives generated a more in-depth understanding of the active site pocket assignment of MeHNL.[69] Interestingly, single mutants of MeHNL at position W128 resulted in improved conversion and enantiomeric excesses for long chain alkyl ethyl ketones as substrates.[73] This result indicated that the equilibrium position was not limiting the reaction for these substrates. The increase in cyanohydrin concentration was likely to be deleterious to the enzyme activity and active site mutation improved the catalyst performance.

Unfavourable thermodynamics remain the main limitation toward a generally applicable strategy for the HNL-catalyzed synthesis of cyanohydrins from ketones. Kinetic resolution provides a valuable alternative as reported for acetophenone.[74] Interestingly, the enantioselective dehydrocyanation of racemic cyanohydrins from ketones could be carried out in the presence of an aldehyde. The kinetic resolution allowed the generation of HCN *in situ* resulting in the hydrocyanation of the aldehyde in one pot.[75,76] This strategy afforded a mixture of the enantio-enriched cyanohydrins from the respective ketone and

aldehyde. Moreover, the use of a ketone cyanohydrin as cyanide source is less hazardous than that of HCN. This method has been applied successfully with acetone cyanohydrin as cyanide donor for the synthesis of cyanohydrins from aldehydes.[42] However, for the direct synthesis of cyanohydrins from ketones, the thermodynamics of the system with acetone cyanohydrin as cyanide source are even less favourable than with HCN. Furthermore the reaction rates are typically lower with acetone cyanohydrin when compared to HCN.[77] Recently, the successful kinetic resolution of acetates of cyanohydrins from ketones catalyzed by *Subtilisin A* or *Candida rugosa* lipase has been reported by the BOC group in TUDelft as a promising alternative.[78]

Outline of the Thesis

In this thesis I report my investigations on the HNL-catalyzed synthesis of cyanohydrins from ketones.

In order to develop general procedures for the direct synthesis of cyanohydrins from ketones, the main challenge resides in unfavourable thermodynamics. Removing the product *in situ* with subsequent derivatization of the cyanohydrin would shift the equilibrium. This type of *in-situ* cascade reactions[79] has been reported for the conversion of benzaldehyde to mandelic acid.[80] In this case, the derivatization is nevertheless irreversible since the cyanohydrin intermediate cannot be regenerated. Successful derivatization of benzaldehyde into the ethyl carbonate derivative of mandelonitrile was also reported.[81] This type of derivatization was particularly attractive since the cyanohydrin can potentially be recovered by decomposition of the carbonate.

Water in the reaction medium leads to the decomposition of most reagents that would be suitable for derivatization. An organic medium is therefore

required for the implementation of cascade reactions. The biocatalyst stability in organic media is critical here and Chapter 2 concentrates on the catalytic performances in organic media of immobilized HNL as Cross-Linked Enzyme Aggregates (CLEA®) and as sol-gels. In Chapter 3, I will discuss the development of a highly enantioselective CLEA from the HNL from *Linum usitatissimum* (*Lu*CLEA) and its application for the preparation of 2-butanone cyanohydrin in an organic medium. Chapter 4 focuses on the development of one-pot, multistep strategies based on HNL-CLEA catalysis in organic media using benzaldehyde as model substrate. This study provides a general understanding of the stability of various derivatization reagents under the conditions of the enzymatic reaction. Furthermore Chapter 4 highlights some of the key advantages of organic solvents as reaction media for the straightforward implementation of multistep strategies.

In Chapter 5, I report an α,β-unsaturated ketone as a new substrate recognized by HNLs. For the preparation of the corresponding cyanohydrin the kinetic resolution was the method of choice since the equilibrium is shifted toward the ketone. An interesting type of rearrangement of cyanohydrin acetates from α,β-unsaturated ketone is also discussed. One of the key bottlenecks in kinetic resolution remains that the racemic cyanohydrin needs to be prepared which can be challenging as we will see for the ketones studied in Chapter 5. To conclude this thesis, I will report in Chapter 6 the development of straightforward, yet expedient and cost-effective procedures for the preparation of racemic cyanohydrin from unreactive ketones.

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CHAPTER 2

Immobilized HNLs for Enantioselective Synthesis of Cyanohydrins: Sol-Gels and CLEAs

Abstract: The hydroxynitrile lyases (HNLs) from *Prunus amygdalus* (*Pa*HNL), *Manihot esculenta* (*Me*HNL), and *Hevea brasiliensis* (*Hb*HNL) were successfully immobilized in sol-gels. The cross-linked enzyme aggregate (CLEA) of *Hb*HNL was also prepared. These immobilized enzymes and the commercial *Pa*HNL- and *Me*HNL-CLEAs were employed for the enantioselective synthesis of cyanohydrins. The sol-gels were highly efficient at low catalyst loading and particularly stable towards the organic solvent (diisopropyl ether) and substrate/product deactivation. The stabilization effect was inconsistent for CLEAs of different HNLs and significant deactivation of *Pa*HNL- and *Hb*HNL-CLEAs in diisopropyl ether was observed. In contrast commercial *Me*HNL-CLEA proved to be a remarkably robust and efficient biocatalyst in diisopropyl ether.

Adv. Synth. Catal. **2006**, *348*, 1645 – 1654

Introduction

The stability of enzymes in organic solvents can be greatly enhanced by immobilization. Moreover the catalyst can be filtered off easily and no extraction step is required to recover the product of the reaction from the liquid phase. It is also particularly beneficial in the HNL-catalyzed enantioselective cyanohydrin synthesis since the racemic, non-catalyzed, addition of HCN to carbonyl compounds can reduce the enantiopurity of the product significantly. The rate of this competing process depends on the water content and the pH of the water phase in the reaction system.[1,2] By immobilizing HNLs it might become possible to avoid an organic/water biphasic reaction mixture and the racemic background reaction should ideally be completely suppressed. Although several immobilization methods have been employed for HNLs, each having their particular advantages, the reaction conditions under which they have been tested were not always identical. Here, we compare different forms of immobilized HNLs in diisopropyl ether (DIPE), a solvent used industrially for a *Hb*HNL-catalyzed reaction.[3]

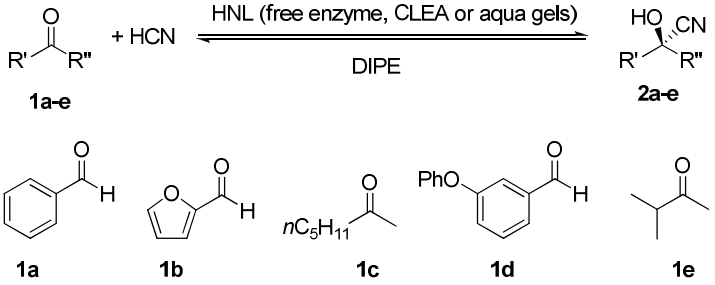
In order to improve the HNLs robustness, immobilization onto supports such as celite,[1] cellulose[1, 4–6] or nitrocellulose[5,6] has been studied. Our attention focuses on immobilization strategies where the HNL can be recycled efficiently. Cross-linked enzyme crystals (CLECs) of the HNL from *Manihot esculenta* (*Me*HNL) were reported earlier as stable and recyclable biocatalysts for the addition of hydrogen cyanide to carbonyl compounds.[7] However, challenges inherent to the preparation of crystals from proteins have limited the range of applications.[8] Attempts to overcome these limitations on a large scale have been reported[9] but a recently established technology based on the cross-linking of enzyme aggregates rather than crystals is now considered a more viable alternative for immobilization.[10,11] Cross-linked enzyme

aggregates (CLEAs) of the HNLs from *Prunus amygdalus* (*Pa*HNL) and *Manihot esculenta* (*Me*HNL) are even commercially available biocatalysts for the enantioselective synthesis of (*R*)- or (*S*)-cyanohydrins, respectively. Poly-(vinyl alcohol) hydrogels (Lentikats®) of *Pa*HNL have been reported as efficient and robust catalysts for the synthesis of (*R*)-mandelonitrile.[12] Furthermore, the *Pa*HNL-CLEA-catalyzed synthesis of cyanohydrins derived from substituted aromatic aldehydes was recently described in microaqueous (2% v/v aqueous buffer in organic solvent) and biphasic systems. Under these conditions the biocatalyst could be recycled up to 10 times.[13]

Another successful immobilization technique is the encapsulation of enzymes in sol-gels. Encapsulation of the HNL from *Hevea brasiliensis* (*Hb*HNL) in a sol-gel matrix was reported recently and the system proved to be efficient for the synthesis of a range of cyanohydrins.[14] Capsules of the sol-gel matrix were filled with buffer solution, thereby forming an "aqua gel" with the enzyme maintained in an aqueous environment. Although some loss of activity was observed upon recycling, the "aqua gel" was still catalytically active in buffer-saturated DIPE.

In this Chapter we compare the catalytic performance of "aqua gels" and cross-linked enzyme aggregates (CLEAs) of (*S*)-selective *Hb*HNL, (*S*)-selective *Me*HNL and (*R*)-selective *Pa*HNL for HCN addition to a range of carbonyl compounds, including the industrially relevant *m*-phenoxybenzaldehyde **1d** (Figure 1).

Figure 1. Substrates investigated.



Results and Discussion

1. Immobilization

When immobilizing a homogeneous catalyst two targets have to be achieved: a high percentage of the catalyst has to be immobilized in its active form, and the immobilized catalyst, here the enzyme, has to be stable. Aqua gels of *Hb*HNL were first prepared according to the reported procedure[14] and 58% of the activity was recovered when starting from a 3.6 kU/mL stock solution, in line with the literature value. The residual methanol in the gel precursor and diffusion limitations are believed to be responsible for the decrease in activity.[14] When diluted *Hb*HNL was encapsulated using the same amount of precursor as in the original procedure, the recovered activity was found to be significantly lower (Table 1). However, it has earlier been observed that the supposedly low activity of an immobilized enzyme in aqueous buffers does not effectively represent its catalytic activity in organic solvents.[13] Indeed, this drop in activity when encapsulating approximately 13 times less *Hb*HNL in the aqua gel is consistent with a diffusion-limited system where the catalyst is immobilized but not accessible enough for a fast activity test such as the decomposition of dilute *rac*-mandelonitrile **2a** in aqueous buffers. Aqua

gels of *Me*HNL and *Pa*HNL were prepared according to the procedure reported for *Hb*HNL, and the recovered activities were again low (Table 1).

Table 1. Enzyme activity recovered upon encapsulation in aqua gels according to activity test described in the Experimental Section.

	***Hb*HNL**	***Hb*HNL**	***Me*HNL**	***Pa*HNL**
Enzyme Stock Solution [U/ml]	3,600	274	230	300
Activity Recovery in Aqua Gel [%]	58	22	8.5	15

When the CLEA of *Hb*HNL (2.61 kU/g) was prepared according to the procedure developed for *Pa*HNL[13] the activity recovered after immobilization was 16%, which is somewhat higher than the recovery value reported for *Pa*HNL-CLEA (9.6%).[13] As described for the *Pa*HNL-CLEA and proposed for the aqua gels above, the low activity is most likely due to diffusion limitations. To compare the different enzymes and the effects of the carriers on them, the aqua gels of *Hb*HNL, *Pa*HNL and *Me*HNL, as well as the CLEA of *Hb*HNL, were used at loadings of 6 U/mmol substrate in the catalytic experiments. Commercially available CLEAs from *Pa*HNL (4.61 kU/g) and *Me*HNL (1.02 kU/g) were also included into the study and standardized at 6 U/mmol of substrate. This amount of catalyst was relatively low when compared to loadings typically used for these enzymes.

The aqua gel-catalyzed reactions were carried out in diisopropyl ether (DIPE) saturated with 50 mM citrate/phosphate buffer (pH 5.0 to suppress the undesired racemic background reaction) to prevent a possible drying effect of the solvent on the catalyst capsules. For the same reason the hydrogen cyanide stock solution in DIPE was also saturated with aqueous buffer. This buffer (pH 5.5) was selected in order to stabilize the hydrogen cyanide solution upon storage and again to avoid the background reaction (see Experimental

Section). The *Pa*HNL-CLEA-catalyzed addition of HCN to a range of aldehydes has been described in microaqueous media (2% v/v buffer in an organic solvent) but under those conditions[13] a water layer can still be observed. In the study reported here the CLEAs were suspended in commercial DIPE in order to avoid the extraction step during the reaction work-up. Under these conditions the organic solvent still contained trace amounts of buffer from the HCN stock solution but the reaction medium was a single liquid phase. Thus, in both the aqua gel and the CLEA system a single organic phase was present. In the case of the CLEAs the enzyme is surrounded by DIPE providing a true one-phase system, in the case of the aqua gel the enzyme is in the aqueous buffer inside the aqua gel and the system is comparable to a biphasic medium. For comparison the reaction was also studied with the free enzymes (6 U/mmol substrate); in this case a biphasic buffer-DIPE system had to be used (see Experimental Section).

2. Catalytic Performances on various substrates

2.1. Benzaldehyde

As a first model reaction the enantioselective addition of HCN to benzaldehyde was studied in DIPE. Almost enantiopure mandelonitrile (**2a**) was obtained at excellent conversions within 4 h when the reaction was catalyzed by free HNLs. Remarkably the aqua gels of all three HNLs catalyzed the same reaction in a much shorter period of time with the same enantioselectivity (Table 2).

Table 2. Conversion ratios and *ee*s (parentheses) at optimum reaction times in the synthesis of mandelonitrile (**2a**) catalyzed by free HNLs and the corresponding aqua gels.

(*S*)-*Hb*HNL		**(*S*)-*Me*HNL**		**(*R*)-*Pa*HNL**	
Free[a]	Aqua Gel[b]	Free[a]	Aqua Gel[b]	Free[a]	Aqua Gel[b]
4h: 97(97)	0.5h: 97(99)	4h: 97(98)	0.5h: 96(99)	4h: 98(97)	2h: 97(97)

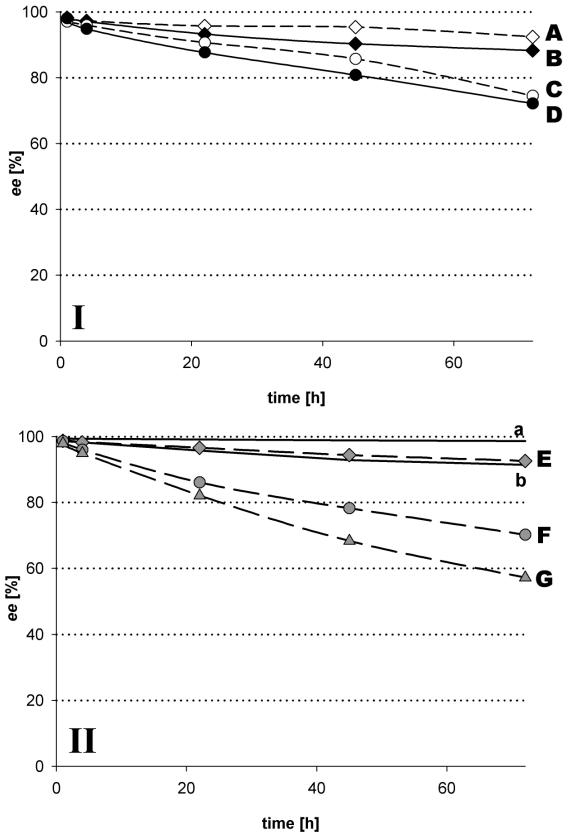
Reaction conditions: Benzaldehyde (0.5 mmol/mL DIPE), HCN (3 equivs.), and the catalyst (6 U/mmol) were shaken at room temperature and the reaction was monitored by GC. [a] The catalyst stock solution was diluted with citrate/phosphate buffer (50 mM, pH 5.0) to a DIPE:aqueous media ratio of 5:1. [b] DIPE saturated with citrate/phosphate buffer (50 mM, pH 5.0).

Under these standard reaction conditions, with relatively low catalyst loading (6 U/ mmol), both the free enzymes and the HNL aqua gels operated well. However, initial rates were higher for HNL aqua gels than for the corresponding free enzyme. Clearly the diffusion limitation that indicated a low enzyme loading in the aqueous activity test (Table 1) was giving misleading results and much less enzyme was deactivated during the immobilization than indicated by this test. Furthermore, the increase in surface area of the aqueous phase for encapsulated HNLs, in comparison with the aqueous environment of homogeneous enzymes, is also believed to be responsible for this effect.

When the reaction was allowed to proceed over an extended period of time, racemization of mandelonitrile **2a** was observed in all cases. This might be due to the chemical background reaction. However, these enzymes do not only catalyze the formation of one enantiomer, they also catalyze its degradation and thus speed up racemization significantly. Rates of racemization for the free enzyme-catalyzed reactions were comparable to the rates observed in a biphasic system for the non-catalyzed racemization of (*S*)-mandelonitrile (Figure 2), demonstrating a deactivation of the enzymes. In the presence of

HNL aqua gels, racemization rates were significantly higher than for the free enzymes indicating the high stability of the aqua gel immobilized enzymes. The racemization of (*S*)-mandelonitrile in the presence of enzyme-free aqua gels proceeded at rates comparable to the non-catalyzed reaction in a biphasic system. Thus the racemization was enzyme induced.

Figure 2. The *ee*s in the HNL-catalyzed synthesis of mandelonitrile. Results in **I**: **A**: free *Pa*HNL, **B**: free *Hb*HNL, **C**: *Pa*HNL aqua gel and **D**: *Hb*HNL aqua gel. **II**: Results of blank racemizations (no enzyme) in DIPE (**a**) and in a biphasic system (**b**); and results from *Me*HNL. **E**: free *Me*HNL, **F**: *Me*HNL aqua gel and **G**: *Me*HNL-CLEA..



Overall the aqua gels of HNLs investigated here had very similar catalytic properties for the synthesis of **2a** although these enzymes come from different plants and are not always structurally related.[15] The performance of cross-linked enzyme aggregates of these HNLs, however, varied greatly. The synthetic activity of *Me*HNL-CLEA was comparable to the results obtained for the free enzyme and *Me*HNL aqua gel while the CLEAs from *Pa*HNL and *Hb*HNL suffered from a noticeable loss of activity under the conditions used and both enzymes catalyzed the reaction very slowly (Table 3). *Hb*HNL and *Pa*HNL are known to be unstable in the absence of water[16,17] and the decrease in activity might be due to the "drying" effect of the reaction medium (DIPE with traces of buffer from the HCN solution) on the catalyst. The rate of racemization for *Me*HNL-CLEA was even higher than observed for the aqua gel of the same enzyme (Figure 2), indicating that the *Me*HNL-CLEA is particularly robust in this single-phase organic reaction medium.

Table 3. Conversion ratios and *ee*s (parentheses) in the synthesis of mandelonitrile catalyzed by CLEAs of different HNLs.

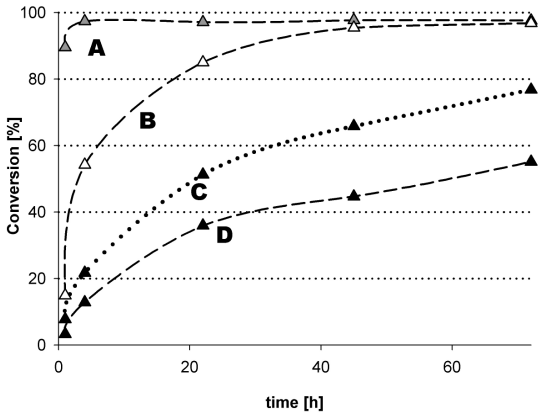
(*S*)-*Hb*HNL CLEA	**(*S*)-*Me*HNL CLEA**	**(*R*)-*Pa*HNL CLEA**
72h: 55(67)	2h: 96(97)	72h: 97(99)

Reaction conditions: Benzaldehyde (0.5 mmol/mL DIPE containing traces of water from the HCN solution), HCN (3 equivs.), and the respective CLEA (6 U/mmol) were shaken at room temperature and the reaction was monitored by GC.

Full conversion was achieved with *Pa*HNL-CLEA over an extended period of time (Figure 3). (*R*)-Mandelonitrile, being the natural substrate of *Pa*HNL, was obtained in high selectivity over the course of the reaction. The CLEA of *Hb*HNL was most dramatically affected by the reaction conditions. The reaction rate and catalyst selectivity were significantly lower than for all other catalysts. When it was employed under microaqueous conditions (Figure 3) its

performance improved significantly, but *Hb*HNL-CLEA remained very sensitive toward deactivation by the organic solvent.

Figure 3. Conversion ratios in the HNL-CLEA catalyzed synthesis of mandelonitrile. **A**: *Me*HNL-CLEA, **B**: *Pa*HNL-CLEA, **C**: *Hb*HNL-CLEA (2% aqueous suspension: "microaqueous"), **D**: *Hb*HNL-CLEA.



The loss of activity observed for CLEAs of *Pa*HNL and *Hb*HNL in DIPE with traces of buffer from the HCN solution was consistent for all the substrates investigated and these catalysts will not be included further in this study. Cross-linked enzyme aggregates are carrier-free biocatalysts where the protein is directly exposed to the reaction media. Relative robustness of HNLs from different sources is therefore revealed to a greater extent in a CLEA than upon encapsulation in an aqua gel where the enzyme is maintained in an aqueous buffer environment. The catalytic performance of *Me*HNL-CLEA has been reported.[18] The remarkable performance of this immobilized form made it a good candidate for a comparison with the aqua gels of HNLs and the free enzymes.

2.2. Furfural

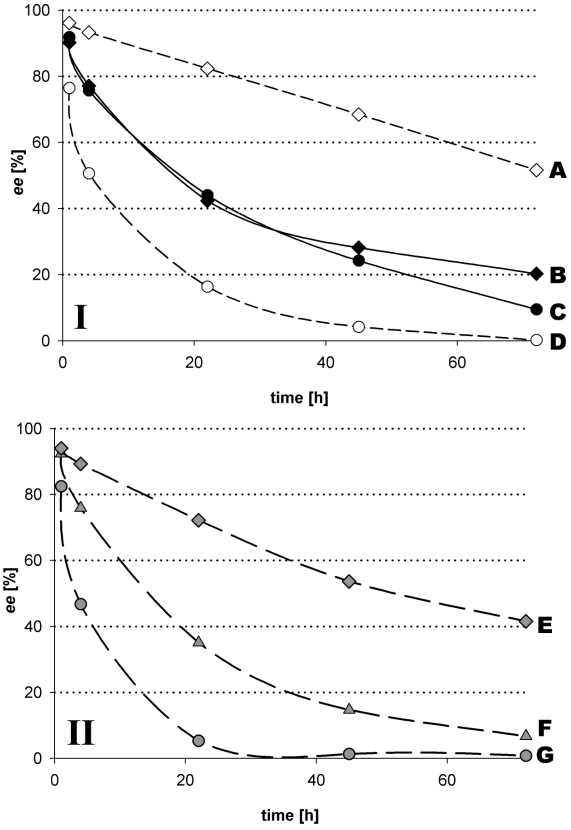
The synthesis of furfural cyanohydrin (**2b**) was achieved within 30 min at high conversion ratios and *ee*s when catalyzed by HNL-aqua gels, free enzymes and the CLEA of *Me*HNL (Table 4).

Table 4. Conversion ratios and *ee*s (parentheses) after 30 min in the synthesis of furfural cyanohydrin

(*S*)-*Hb*HNL		**(*S*)-*Me*HNL**			**(*R*)-*Pa*HNL**	
Free	Aqua Gel	Free	Aqua Gel	CLEA	Free	Aqua Gel
89(94)	89(94)	90(95)	95(87)	94(94)	91(96)	95(82)

Enantiomeric excesses at full conversion were slightly lower for **2b** than for **2a**. Rapid racemization of **2b** accounted for the lower *ee* values obtained. Stabilization of free *Pa*HNL and *Me*HNL upon encapsulation in an aqua gel matrix was observed (Figure 4). This stabilization is much less pronounced for *Hb*HNL. While the free enzyme catalyzed the racemization more efficiently than the other two free HNLs its aqua gel displays the lowest racemization rate.

Figure 4. The *ee*s in the HNL-catalyzed synthesis of furfural cyanohydrin. Results in **I** are presented for *Pa*HNL and *Hb*HNL. **A**: free *Pa*HNL, **B**: free *Hb*HNL, **C**: *Hb*HNL aqua gel and **D**: *Pa*HNL aqua gel. Results for *Me*HNL are presented in **II**. **E**: free *Me*HNL, **F**: *Me*HNL-CLEA and **G**: *Me*HNL aqua gel.



HNLs are susceptible to deactivation/inhibition not only by solvents, but also by the substrate[2,19] and by the product formed. This effect could not be observed in the case of benzaldehyde (**1a**) since the enzyme loadings were standardized according to their catalytic properties in aqueous media for the decomposition of mandelonitrile (**2a**). The extent of substrate/product deactivation/inhibition was therefore leveled out and accounted for in the reaction studied earlier. Using furfural (**1b**), the relative stability of the

individual enzyme toward a specific substrate became apparent. Moreover the greater solubility of this aldehyde in aqueous media is believed to enhance this effect. Indeed, whilst mandelonitrile is sparingly soluble in water,[20] the solubility of furfural is significant[21] (*ca.* 8.2 wt%). The results obtained suggest that the substrate/product deactivation/inhibition phenomenon is significant for *Pa*HNL and *Me*HNL whereas free *Hb*HNL remained active over the course of the reaction (3 days). Furthermore, conversion ratios reported by Griengl et al. for this substrate in biphasic[22] and aqueous[23] media by *Hb*HNL were significantly different (95% and 55%, respectively). This trend seems to indicate that greater concentrations of **2b** in the aqueous phase deactivate or inhibit *Hb*HNL. Aqua gel encapsulation of *Pa*HNL and *Me*HNL improved the stability of these enzymes. The stabilization effect upon immobilization observed for *Me*HNL was also noticed for the CLEA of this enzyme. The CLEA immobilization strategy seems efficacious in preventing substrate/product deactivation/inhibition.

2.3. Hexanal

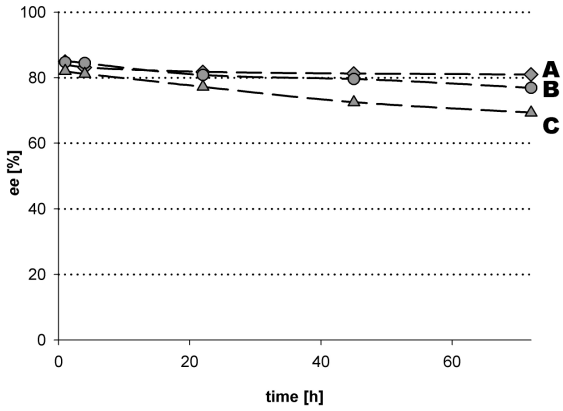
As a representative alkyl substrate the addition of hydrogen cyanide to hexanal was studied. The free enzyme, aqua gel and CLEA form of *Me*HNL achieved full conversion about as fast as for benzaldehyde (Table 5).

Table 5. Conversion ratios and *ee*s (parentheses) at optimum reaction times in the synthesis of hexanal cyanohydrin

(*S*)-*Hb*HNL		**(*S*)-*Me*HNL**			**(*R*)-*Pa*HNL**	
Free	Aqua Gel	Free	Aqua Gel	CLEA	Free	Aqua Gel
4h: 91(94)	2h: 92(94)	3h: 93(84)	3h: 96(85)	3h: 92(81)	3h: 91(87)	3h: 88(85)

Enantiomeric excesses obtained indicate a trend in selectivity for HNLs from different sources for this substrate. Selectivity in the *Hb*HNL-catalyzed reactions was as high as 94% whereas the *Pa*HNL- and *Me*HNL-catalyzed reactions reached *ee* values of only 87% and 85%, respectively. The racemization of **2c** was very limited, as illustrated for *Me*HNL (Figure 5), and could not account for the lower *ee*s observed.

Figure 5. The *ee*s in the *Me*HNL-catalyzed synthesis of hexanal cyanohydrin. **A**: free *Me*HNL, **B**: *Me*HNL aqua gel, **C**: *Me*HNL-CLEA. Racemization rates upon the catalysis of *Hb*HNL and *Pa*HNL (free enzyme and aqua gel) were in the range presented here for *Me*HNL.).



Catalyst selectivity for the addition of hydrogen cyanide to hexanal is clearly higher for *Hb*HNL than *Pa*HNL and *Me*HNL under the conditions used. The enantioenriched mixtures of **2c** racemized at lower rates than those observed for **2a** in the same conditions and only a slight influence was observed for the immobilized enzymes *vs.* the free enzymes. The general trend of enzyme stabilization upon immobilization was consistent with the results obtained for the other substrates.

2.4. *m*-Phenoxybenzaldehyde

The (*S*)-enantiomer of cyanohydrin **2d**, derived from *m*-phenoxybenzaldehyde, is of commercial interest for the preparation of pyrethroid insecticides.[24,25] High conversions and *ee*s have been reported for the addition of hydrogen cyanide to **1d** but the reaction had to be performed over 6 days[26] (50 U/mmol of HNL from *Sorghum bicolor*) or at high catalyst loadings to shorten the reaction time[22] (15 min using 1000 U/mmol of *Hb*HNL). Very good results have also been reported for (*S*)-**2d** using mutant strains of *Me*HNL.[27] In the *Hb*HNL aqua gel-catalyzed formation of **2d**, using acetone cyanohydrin as the cyanide source,[14] high conversion ratios were obtained but the *ee* of the final product was just over 40%, most likely due to very long reaction times. The reaction time was also relatively long in our investigations (2–3 days) but encapsulated HNLs afforded the corresponding cyanohydrin in excellent conversion ratios and *ee*s (Table 6).

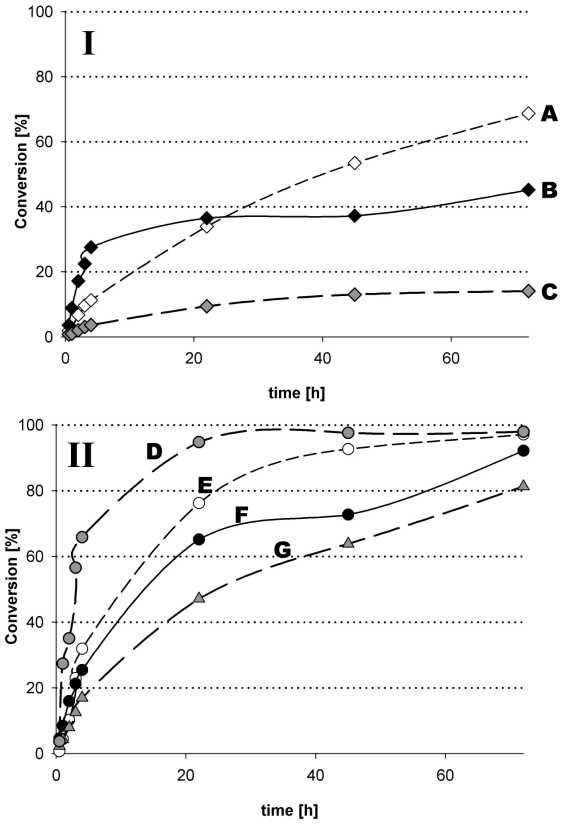
Table 6. Conversion ratios and *ee*s (parentheses) after 72h reaction in the synthesis of *m*-phenoxybenzaldehyde cyanohydrin.

(*S*)-*Hb*HNL		**(*S*)-*Me*HNL**			**(*R*)-*Pa*HNL**	
Free	Aqua Gel	Free	Aqua Gel[a]	CLEA	Free	Aqua Gel
45(82)	92(98)	14(75)	98(97)	81(83)	68(99)	97(99)

[a] Conversion ratio and *ee* after 45h reaction.

The use of hydrogen cyanide evidently improved the performance of the encapsulated catalyst considering that the enzyme loading was much lower than described earlier.[14] Free HNLs catalyzed the reaction but the performance of the enzymes was strongly dependent on the enzyme source (Figure 6).

Figure 6. Conversion ratios in the HNL-catalyzed synthesis of *m*-phenoxybenzaldehyde cyanohydrin. Results in **I** are presented for free enzymes. **A**: *Pa*HNL, **B**: *Hb*HNL, **C**: *Me*HNL. The respective aqua gels and *Me*HNL-CLEA are presented in **II. D**: *Me*HNL aqua gel, **E**: *Pa*HNL aqua gel, **F**: *Hb*HNL aqua gel and **G**: *Me*HNL-CLEA.



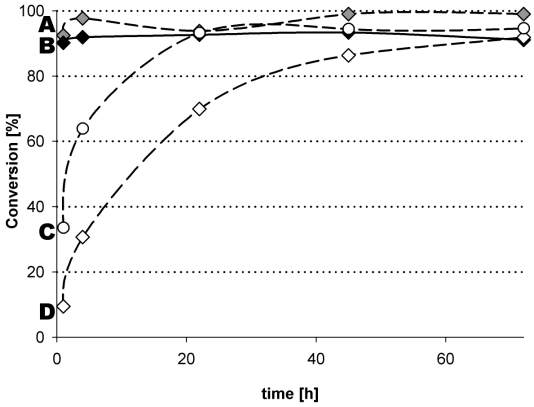
In parallel with the results obtained for furfural (**1b**) the stability of individual enzyme toward the substrate/product of the reaction became apparent. High initial rates were obtained using free *Hb*HNL, but the reaction became very sluggish upon accumulation of the product in the reaction medium, suggesting a cyanohydrin-induced deactivation/inhibition. Free *Me*HNL showed very low activity under the conditions used for the synthesis of

2d. It is noteworthy that the two oxygen-containing substrates investigated (**1b** and **1d**) gave better results for free *Hb*HNL than free-*Me*HNL. On the other hand, the catalytic performances of encapsulated *Me*HNL were in both cases greater than for *Hb*HNL. The stabilization upon encapsulation toward substrate/product deactivation/inhibition for these compounds was therefore more efficient for *Me*HNL than for *Hb*HNL. CLEA immobilization also improved the stability of this enzyme but its overall performance was inferior to that of the aqua gel form. Furthermore, the solubility of *m*-phenoxybenzaldehyde in aqueous media is very limited and the biphasic system used for the free-enzyme-catalyzed reaction did not favor the kinetics of the reaction when compared to the organic media used for immobilized forms. Similar results were obtained for the *Pa*HNL-catalyzed synthesis of (*R*)-**2d**.

2.5. 3-Methyl-2-butanone

Conversions greater than 90% were achieved in the synthesis of 3-methyl-2-butanone cyanohydrin (**2e**) but poor selectivity (*ee*<40%) was observed under the conditions used. Literature results for the *Hb*HNL aqua gel-catalyzed formation of **2e**, using acetone cyanohydrin as a cyanide source,[14] were significantly lower in terms of conversion (below 30%). HCN as cyanide source shifted the equilibrium favourably but catalyst selectivity in the relevant literature[20] was nonetheless higher (>70% *ee*) than in our results. The low catalyst loading was considered unsuitable for this ketone and no acceptable enantiopurity could be achieved for **2e.** Free *Me*HNL and *Hb*HNL were significantly more active than free *Pa*HNL for this substrate (Figure 7).

Figure 7. Conversion ratios in the HNL-catalyzed synthesis of 3-methyl-2-butanone cyanohydrin. **A**: free *Me*HNL, **B**: free *Hb*HNL, **C**: *Pa*HNL aqua gel, **D**: free *Pa*HNL.



Aqua gel encapsulation of *Pa*HNL improved its catalytic activity, but the reaction was still sluggish *vs.* free *Me*HNL and *Hb*HNL. The latter two (*S*)-selective enzymes have ketone-derived cyanohydrins as natural substrates, while mandelonitrile is the natural substrate of *Pa*HNL. Hence, the catalytic activity of HNLs from different sources toward **2e** is in line with the natural substrate preferences of the individual enzymes.

Conclusion

The biocatalysts investigated in this Chapter were active for the addition of hydrogen cyanide, in DIPE, to five structurally different carbonyl compounds. Catalytic performance and robustness were strongly influenced by the reaction media, the substrate/product, the enzyme source and consequently the structure of the enzyme as well as the immobilization method. Encapsulation of HNLs in an aqua gel matrix stabilized them against deleterious effects of the

reaction media and substrate/product deactivation/inhibition. Moreover, it could be demonstrated that a fast and highly enantioselective formation of the desired cyanohydrins could be achieved with much lower enzyme loadings than reported earlier.[13,14,28] However, the reaction conditions used here were not suitable for the preparation of 3-methyl-2-butanone cyanohydrin in high enantiomeric excess and efforts are currently being made to address this limitation.

The stability of cross-linked enzyme aggregates of HNLs in DIPE containing only traces of water was strongly dependent on the enzyme structure. The CLEA prepared from *Hb*HNL as well as the commercial CLEA from *Pa*HNL did not allow an application of these enzymes in a single organic phase. In contrast the *Me*HNL CLEA showed outstanding stability in DIPE. This opens up new opportunities for the application of this enzyme.[18]

Experimental Section

General Remarks

CAUTION: *All procedures involving hydrogen cyanide were performed in a well-ventilated fume hood equipped with a HCN detector. HCN-containing wastes were neutralized using commercial bleach and stored independently over a large excess of bleach for disposal.*

Enzymes: The hydroxynitrile lyase (HNL) from *Prunus amygdalus* (*Pa*HNL, [EC 4.1.2.10], Juelich Fine Chemicals, 300 U/mL) was commercially available in 50% glycerol. More concentrated enzyme solutions from *Hevea brasiliensis* (*Hb*HNL, [EC 4.1.2.39], DSM, 3.6k U/mL), and *Manihot esculenta* (*Me*HNL, [EC 4.1.2.39], Juelich Fine Chemicals, 2.3 kU/mL) were diluted to 274 U/mL and 230 U/mL, respectively, using 25 mM potassium phosphate buffer (pH 6.5).

CLEAs of *Pa*HNL (4.61 kU/g) and *Me*HNL (1.02 kU/g) were prepared according to literature procedures.[13,18]

Chemicals: Diethyl ether solutions of benzaldehyde (Fluka, 99%+), (±)-mandelonitrile (Acros Organics, technical grade, distilled before use), furfural (Acros Organics, 99%), hexanal (Aldrich, 98%), and *m*-phenoxybenzaldehyde (Acros Organics, 97%) were treated with saturated sodium bicarbonate solution prior to each use to remove traces of acid. The organic phase was dried and the solvent was removed under reduced pressure. (*S*)-Mandelonitrile (96% purity, containing 4 mol% benzaldehyde; 99.3% ee) was prepared according to a literature procedure.[18] Ethylene glycol dimethyl ether (glyme, Aldrich, 99.5%), dichloromethane (Aldrich, Anhydrous 99.8%), pyridine (Aldrich, Anhydrous, 99.8%), acetic anhydride (Acros Organics, 99%+), dodecane (Acros Organics, 99%), methyltrimethoxysilane (MTMS, Aldrich, 98%), tetramethoxysilane (TMOS, Fluka, 99%+), glutaraldehyde (Fluka, 25% in water, *ca.* 2. 6M), and 3-methyl-2-butanone (MIPK, Aldrich, 99%) were used as supplied, without further purification. Diisopropyl ether (DIPE, Acros Organics, 98%+, stabilized with 2,6-di-*tert*-butyl-*p*-cresol) was used without further treatment unless otherwise specified. Aqueous buffers were prepared from analytical grade salts and stabilized with 0.09% sodium azide.

Analytical Methods: The course of the reaction was followed by chiral gas chromatography on a Shimadzu Gas Chromatograph GC-14B equipped with a FID detector and a beta-cyclodextrin column (CP-Chirasil-Dex CB 25 m x 0.25 mm). Derivatization of reaction samples (20 µL aliquots) into cyanohydrin acetates, and GC analysis were performed as reported in the literature.[14] Shorter retention times are obtained with helium as carrier gas as compared to nitrogen (Table 7). Depending on the reaction scale, 200 µL or 40 µL additions of *n*-dodecane were used as internal standards to determine conversions and yields. Enantiomeric excess values were calculated from the areas of the

respective cyanohydrin acetate peaks. UV measurements were carried out at 25.0°C on a Varian CARY 3 spectrophotometer.

Table 7. GC retention times (in min) with helium as carrier gas.

	Substrate	Dodecane	(*R*)-Acetate	(*S*)-Acetate
1a	1.16	1.74	4.04	4.52
1b[a]	1.21	3.84	4.91	4.46
1c	1.67	6.92	7.28	7.61
1d	6.74	1.73	9.84	10.06
1e	1.18	18.85	15.37	15.59

[a] CIP rules invert the designation of the respective enantiomers for furfural cyanohydrin. Temperature as ref. [14].

Enzyme Activity Measurements

Enzymatic activity of free enzymes[19] and aqua gels[14] was measured according to reported literature procedures. CLEAs were suspended in 25 mM potassium phosphate buffer (pH 6.5) and samples of this suspension were used to calculate the activity according to the procedure reported for the free enzyme.[19] UV measurements were performed with continuous stirring in order to keep the CLEA suspended.

Preparation of *Hb*HNL-CLEA

*Hb*HNL-CLEA was prepared based on the reported procedure for *Pa*HNL.[13] *Hb*HNL stock solution (frozen at -20°C) was diluted to 1.8 kU/mL in 25 mM potassium phosphate buffer (pH 6.5). The dilute sample (4.5 mL) was allowed to warm up slowly from -20°C to 0°C (ice bath) and glyme (4.5 mL) was then added. Precipitation was allowed to proceed for 15 min while stirring at 0°C and glutaraldehyde (1.5 mL, 25% in water) was then added. The mixture was

stirred at 0°C for 17 h. The CLEA was filtered and rinsed thoroughly with acetonitrile and diethyl ether. After vacuum drying for 4 h, *Hb*HNL-CLEA (496 mg, 2.61 kU/g as determined by the activity test described above) was stored at -20°C.

Hydrogen Cyanide (HCN): 2M Solution in DIPE

Sodium cyanide (49 g, 1.0 mol) was dissolved in a mixture of water (100 mL) and diisopropyl ether (DIPE) (250 mL) at 0°C. The biphasic system was stirred vigorously for 15 min and 30% aqueous HCl (100 mL) was added slowly. This mixture was allowed to warm slowly to room temperature (at least 25 min). The phases were separated and 150 mL of DIPE was added to the organic layer. The combined organic phases were stirred and residual water was separated. This procedure was repeated with another 100 mL of DIPE. The 2M standard HCN solution[29] was kept over citric acid buffer (pH 5.5) in the dark.

Free HNL-Catalyzed Synthesis of Cyanohydrins

The HNL (30 units) from *Prunus amygdalus* (300 U/mL), *Manihot esculenta* (230 U/mL) or *Hevea brasiliensis* (274 U/mL) was diluted in 50 mM citrate/potassium phosphate buffer (pH 5.0) to a total volume of 2 mL. Diisopropyl ether (2.5 mL) was then added followed by the carbonyl compound of interest (5 mmol) and *n*-dodecane (200 µL). An analytical sample representative of initial conditions (5 µL) was drawn from the organic layer and diluted in DIPE (1 mL) for GC analysis. The reaction was initiated by addition of 2M HCN in DIPE (7.5 mL, 3 equivs.) and monitored by chiral GC over three days while shaking the sealed flask at room temperature.

HNL Aqua Gel-Catalyzed Synthesis of Cyanohydrins

500 µL of *Pa*HNL (300 U/mL), *Me*HNL (230 U/mL) and *Hb*HNL (274 U/mL) were encapsulated into aqua gels using 500 µL of precursor prepared according to the literature.[14] The exchange of aqueous buffers in the gels was allowed to proceed over two days. Gels were then ground into a fine powder and used immediately. The scale of the reaction (Table 8) was adapted from the activity recovery (Table 1) to suit 6 U/mmol catalyst loading. The *Hb*HNL sol gel reaction is given as a representative procedure.

Table 8. Reaction conditions for the synthesis of cyanohydrins catalyzed by aqua gels.

	***Hb*HNL**	***Me*HNL**	***Pa*HNL**
Residual activity in aqua gel[a] [Units]	30	9.8	22.5
Reaction Scale[b] [mmol substrate]	5	1.6	3.75
Catalyst Loading [U/mmol]	6	6.1	6

[a] Calculated for a gel prepared from 500 µL of precursor and 500 µL of HNL dilute solution. [b] All other reaction parameters were scaled accordingly.

The aqua gels were ground into a fine powder and DIPE (2.5 mL) saturated with 50 mM citrate/potassium phosphate buffer (pH 5.0) was added. The substrate (5 mmol) and *n*-dodecane (200 µL) were dissolved into the mixture. A GC sample was taken to determine initial conditions and the reaction was initiated by addition of 2M HCN in DIPE (7.5 mL, 3 equivs.). The reaction was monitored by chiral GC over three days while shaking the sealed flask at room temperature.

HNL-CLEA-Catalyzed Synthesis of Cyanohydrins

The procedure given here for *Hb*HNL-CLEA was scaled accordingly for individual CLEAs to suit 6 U/mmol loading of catalyst. *Hb*HNL-CLEA (30 units, 2610 U/g) was suspended in commercial DIPE (2.5 mL). The starting material (5 mmol) and *n*-dodecane (200 µL) were added to the mixture and a GC sample was taken to determine initial conditions. The reaction was initiated by addition of 2M HCN in DIPE (7.5 mL, 3 equivs.) and monitored by chiral GC over 3 days while shaking in a sealed flask at room temperature. The *Hb*HNL-CLEA-catalyzed synthesis of mandelonitrile in microaqueous media was performed by first suspending the catalyst (30 units) in 200 µL of 50mM citrate/potassium phosphate buffer (pH 5.0) and the procedure was followed accordingly.

Racemization of (*S*)-Mandelonitrile

The non-enzymatic racemization of (*S*)-mandelonitrile was based on the conditions at full conversion of the synthetic reaction. Conditions in the biphasic system used for the free enzyme were reproduced by adding HCN (2 equivs., 5 mL) to a mixture of DIPE (4 mL) and 2 mL of 50 mM citrate/potassium phosphate buffer (pH 5.0). The reaction was initiated by addition of (*S*)-mandelonitrile (1 mL) from a 5 mmol/mL stock solution in DIPE:dodecane (4:1) and monitored by chiral GC. The racemization reaction described above was repeated in commercial DIPE in order to model the conditions of the CLEA-catalyzed reaction. The influence of the carrier in aqua gels was evaluated from the rate of racemization of (*S*)-mandelonitrile in the presence of enzyme-free aqua gels prepared from 500 µL of precursor and 500 µL of 50mM citrate/potassium phosphate (pH 5.0). Enzyme free gels were ground into a fine powder and DIPE (4 mL) saturated with 50 mM citrate/potassium phosphate buffer (pH 5.0) was added followed by HCN (5

mL, 2 equivs.). The reaction was initiated by addition of the (*S*)-mandelonitrile stock solution described above (1 mL) and monitored by chiral GC over three days while shaking in a sealed flask at room temperature.

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CHAPTER 3

Linum usitatissimum HNL CLEA: A Recyclable Enantioselective Catalyst

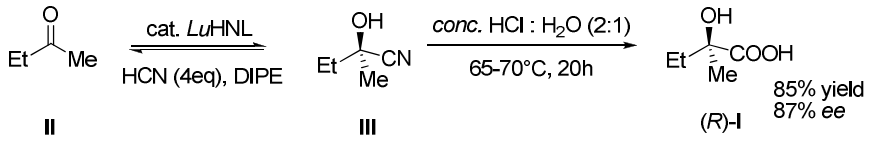
Abstract: An immobilized form of the hydroxynitrile lyase from *Linum usitatissimum* (*Lu*HNL) as cross-linked enzyme aggregates (CLEA) with high specific activity (303.5U/g) and recovery (33%) was developed. Molecular imprinting using 2-butanone as additive in the immobilization process improved the synthetic activity of the biocatalyst. *Lu*CLEA could be partially recycled for the synthesis of (*R*)-2-butanone cyanohydrin in preparative scale over two batches. The enantioenriched cyanohydrin obtained was further hydrolyzed to give (*R*)-2-hydroxy-2-methylbutyric acid in 85% yield (from 2-butanone) and 87% *ee*.

Adv. Synth. Catal. **2008**, *350*, 2329 – 2338

Introduction

The enantioselective synthesis of quaternary stereogenic carbon atoms is a major challenge in organic chemistry. In particular chiral tertiary alcohols are at the centre of attention. Due to their importance in the pharmaceutical industry and the drive towards environmentally benign synthesis,[1,2] catalytic routes are the focus of current research.[3] 2-Hydroxy-2-methyl-butyric acid (**I**) is such a building block. It is, for two reasons, a particularly interesting synthetic challenge. (1) Its *S*-enantiomer is used for the preparation of a COX-2 specific inhibitor,[4] while its *R*-enantiomer forms part of several biologically active natural products, such as the Clerodendrins,[5-7] Protoveratrine A[8,9] and Germinalinine.[10] (2) The synthesis of **I** via the enantioselective addition of cyanide to the prochiral butanone and subsequent hydrolysis involves a daunting stereo-differentiation between a methyl and an ethyl group (Scheme 1). While chemical catalysis has so far proven rather unsuccessful for this challenge,[11-14] enantioselective biocatalysis using hydroxynitrile lyases (HNL) is the way forward to prepare both the *R* and *S*-enantiomer of **I**.[11,12,15]

Scheme 1. Synthetic approach toward (*R*)-enantioenriched 2-hydroxy-2-methylbutyric acid: (*R*)-**I**.



Recently a HNL-based pathway to *S*-**I** was described in which butanone was "disguised", i.e. an auxiliary was introduced in order to ease stereodifferentiation.[16] This elegant approach has the drawback that its atom economy is poor,[1,2] comparable to the chemical chiral auxiliary route.[17] For

the *R*-enantiomer of **I**, *Prunus amygdalus*, *Prunus mume*, and *Linum usitatissimum* HNL have been employed. However, *Prunus amygdalus* and *Prunus mume* enzymes displayed moderate enantioselectivity[18,19] while the *Linum usitatissimum* HNL (*Lu*HNL) was reported to give good to very good enantiopurities of the cyanohydrin intermediate.[20,21] In Chapter 2, we described that immobilizing the HNLs from *Prunus amygdalus*, *Hevea brasiliensis* and *Manihot esculenta* as CLEAs can improve their stability and ease their recycling.[22-24] In particular their application in organic solvents became possible. All these parameters are essential for the repeated and sustainable application of enzymes in a green process.[1,2,25-27] In this third Chapter, we describe the preparation of a CLEA from *Lu*HNL (*Lu*CLEA) and its application in the synthesis of *R*-**I**.

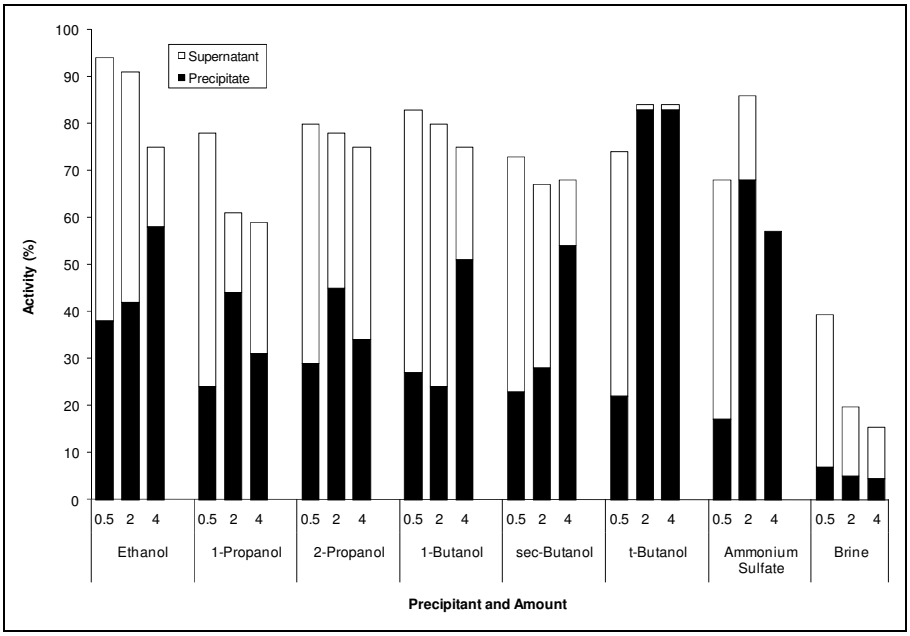
Results and Discussion

1. Development of *Lu*CLEA

1.1. Enzyme Precipitation

The selection of precipitation parameters (nature and amount of co-solvent, duration, and temperature) is a critical step in the preparation of active CLEAs with a high recovery of enzyme activity.[24,28] A rapid evaluation of *Lu*HNL sensitivity towards temperature and length of exposure to a co-solvent prompted us to select conditions that allow fast precipitation at 0°C (data not shown). A screening procedure could then be developed to determine the best co-solvents from a range of alcohols and saturated aqueous salt solutions in various amounts (Figure 1).

Figure 1. Activity recovery (% of commercial enzyme solution used) in the precipitate (black) and supernatant (white) after enzyme precipitation using alcohols and saturated saline solutions[a].

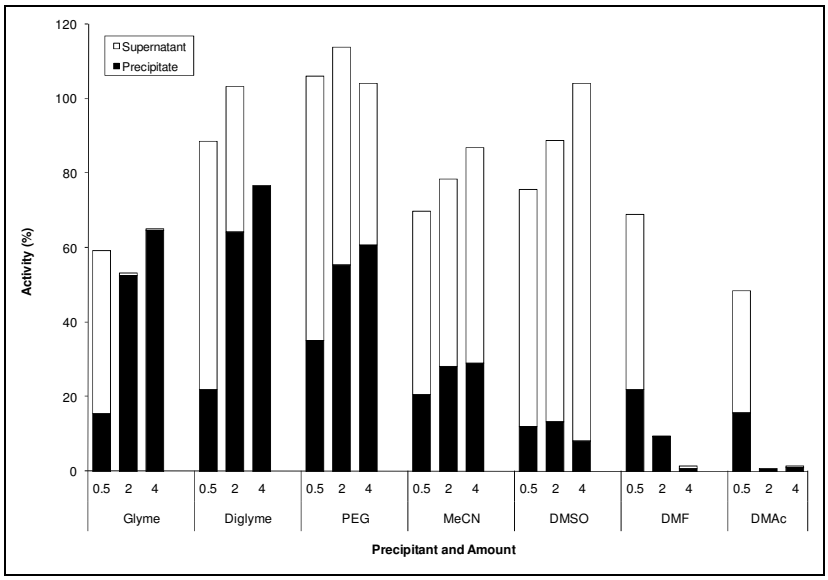


[a] The amount of precipitant is expressed as a factor (in volume) of the commercial enzyme solution used.

Ideally, the co-solvent should provide 100% activity recovery (no deactivation) in the form of an aggregate (no enzyme left in solution). One particular solvent: *t*-butanol, in amounts greater than twice (v/v) the volume of commercial *Lu*HNL solution gave very satisfying results in this regard. Saturated aqueous ammonium sulfate (sat. $(NH_4)_2SO_4$) also provided a fair amount of precipitate with little deactivation and soluble enzyme left when it was used in amounts greater than twice the initial volume of enzyme solution. The other alcohols screened here did not provide efficient precipitation since a large amount of enzyme was left in the solution. Brine had a severe deleterious effect on the enzyme activity. We continued this screening of co-solvents with a range of water-miscible organic solvents and PEG (Figure 2). Glyme and

diglyme stood out as relatively good candidates for precipitation in this screening. The extent of deactivation was particularly significant with *N*,*N*-dialkylamide type solvents such as dimethylformamide (DMF) and dimethylacetamide (DMAc) while PEG, DMSO and acetonitrile left a significant amount of active enzyme in solution.

Figure 2. Activity recovery (%) in the aggregate and the supernatant after enzyme precipitation using organic solvents and PEG[a].



[a] The amount of precipitant is expressed as a factor (in volume) of the commercial enzyme solution used.

Interestingly the influence of precipitants on the activity of *Lu*HNL was consistent with earlier investigations on suitable water-miscible co-solvent in the *Lu*HNL catalyzed conversion of 2-butanone into its corresponding cyanohydrin.[20] Based on the results obtained in the precipitation study, four co-solvents (*t*-butanol, sat. $(NH_4)_2SO_4$, glyme and diglyme) were selected for the cross-linking study.

1.2. Cross-Linking of Aggregates

Although precipitation aims at shaping the physical state of the aggregates while maintaining the enzyme activity,[29] the cross-linking step is no less important to the successful preparation of an active CLEA. Efficient cross-linking will indeed "lock" the enzyme into its active state and prevent redissolution (leaching) during reaction.[26] Moreover cross-linking determines the particle size and contributes to making the biocatalyst more robust toward deleterious effects such as substrate/product inhibition or organic solvent deactivation during the reaction.[23] Traditionally, glutaraldehyde is preferred as a cross-linking agent as it is commercially available and inexpensive. We investigated the activity recovery in the CLEA after cross linking using 5%, 10%, 20% and 30% (v/v of the commercial enzyme solution used) of glutaraldehyde for each of the four co-solvents selected in the precipitation study. The amount of each precipitant used was also screened in order to fine tune this parameter when combined with the cross-linking step.

When glyme was used as a precipitant, no satisfactory results could be obtained and this co-solvent was not considered further. The optimized conditions using the other three co-solvents (Table 1) also indicated that diglyme was the least preferable and we did not consider this precipitant further.

Table 1. Cross-linking study; Optimized results for each precipitant.

Co-solvent[a]	**Cross-linker**[b]	**Activity Recovery**[c]
t-Butanol (2)	10	19.5 (0)
sat. $(NH_4)_2SO_4$ (4)	10	53 (8)
Diglyme (3)	5	17 (3)

[a] Precipitant and amount (parentheses) as a factor of the volume of commercial enzyme solution used. [b] Amount of glutaraldehyde 25% aqueous solutions as a percentage (v/v) of the volume of commercial enzyme solution used. [c] Activity recovery (%) in the CLEA based on the initial amount of enzyme used (in units). The number in parentheses indicates the percentage of activity present in the buffer used to wash the CLEA (enzyme not immobilized).

The CLEAs prepared using optimized amounts of *t*-butanol and sat. $(NH_4)_2SO_4$ as precipitants and glutaraldehyde as cross-linker (Table 1) were selected for further study. We also noticed that although *Lu*HNL shares several structural homologies with the Zn^{2+}-containing alcohol dehydrogenases (ADHs).[21,30] efficient cross-linking could be achieved using glutaraldehyde while dextran polyaldehyde had to be used to prepare CLEAs of the ADH from *Lactobacillus brevis* since glutaraldehyde deactivated the enzyme.[31] This observation further highlights the need to optimize the conditions for CLEA preparation independently of results obtained for similar enzymes.

1.3. Enzyme Aggregates (EAs) and other HNLs

The results obtained so far in the study seem to indicate that the relatively low activity recovery in the CLEA is mostly due to the cross-linking step. For instance, when *t*-butanol was used as precipitant the recovered activity was greater than 80% after precipitation and below 20% after cross-linking. Since we aimed at developing an immobilized version of *Lu*HNL that can perform in buffer-saturated organic solvent as we reported earlier for *Me*CLEA,[23] a simple aggregate of *Lu*HNL (without cross-linking) could also be considered as

an immobilized catalyst as it would be insoluble in the reaction media. Enzyme aggregates of *Lu*HNL (*Lu*EA) were therefore prepared using *t*-butanol and sat. $(NH_4)_2SO_4$ in a scale-up (factor 10) of the optimized conditions for precipitation. The preparation of CLEAs from these two precipitants was also scaled up accordingly and a significant drop in activity recovery was observed (Table 2) when compared to the results obtained on a smaller scale as described in the cross-linking study. The specific activity and activity recovery for CLEAs of *Pa*HNL and *Me*HNL were also measured for comparative purposes (Table 2).

Table 2. Specific activity and activity recovery of CLEAs and EAs.

Biocatalyst	Precipitant	X-linking	Activity[a]	Recovery[b]
*Lu*CLEA(Am.Sulf)	sat. $(NH_4)_2SO_4$	Yes	180.5 U/g	20%
*Lu*EA(Am.Sulf)	sat. $(NH_4)_2SO_4$	No	9.9 U/g	5%
*Lu*CLEA(*t*-Bu)	*t*-butanol	Yes	110.9 U/g	6%
*Lu*EA(*t*-Bu)	*t*-butanol	No	221.9 U/g	9%
*Pa*CLEA[c]	Glyme	Yes	Not active	*nd*
*Me*CLEA[c]	sat. $(NH_4)_2SO_4$	Yes	847.1 U/g	36%

[a] Specific activity per g of solid measured according to the standard procedure for *Lu*HNL. [b] Activity recovery (%) in the catalyst based on the initial amount of enzyme used (in units). [c] Prepared according to literature procedure.[22,24] *nd*: not determined.

Better recovery was obtained when the CLEA was prepared from sat. $(NH_4)_2SO_4$ than from *t*-butanol as a co-solvent as observed on a smaller scale. *Lu*EA(Am.Sulf) was poorly active which was attributed to a large excess of salt ($(NH_4)_2SO_4$) in the catalyst. The EA was indeed washed with acetonitrile since an aqueous buffer would redissolve the enzyme aggregate. As a result some of the salt precipitated upon addition of MeCN resulting in low specific activity (< 10U/g) and overall recovery (5%). To the contrary, the specific activity of *Lu*EA(*t*-Bu) was twice as high as for *Lu*CLEA(*t*-Bu) and the activity recovery was also improved. *Pa*CLEA was not active in the conditions of the standard

activity test which was attributed to the relatively low pH (4.1). On the other hand, the very high specific activity and activity recovery measured for *Me*CLEA are consistent with an earlier report on the robustness and high activity of this biocatalyst.[23]

1.4. Molecular Imprinting

The use of surfactants and crown ethers as additives in the preparation of CLEAs has been reported[28] and very significant improvements of the biocatalyst activity could be observed in some instances. These additives were selected in order to stabilize the enzyme leading to a more robust biocatalyst and to modify the enzyme conformation into a more active state that could be "locked" by immobilization. Our approach to additives for the development of immobilized *Lu*HNL was closer to the concept of molecular imprinting[32-34] where the enzyme could potentially be immobilized in its enzyme-substrate complex conformation. A cyanohydrin as substrate for this purpose would be problematic since its decomposition into the corresponding carbonyl compound and HCN would be difficult to prevent without complete inhibition of the enzyme. However, in the absence of HCN, a carbonyl compound that can bind to the active site of *Lu*HNL in solution and be washed away easily after immobilization would be suitable. Moreover the additive should be water-soluble, preferably inexpensive and a low boiling point would be an advantage in order to remove traces in vacuum. Considering these requirements we selected 2-butanone as an additive and studied its influence on the preparation of CLEAs and EAs (Table 3).

Table 3. Influence of 2-butanone as additive on the immobilization process.

Biocatalyst	2-Butanone	Activity (Difference[a])
*Lu*CLEA(Am.Sulf)	Without	180.5 U/g
	With	195.1 U/g (+8%)
*Lu*EA(Am.Sulf)	Without	9.9 U/g
	With	na[b]
*Lu*CLEA(*t*-Bu)	Without	110.9 U/g
	With	160.1 U/g (+44%)
*Lu*EA(*t*-Bu)	Without	221.9 U/g
	With	71.6 U/g (-68%)

[a] Difference in specific activity between the biocatalyst prepared with and without 2-butanone as additive (see Experimental Section). [b] not applicable (catalyst not active when 2-butanone was used as additive).

We used a standard 1µL of 2-butanone per unit of *Lu*HNL as a reference and observed significant differences in the specific activities of *Lu*EA(*t*-Bu) and *Lu*CLEA(*t*-Bu). A drop in activity was indeed observed for the EA whereas the CLEA specific activity increased very significantly. Since the EA redissolves in the aqueous buffer of the standard activity test thereby restoring the free enzyme conformation (non-complexed) one would not expect a significant difference in activity. In contrast, a CLEA retains the structural features imposed during immobilization even in an aqueous buffer. Hence, we conclude that the enzyme-substrate complex was indeed formed but the benefits of the additive could only be retained in the biocatalyst after "locking" the structure by cross-linking. The additive had little effect on the activity of *Lu*CLEA(Am.Sulf) which suggests that *t*-butanol also plays a role in coordinating to the active site during immobilization but the enzyme conformation obtained with *t*-butanol can be modified further to the enzyme-2-butanone complex allowing immobilization in an even more active form. The

deleterious effect of the additive was observed on *Lu*EA(Am.Sulf) that was rendered totally inactive.

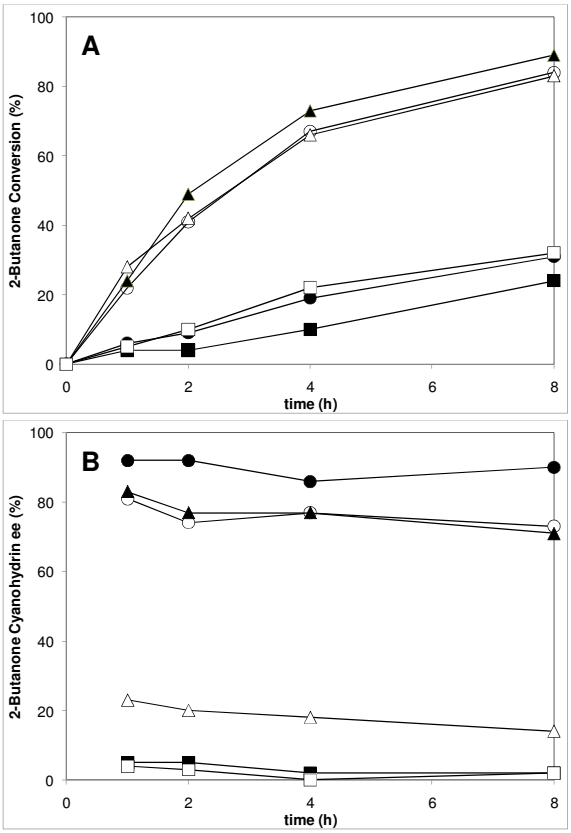
These investigations on the parameters that influence immobilization of *Lu*HNL in the form of an EA or CLEA led us to select five immobilized forms for synthetic applications: *Lu*CLEA(Am.Sulf) (no additive), *Lu*CLEA(*t*-Bu) (with and without additive), and *Lu*EA(*t*-Bu) (with and without additive). *Me*CLEA was also selected as a standard for a robust and active biocatalyst.

2. Synthetic Application

2.1. Catalyst Selection

The stereodifferentiation in 2-butanone is a challenge, in particular since the racemic non-catalyzed addition of HCN to the ketone strongly depends on the reaction conditions. In an uncatalyzed control experiment using a biphasic system (DIPE : Buffer pH=4.1) the conversion of 2-butanone into its racemic cyanohydrin was indeed 16% in 2h. When the control experiment was performed in buffer saturated DIPE the conversion of 2-butanone was still 9% after 48h. We therefore decided to screen the immobilized forms of *Lu*HNL for the synthesis of 2-butanone cyanohydrin in buffer saturated DIPE as suggested in Scheme 1. The conversion of 2-butanone (**II**) and the *ee* of the cyanohydrin formed (**III**) were monitored to evaluate the synthetic activity and selectivity of the catalyst respectively (Figure 3).

Figure 3. *Lu*HNL and *Me*CLEA-catalyzed conversion of 2-butanone (**A**) and *ee* of the formed cyanohydrin (**B**).



Reaction Conditions: 2-butanone (0.50 mmol), HCN (4 eq), biocatalyst (2.2 U), RT, in buffer sat. DIPE (1.00 mL). ●: *Lu*CLEA(Am.Sulf), ○: *Lu*CLEA(*t*-Bu), ▲: *Lu*CLEA(*t*-Bu) prepared using 2-butanone as additive, △: *Me*CLEA, ■: *Lu*EA(*t*-Bu), □: *Lu*EA(*t*-Bu) prepared using 2-butanone as additive.

The catalysts could be clearly divided into two categories based on their synthetic activity (Figure 3, A). *Lu*CLEA(*t*-Bu) (with and without additive) performed exceptionally well under the above selected conditions, matching the 2-butanone conversion profile obtained with *Me*CLEA. *Lu*CLEA(*t*-Bu) prepared in the presence of 2-butanone performed even better than the standard *Me*CLEA (Figure 3, A). *Lu*CLEA(Am.Sulf) and the EAs performed

poorly under these conditions, the EA prepared with 2-butanone being slightly more active. We investigated the effect of organic solvents on *Lu*CLEA(Am.Sulf) by washing the freeze-dried catalyst with acetonitrile, ethyl acetate and then diethyl ether and only 31% of the activity could be recovered. The specific activity of *Lu*CLEA(Am.Sulf) had also decreased greatly to 39.8U/g after washing. These results indicated a severe deleterious effect of the media for this catalyst.

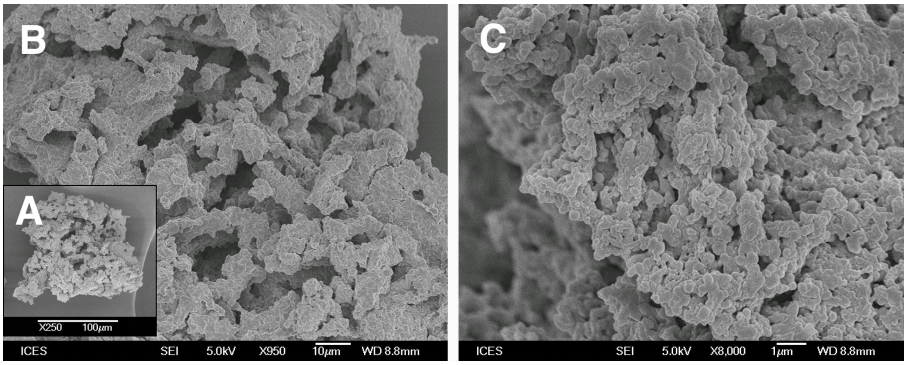
Next to its excellent activity, *Lu*CLEA(*t*-Bu) (with and without additive) also showed good selectivities (Figure 3, B). 2-butanone as additive did not seem to affect the *ee* profile. This indicates that, the imprinting strategy only affected the catalyst activity but not its selectivity. Difficult stereodifferentiation for this substrate was reflected in the low selectivity observed for *Me*CLEA (*ee* around 20% under the reaction conditions) which was consistent with earlier reports for this enzyme-substrate combination.[35]

This evaluation of the synthetic activity highlighted the importance of selecting an immobilized biocatalyst not only from the results in the standard activity test but from its performance under actual reaction conditions. The standard activity test proved to be a fast and efficient method to narrow the range of suitable conditions for the development of immobilized *Lu*HNL but only the evaluation of the synthetic activity allowed us to select *Lu*CLEA(*t*-Bu) prepared using 2-butanone as additive (referred to as *Lu*CLEA from here on) as the best candidate for synthetic application in buffer saturated organic solvent.

SEM analysis of *Lu*CLEA (Figure 4) revealed relatively large particles of ca. 100µm (Figure 4, A). The internal structure of *Lu*CLEA is organized as a network of "branches" (Figure 4, B) separated by pores of about 10µm. Enlargement of these "branches" showed aggregates in the range of 500nm

(Figure 4, C). The amalgams of these aggregates created a second level of pores of about 1µm.

Figure 4. SEM photographs of *Lu*CLEA.



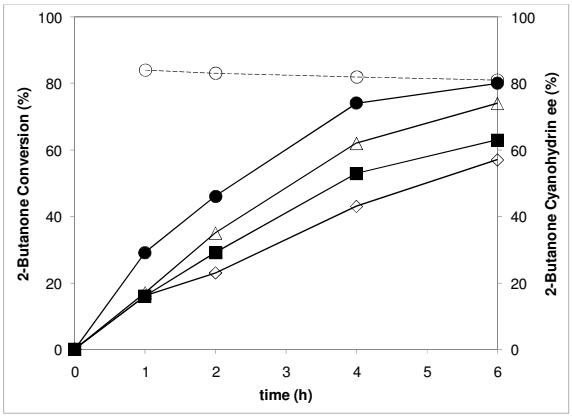
A: x250, **B**: x950, and **C**: x8,000.

This arrangement is typical of CLEAs of HNLs such as the highly active *Me*HNL.[24] The structure maximizes the catalyst surface available for reaction (amalgams of small aggregates separated by pores) while minimizing the diffusion effect within the catalyst (branch-like sub-structure).

2.2. Catalyst Recycling

We investigated the recycling ability of *Lu*CLEA on a suitable scale to minimize catalyst loss during filtration between cycles. The catalyst loading was increased to 8 U/mmol and the temperature set at 30°C. The reaction was monitored over 6h under these conditions for four consecutive cycles (Figure 5).

Figure 5. *Lu*CLEA recycling experiment in the preparation of 2-butanone cyanohydrin (5 mmol scale).



Reaction Conditions: 2-butanone (5.0 mmol), HCN (4 eq), *Lu*CLEA (40 U), 30°C, in buffer sat. DIPE (10 mL). ●: Conversion of 2-butanone (fresh catalyst), ○: *ee* of 2-butanone cyanohydrin (fresh catalyst). △: Conversion (Cycle 1), ■: Conversion (Cycle 2), ◇: Conversion (Cycle 3).

When the reaction was catalyzed by fresh catalyst, good conversion and selectivity was achieved in a relatively short time (6h). *Lu*CLEA activity decreased with each cycle and therefore could not be reused directly for repeated batches. In an attempt to further improve the overall selectivity, the reaction was performed with fresh catalyst at 5°C under otherwise identical conditions (data not shown) but the reaction became extremely slow (7% conversion after 2h) while the selectivity improved only very slightly (84% *ee* after 2h). At 30°C, racemization of 2-butanone cyanohydrin was not significant (Figure 5). The catalyst selectivity was nonetheless maintained upon recycling (Table 4).

Table 4. 2-Butanone cyanohydrin obtained upon recycling: isolated yield after 6h, *ee*, and residual substrate content.

Catalyst	Yield[a]	*ee*[b]	Residual Substrate[c]
Fresh CLEA	84	81	<1%
Cycle 1	76	80	<1%
Cycle 2	64	81	<1%
Cycle 3	56	78	<1%

[a] Isolated yield (%). [b] *ee* of the isolated product. [c] in mol% as determined by ^{1}H NMR.

A straightforward filtration and evaporation under reduced pressure yielded the crude product which contained less then 1% of residual substrate (Table 4.). The loss of activity upon recycling without decrease in selectivity was low enough to consider adding a fraction of fresh catalyst between batches in order to compensate for this effect. This approach is particularly suitable for a carrier-free immobilized catalyst with very high volumetric activity such as CLEAs since the catalyst accounts for a small percentage of the overall reaction volume.

2.3. Synthesis of (R)-2-hydroxy-2-methylbutyric acid

Our approach for the preparation of (*R*)-2-hydroxy-2-methylbutyric acid (**(*R*)-I**) in high enantiopurity (Scheme 1) involved the direct synthesis of the cyanohydrin (**III)** from 2-butanone (**II**) catalyzed by *Lu*CLEA followed by acid hydrolysis to the corresponding α-hydroxy acid (**I**) as reported for *Pa*HNL.[18]

We first optimized *Lu*CLEA preparation on 90mL scale and noticed that freeze drying affected the biocatalyst activity. From results obtained in earlier samples it was clear that the CLEA could not be freeze-dried overnight and should be promptly stored at -20°C when the biocatalyst is sufficiently dry. We investigated this effect by measuring the specific activity of the CLEA as a function of the freeze drying duration and established that a ratio of 1 g CLEA

obtained per 12 mL of commercial *Lu*HNL solution gave a sufficiently dry catalyst with very high specific activity (303.5 U/g) and recovery (33%).

*Lu*CLEA was then used in the synthesis of (*R*)-2-butanone cyanohydrin on 80 mmol scale and the reaction was complete in 3h at 30°C. Following the conclusions of the recycling experiments, *Lu*CLEA was recycled for a second batch and a portion of fresh catalyst (20% of the original loading) was added to compensate for the loss of activity. The second batch was equally fast, selective and high yielding thereby allowing the preparation of (*R*)-2-butanone cyanohydrin in 160 mmol scale over two 3h batches using an overall catalyst loading of 4.8 U/mmol. The *ee* of the cyanohydrin formed was also improved significantly to 87% which was attributed to the optimized catalyst used here. This *ee* value comes close to a literature report for *Lu*HNL immobilized on nitrocellulose on an analytical scale (*ee* = 95%). Due to the analytical conditions a ten times higher catalyst loading (50 U/mmol) could be employed. This helped to suppress the fast racemic chemical reaction even better than under the conditions described here.[21] The crude cyanohydrin easily obtained by filtration of the catalyst and evaporation of the volatiles under reduced pressure was subjected to acid hydrolysis to give (*R*)-2-hydroxy-2-methylbutyric acid in 85% isolated yield (from 2-butanone) and 87% *ee*. The optical purity of the acid can readily be improved by a very efficient crystallization using enantiopure 1-(1-naphthyl)ethylamine as a resolving reagent[4].

The *Lu*CLEA based approach towards enantiopure acid **I** described here is highly atom efficient and generates little waste, even when a recrystallisation is taken into account. Moreover, *Lu*CLEA can be recycled without loss of enantioselectivity and modest loss of activity. When compared to the substrate engineering approach described earlier[16] where overall yields of 45% and 51% were reported for the preparation of (*S*)-**I** in 99% *ee*, the direct synthesis

of the cyanohydrin from 2-butanone catalyzed by *Lu*CLEA towards (*R*)-**I** proved to be high yielding with a good atom economy. Moreover relatively high catalyst loadings were required in the substrate engineering approach (>150 U/mmol) to achieve good conversion while our approach required less than 5 U/mmol of *Lu*CLEA, which corresponds to less than 15 U/mmol of initial commercial *Lu*HNL solution.

Conclusion

The CLEA immobilization strategy was applied successfully to the sole representative of the zinc-dependent alcohol dehydrogenases group among HNLs: *Lu*HNL. Various strategies were attempted to develop this catalyst including the first report of molecular imprinting as a tool to improve the activity of a CLEA. *Lu*CLEA with high specific activity (303.5U/g) could be prepared in good activity recovery (33%) on multigram scale.

(*R*)-2-butanone cyanohydrin was synthesized in preparative scale over two batches upon the catalysis of *Lu*CLEA using only a small portion of fresh catalyst (20%) between batches to compensate for the loss of activity upon recycling. After hydrolysis, (*R*)-2-hydroxy-2-methylbutyric acid was obtained in 85% isolated yield (from 2-butanone) and 87% *ee*.

Experimental Section

General Remarks

CAUTION: *All procedures involving hydrogen cyanide were performed in a well-ventilated fume hood equipped with a HCN detector. HCN-containing wastes were neutralized using commercial bleach and stored independently over a large excess of bleach for disposal.*

Enzymes: Solutions of the Hydroxynitrile Lyases from *Prunus amygdalus* (*Pa*HNL, 690U/mL), *Manihot esculenta* (*Me*HNL, 2.23kU/mL), and *Linum usitatissimum* (*Lu*HNL, 114U/mL or 76.9U/mL) were purchased from Codexis. The CLEAs from *Pa*HNL[22] and *Me*HNL[24] were prepared according to literature procedures.

Chemicals: A standard solution of HCN (2M in DIPE) was prepared as previously reported.[23] Acetone cyanohydrin was prepared according to literature procedure.[36] Racemic 2-butanone cyanohydrin was prepared via its TMS derivative according to literature procedure.[37] Other chemicals and reagents were purchased from commercial sources and used without further purification unless otherwise specified

Analytical Methods: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Avance Ultrashield spectrometer. Absorbance was measured using a Shimadzu Biospec-1601 UV spectrometer. For SEM analysis, the sample was coated with gold and the pictures were taken on a Jeol JSM-6700M field emission scanning electron microscope. Chiral gas chromatography (GC) was performed using an Agilent Technologies 6890N chromatograph equipped with a β-Dex 225 column and a FID detector. High performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1100 series chromatograph equipped a diode array detector. Specific conditions for chromatography and retention times are given in the experimental section for the respective compounds.

Standard Enzyme Activity Test

The activity of soluble enzyme was measured according to a modified version of the literature procedure.[20] The reaction was performed in a screw cap vial using a dilute solution of commercial *Lu*HNL in phosphate buffer (5mM, pH=6.5). The dilute solution (50μL) was added to citrate/phosphate

buffer (438µL, 0.2M Na_2HPO_4, 0.1M citric acid, pH=4.1) followed by 12µL of a solution of acetone cyanohydrin (10% v/v) in 0.1M citric acid. The reaction mixture was incubated for 10min at 30°C and quenched with 0.01M aqueous HCl (500µL). A sample (10µL) was then diluted in water (9.99mL) and the cyanide concentration was measured using a commercially available cyanide test (Merck, Spectroquant cyanide test). The test was calibrated using standard aqueous solutions of sodium cyanide. A reference reaction (without enzyme) was performed in parallel and the activity of the soluble enzyme was determined in µmol of acetone cyanohydrin hydrolyzed per min. The CLEA and EA activities were measured according to the same procedure.

Precipitation Study

Phosphate buffer (75µL, 0.1M, pH=7) was added to a commercial solution of *Lu*HNL (225µL, 114U/mL) and the mixture was kept shaking at 0°C for 5min. The co-solvent (113µL, 450µL, or 900µL depending on the experiment) was then added and the mixture was kept shaking at 0°C for an additional 10min. The aggregates were separated from the supernatant by centrifugation (10 000rpm, 5min) and the supernatant was diluted in 500µL of phosphate buffer (5mM, pH=6.5) before measuring the activity. The aggregates were dissolved in 1.50mL of phosphate buffer (5mM, pH=6.5) and the activity was measured.

Cross-Linking Study

The cross-linking experiments were conducted as described for the precipitation and after 10min at 0°C a 25% aqueous glutaraldehyde solution (11µL, 22.5µL, 45µL, 67.5µL depending on the experiments) was added to the mixture. The suspension was kept shaking at 0°C for 1h and the CLEAs were separated by centrifugation (10,000rpm; 5min). The CLEAs were resuspended

in 1.50mL of phosphate buffer (5mM, pH=6.5) and after centrifugation the activity of the supernatant was measured to determine the efficiency of the cross linking. The CLEAs were then suspended in 1.50mL of fresh phosphate buffer (5mM, pH=6.5) and the activity was measured accordingly.

EAs and CLEAs Specific Activity and Recovery

Enzyme Aggregates (EAs): Commercial *Lu*HNL (900μL, 69.2U) was diluted in phosphate buffer (300μL, 0.1M, pH=7) and the solution was shaken in an ice/water bath. After 5min, sat. aqueous $(NH_4)_2SO_4$ (3.6mL) for *Lu*EA(Am.Sulf) or *t*-butanol (1.8mL) for *Lu*EA(*t*-Bu) was added and shaking in ice was continued for 10min. The suspension was then centrifuged (10,000rpm, 5min) washed with acetonitrile and centrifuged again. The enzyme aggregates were freeze dried and the activity was measured according to the standard activity test.

Cross-Linked Enzyme Aggregates (CLEAs): Precipitation of the enzyme was performed as described for the EAs. A 25% aqueous glutaraldehyde solution (90μL) was added to the suspension and shaking in ice was continued for 1h. The CLEAs were centrifuged (10,000rpm, 5min), washed with phosphate buffer (5mM, pH=6.5), and freeze dried. The CLEA's activity was measured according to the standard activity test.

PaCLEA and MeCLEA: The biocatalysts were prepared as reported.[22,24] The activity of commercial *Pa*HNL, *Me*HNL and the corresponding CLEAs was measured according to the standard activity test for *Lu*HNL.

Molecular Imprinting Study

EAs and CLEAs with 2-butanone as additive were prepared as described above from a solution of commercial *Lu*HNL solution (900μL, 69.2U) in phosphate buffer (300μL, 0.1M, pH=7) and 2-butanone (69.2μL). The activity

of the biocatalysts obtained was measured according to the standard activity test. The difference in specific activity (in percentage) between the catalyst prepared with and without additive was calculated according to Equation 1.

Equation 1. Influence of molecular imprinting on immobilization.

$$\text{Activity Difference (\%)} = 100\text{x}[\text{Activity}_{(\text{with additive})} - \text{Activity}_{(\text{without additive})}]/\text{Activity}_{(\text{with additive})}$$

Synthetic Activity

Selected CLEAs and EAs were loaded (2.2 U) in a screw-cap vial and a solution of HCN 2M in DIPE (1.00 mL) was added. The HPLC internal standard, biphenyl (*ca.* 1 mg), was added to the mixture upon stirring to ensure complete dissolution. The reaction was started by addition of 2-butanone (44.8µL, 0.50 mmol) and an analytical sample was taken immediately after addition to determine the initial conditions in HPLC. The conversion ratios were determined by HPLC (10µL reaction samples in 1.00mL hexane; Chiralpak AD; mobile phase: Hex:iPA (99:1); flow: 1.5mL/min; UV detection at 280nm; R_t(biphenyl) = 2.64min, R_t(ketone) = 3.45min). The *ee* of the cyanohydrin formed was monitored by chiral GC of the trifluoroacetate derivative in a modified version of the literature procedure[20] (20µL reaction samples in 1.00mL anhydrous dichloromethane, 10µL trifluoroacetic acid anhydride, 10µL anhydrous pyridine; β-Dex 225 column; 80°C; 10psi; R_t(*R*)=13.77min , R_t(*S*)=14.20min).

Recycling Experiments

Commercial *Lu*HNL (9.00mL, 692U) was diluted in phosphate buffer (3.00mL, 0.1M, pH=7) at 0°C and 2-butanone (692µL) was added. The solution was shaken at 0°C for 5min and *t*-butanol (18mL) was added. Shaking at 0°C

was continued for 10min and a 25% aqueous glutaraldehyde solution (900μL) was added to the suspension. After shaking at 0°C for 1h *Lu*CLEA was centrifuged (10,000rpm, 5min), washed with phosphate buffer (5mM, pH=6.5), and freeze dried to give 392.5mg of immobilized biocatalyst (125.9U/g, 7% recovery). *Lu*CLEA (318.0mg, 40U) was then loaded in a jacketed flask and a 2M HCN solution in DIPE (10mL) was added. The temperature was kept at 30°C and biphenyl (*ca.* 2mg) was added to the suspension. 2-butanone (448μL, 5mmol) was then added and the reaction was monitored as described above. After 6h of reaction at 30°C *Lu*CLEA was filtered off, rinsed with fresh DIPE (30mL) and loaded back into the jacketed flask for the next cycle. The combined DIPE phases were evaporated under reduced pressure to give the crude 2-butanone cyanohydrin as a colorless liquid. ^{1}H NMR ($CDCl_3$, 400MHz): δ = 1.10 (t, 3H, J =7.5), 1.59 (s, 3H), 1.81 (m_c, 2H), 3.77 (bs, 1H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 8.4, 26.8, 34.6, 69.1, 121.8.

(*R*)-2-hydroxy-2-methylbutyric acid

Commercial *Lu*HNL (90 mL, 6.92 kU) was diluted in phosphate buffer (30 mL, 0.1 M, pH=7) at 0°C and 2-butanone (6.92 mL) was added. The solution was shaken at 0°C for 5min and *t*-butanol (180 mL) was added. Shaking at 0°C was continued for 10min and a 25% aqueous glutaraldehyde solution (9.00 mL) was added to the suspension. After shaking at 0°C for 1h *Lu*CLEA was centrifuged (10,000 rpm, 5min), washed with phosphate buffer (5mM, pH=6.5), and freeze dried until the weight of immobilized biocatalyst was 7.59 g (303.5 U/g, 33% recovery). *Lu*CLEA (2.11 g, 640 U, 8.0 U/mmol) was then loaded in a jacketed flask and a 2M HCN solution in DIPE (160 mL) was added. The temperature was kept at 30°C and biphenyl (ca. 10 mg) was added to the suspension. 2-Butanone (7.2 mL, 80 mmol) was then added and the reaction was monitored as described above. After 3h at 30°C *Lu*CLEA was filtered off, rinsed with diethyl ether (3x100 mL), loaded back into the jacketed flask, and

resuspended in 2M HCN solution in DIPE (160 mL). An additional loading of fresh *Lu*CLEA (421.9 mg, 128 U, 2.0 U/mmol) was added and a second batch of 2-butanone cyanohydrin was prepared accordingly. The combined ethereal phases from the first batch were evaporated under reduced pressure to give the crude (*R*)-2-butanone cyanohydrin (8.69 g, 86% *ee*). This crude cyanohydrin was stirred at r.t. in a mixture (1:2) of water : conc. HCl while the enzymatic reaction for the second batch proceeded (3h). The crude (*R*)-2-butanone cyanohydrin from the second batch (7.92 g, 87% *ee*) was diluted in 60 mL of water : conc. HCl (1:2) and combined with the reaction mixture of the first batch. The hydrolysis was allowed to proceed upon vigorous stirring at 65-70°C and monitored by ^{1}H NMR of reaction samples (100 µL) extracted in ether (1.00 mL). After 20h the reaction mixture was cooled to r.t., diluted with water (80 mL) and ether (300 mL) was added. After stirring for 10min, the phases were separated and the aqueous layer was extracted with ether (2x300 mL). The combined ethereal layers were dried ($MgSO_4$) and the solvent was removed under reduced pressure to give (*R*)-2-hydroxy-2-methylbutyric acid as a white solid (16.08 g, 136 mmol, 85% yield, 87% *ee*). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.94 (t, 3H, J=7.5), 1.47 (s, 3H), 1.73 (dq, J=7.5, 15.0, 1H), 1.86 (dq, J=7.5, 15.0, 1H), 5.79 (bs, 2H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 7.8, 25.4, 32.9, 75.1, 181.4. The *ee* of the product was determined as described previously[4] (ca. 1 mg product dissolved in iPA (100µL) and diluted in hexane (1.00mL); Chiralpak AD; mobile phase: Hex:iPA:TFA (96:4:0.1); flow: 1.5 mL/min; UV detection at 210 nm; R_t(*R*) = 8.22min, R_t(*S*) = 9.10min).

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CHAPTER 4

Expedient Multistep Syntheses Based on HNL CLEA-Catalysis

Abstract: A set of robust and efficient multistep syntheses toward a range of cyanohydrin derivatives is reported. Starting from benzaldehyde as a model substrate, (*R*)- and (*S*)-mandelonitrile can be obtained in excellent yields and enantiopurities using *Pa*CLEA or *Me*CLEA as biocatalysts in buffer-saturated organic media. Reaction conditions were optimized to further derivatize the cyanohydrin intermediate either in a genuine two-step-one-pot process or after filtration of the biocatalyst and solvent switch as the only isolation steps required.

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Introduction

Chiral cyanohydrins are mostly used as intermediates in synthetic chemistry.[1-4] One of the main drawbacks of cyanohydrins as commercial products is the possible release of HCN upon decomposition.[5] This potential source of hazard incurs additional costs in handling, shipping and storage of bulk quantities. As a result, cyanohydrins are generally seen as in-house intermediates towards more stable and marketable chiral products such as α-hydroxy-amines, α-hydroxy-acids, and α-hydroxy-esters. *O*-protected cyanohydrins are also of particular interest when further derivatization involves a basic medium that would otherwise lead to the decomposition of unprotected cyanohydrins.

The direct enantioselective synthesis of cyanohydrins from inexpensive HCN and readily available prochiral carbonyl compounds is nonetheless remarkably attractive in terms of atom economy. An integrated multi-step synthetic strategy towards cyanohydrin derivatives would therefore answer a need for step economy[6,7] in order to "efficaciously deliver a meaningful supply of target" (a cyanohydrin derivative) as Wender described.[8] Ideally the multi-step synthesis would proceed in one pot. Alternatively, downstream processing of intermediates should be kept to a minimum. The preparation of cyanohydrins esters, THP-protected, and trialkylsilyl derivatives by kinetic resolution of cyanohydrin acetates followed by the corresponding downstream chemistry of the free cyanohydrin has been reported.[9] However, in such kinetic resolutions yields are limited to 50 %.

Both (*R*) and (*S*)-HNLs are naturally occurring, stable and relatively inexpensive enzymes with a wide substrate range. The HNL-catalyzed preparation of cyanohydrins is typically carried out in aqueous buffer or in a biphasic (buffer:organic) type medium. This type of medium is not suitable for

the development of cost-effective multistep syntheses since the reagents required to derivatize the cyanohydrin would decompose in water. Earlier attempts to combine a HNL with a lipase in one pot failed due to the hydrolysis of the acylating reagent. The acetic acid released then denatured the HNL.[10] Purification of the cyanohydrin is therefore carried out by extraction in organic media – a costly and potentially hazardous step due to the residual HCN in the reaction mixture. In order to efficiently implement a multistep strategy towards cyanohydrin derivatives, the HNL-catalyzed formation of cyanohydrins should be carried out in an organic medium.

Engineering the reaction medium for the HNL-catalyzed synthesis of cyanohydrins typically aims at limiting the contribution of the non-catalyzed addition of HCN to the substrate which decreases the practical enantiopurity of the product.[11-17] To this end, the development of immobilized HNLs as Cross-Linked-Enzyme-Aggregates (CLEA®) was particularly successful.[17-20] CLEAs of the HNLs from *Linum usitatissimum* (*Lu*HNL),[21] *Manihot esculenta* (*Me*HNL),[17,22] and *Prunus amygdalus* (*Pa*HNL)[17,23,24] showed high volumetric activity and improved stability in organic media. Similar to earlier observations for *Pa*HNL[25] and other HNLs immobilized on different carriers[13,16,26] the HNL CLEAs were catalytically active in buffer saturated organic solvents.

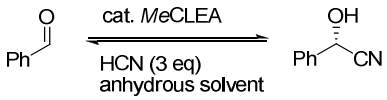
We have developed a range of multistep syntheses to probe the suitability of HNLs immobilized as CLEAs for reaction cascades,[27] in organic media and in one pot.

Results and Discussion

1. Selection of the Reaction Medium

The nature of the medium and the water content strongly influence the catalytic performances of HNLs.[17-26] A rapid screening of organic solvents as media for the conversion of benzaldehyde to (*S*)-mandelonitrile catalyzed by *Me*CLEA (Scheme 1) showed that under anhydrous conditions no product was formed in methyl-*tert*-butyl ether (MTBE), diethyl ether, methylene chloride or toluene. However, some conversion was detected when anhydrous diisopropyl ether (DIPE) and tetrahydrofuran (THF) were used.

Scheme 1. Reaction conditions for solvent selection.

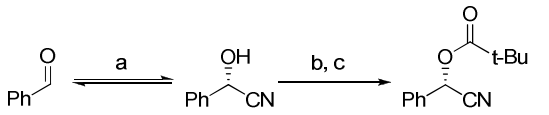


Best results were obtained with *Me*CLEA when the HCN stock solution in DIPE was saturated with aqueous buffer (50mM citrate/phosphate pH=5.5). Water plays an important part in the structure of HNLs and is present even in the active site.[28] Deactivation of the biocatalyst occurs under conditions where the medium can still remove water from the enzyme. The ability of the enzyme to retain structural water molecules therefore dictates the biocatalyst stability in the medium.[19]

2. One-pot esterification of (*R*)- and (*S*)-mandelonitrile

The two-step-one-pot synthesis of (*S*)-mandelonitrile pivaloate from benzaldehyde was selected as a model reaction to investigate the influence of water on the overall process. The esterification of (*S*)-mandelonitrile was catalyzed by 4-DMAP and pyridine (Scheme 2).

Scheme 2. One-pot synthesis of (*S*)-mandelonitrile pivaloate.



a) *Me*CLEA (*cat.*), HCN (3 eq), buffer sat. DIPE, 3h, r.t.
b) Various drying agents (50mg/mmol), 1h, r.t.
c) DMAP (*cat.*), tBuCOCl : pyridine (various amounts), 3h, r.t.

The amount of base was kept lower than the amount of pivaloyl chloride to prevent the base-catalyzed decomposition of mandelonitrile into benzaldehyde. When a slight excess of reagent:base combination (with reference to mandelonitrile) was used (2:1.5) only 43% conversion could be achieved after 3h of esterification. Clearly, residual water had a negative influence on the second step. We therefore investigated a range of drying agents (Table 1) and the conversion improved significantly especially when $MgSO_4$ or molecular sieves were used. After stirring for 1h in the presence of the drying agent, a decrease in the *ee* of the cyanohydrin (from 99% to 97%) was observed when molecular sieves were used. With $MgSO_4$ the enantiopurity of the cyanohydrin could be maintained and the conversion improved consistently with increased amounts of reagent and base (Table 1).

Table 1. Reaction conditions optimisation for mandelonitrile esterification in one-pot.

Drying Reagent[a]	**Pyridine**[b]	**Pivaloyl Chloride**[c]	**Conversion**[d]
None[e]	1.5	2	43%
P_2O_5	1.5	2	59%
Sicapent	1.5	2	18%
$MgSO_4$	1.5	2	66%
Mol. Sieves[f]	1.5	2	65%[g]
$MgSO_4$	2	3	80%
$MgSO_4$	3	4	90%
$MgSO_4$	4	5	>95%

[a] Type of drying agent used (see experimental section) [b] Amount of pyridine in equivalents with reference to benzaldehyde [c] Amount of pivaloyl chloride in equivalents with reference to benzaldehyde [d] Conversion calculated from the area of peaks in GC and corresponding response factors [e] Reference reaction performed without drying reagent [f] Molecular sieves 4Å was used. [g] Some racemization was observed in this experiment.

This result represents a genuine two-step-one-pot conversion of benzaldehyde to the corresponding ester. This strategy answers the lack of general *in-situ* derivatization methods, under which conditions, the stability of the biocatalyst depends strongly on the type of reagent, by-products and water content of the medium.[29,30] All approaches attempted to date, *in-situ*[29] or the combination of HNLs and lipases in organic solvents have had limited success.[10]

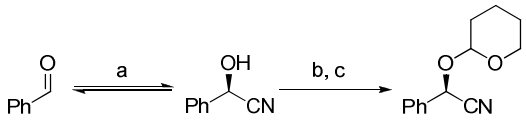
Based on these results we further optimized the reaction conditions of the enzymatic step to reach more than 95% conversion with at least 97% *ee* within 4h at room temperature. This could be achieved using 4g/L CLEA at 52g/L substrate loading and 4eq HCN for both *Me*CLEA and *Pa*CLEA with (*S*)-mandelonitrile and (*R*)-mandelonitrile, respectively.

3. Expedient synthesis of THP-protected (*R*)-mandelonitrile from benzaldehyde and its application in a synthesis of (-)-ephedrine

Ether type protecting groups such as tetrahydropyran (THP) protection are commonly used in cyanohydrin chemistry.[3,6,31,32] For the development of a two-step-one-pot procedure, both HCN and residual water in the medium needed to be removed since the reagent (DHP) reacts with them. Iron(II) sulphate in combination with a drying reagent could be used but conversions remained unsatisfactory and the complexity of the system was greatly increased. We therefore circumvented these difficulties by using a simple filtration of the catalyst through cotton wool followed by evaporation of the volatiles. Although this could no longer be defined as a genuine two-step-one-pot process, this fast and efficient downstream processing (DSP) was very advantageous. *Me*CLEA and *Pa*CLEA can indeed be recycled as described in earlier reports.[22,23] The catalysts could be recycled three times without any loss of activity and selectivity in all the experiments reported here. Moreover, the use of additives was no longer necessary since residual HCN was removed by evaporation. Finally it became possible to switch the solvent to a more appropriate medium for the second step since DIPE was also removed.

The preparation of THP-protected (*R*)-mandelonitrile was thereby achieved with a solvent-less system in the second step of the process using a relatively large excess of DHP but no drying agent to remove residual water from the crude cyanohydrin (Scheme 3).

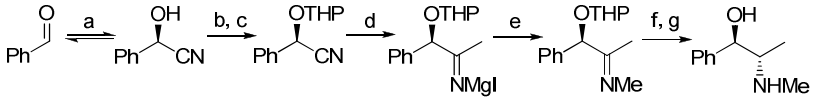
Scheme 3. Multistep synthesis of THP-protected (*R*)-mandelonitrile.



a) *Pa*CLEA (*cat.*), HCN (4 eq), buffer sat. DIPE, 4h, r.t.
b) Filter *Pa*CLEA off, evaporate volatiles
c) DHP (4 eq), *p*TsOH (*cat.*), 0°C to r.t. overnight

Earlier reports on the reduction-transimination-reduction of protected mandelonitrile toward ephedrine started from purified substrate[33,34]. Using our strategy the process did not require purification other than filtration of the biocatalyst and evaporation of the volatiles to prepare the crude ephedrine (which can be recrystallized easily as its hydrochloride salt) from benzaldehyde (Scheme 4). As a part of an overall process the protection step does not require purification and our approach provides for the formation of the final compound in high yield after five chemical conversions and two operational procedures. It also compares favourably with a recently published stepwise approach toward the structurally related thiamphenicol.[35]

Scheme 4. Multistep synthesis of (-)-ephedrine.



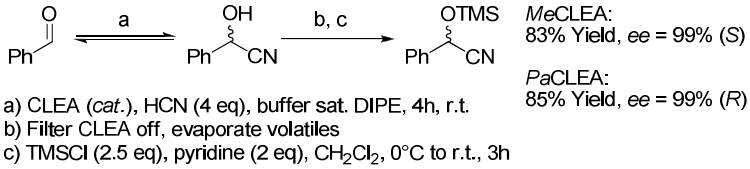
a) *Pa*CLEA (*cat.*), HCN (4 eq), buffer sat. DIPE, 4h, r.t.
b) Filter *Pa*CLEA off, evaporate volatiles
c) DHP (4 eq), *p*TsOH (*cat.*), 0°C to r.t. overnight
d) MeMgI (2.5 eq), Et_2O, 1h, r.t.
e) MeOH (0°C), $MeNH_2$ (10eq), 0°C to r.t., 1h
f) $NaBH_4$ (5.35 eq) -78°C to r.t., 24h
g) H^+/H_2O, 2h, r.t.

44% Yield
ee (hydroxy) >99% (*R*)
de = 78%

4. Two step synthesis of TMS-protected (*R*)- and (*S*)-mandelonitrile

The synthesis of cyanohydrin silyl ethers has been reported extensively, in particular for TMS-protected cyanohydrins using trimethylsilyl cyanide[36] (TMSCN) or sodium cyanide/trimethylsilyl chloride[37] (NaCN/TMSCl) and the corresponding carbonyl compounds. The CLEA-based two-step one-pot approach provides an attractive synthesis of TMS-protected enantioenriched cyanohydrins from hydrogen cyanide and TMSCl as an alternative to TMSCN, as it would again shorten the reaction process. The general procedure for the preparation of (*R*)- and (*S*)-mandelonitrile using *Pa*CLEA or *Me*CLEA, respectively, followed by the DSP described earlier allowed us to use only a moderate excess of chlorotrimethylsilane (TMSCl) and base to achieve good yields and maintain the excellent enantiopurity obtained in the enzymatic step (Scheme 5).

Scheme 5. Multistep syntheses of TMS-protected (*R*)- and (*S*)-mandelonitrile.



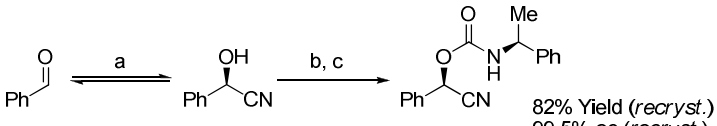
When TMSCN was used as the sole protecting reagent, we observed a slight drop in the enantiopurity of the protected cyanohydrin. This effect was attributed to the racemic (non-catalyzed) addition of TMSCN to residual benzaldehyde and could be suppressed completely when TMSCl was used as the protecting reagent. The slightly lower isolated yields obtained were attributed to the volatility of TMS-mandelonitrile.

From this experiment we concluded that, regardless of the follow-up chemistry investigated, our approach was valid for the synthesis of either (*R*)-mandelonitrile derivatives using *Pa*CLEA or (*S*)-mandelonitrile derivatives using *Me*CLEA. Since the use of *Me*CLEA in a buffer-saturated organic solvent has been described in Chapter 2 we selected *Pa*CLEA as biocatalyst for further elaboration of our CLEA-based multistep syntheses.

5. Carbamoylation of (*R*)-Mandelonitrile

The carbamoylation of chiral alcohols using isocyanates and copper bromide has been reported earlier for the determination of enantiopurity by NMR.[38] This derivatization method employs a different type of reagent to the protecting group chemistry (typically base or acid catalyzed) discussed earlier and was therefore suitable to further probe the robustness of our multistep approach toward cyanohydrin derivatives. Coupling of freshly prepared isocyanate[39] with (*R*)-mandelonitrile in THF proceeded smoothly toward the corresponding carbamate. After purification by crystallisation good yields and excellent enantiopurity were obtained (Scheme 6). Only a slight excess of reagent was necessary in this case but the removal of HCN was essential for the reaction to proceed to completion under these conditions.

Scheme 6. Multistep syntheses of (1-(*S*)-phenyl-ethyl) carbamic acid (*R*)-cyano-phenyl-methyl ester.



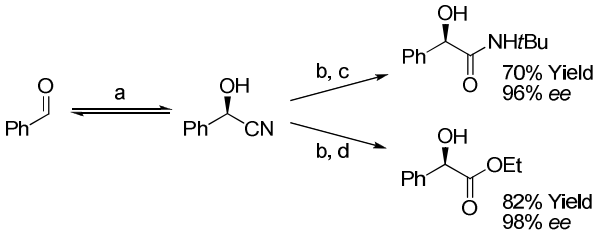
a) *Pa*CLEA (*cat.*), HCN (4 eq), buffer sat. DIPE, 4h, r.t.
b) Filter *Pa*CLEA off, evaporate volatiles
c) CuBr.Me_2S (1.5 eq), (*S*)-(1-isocyanatoethyl)benzene (1.5 eq), THF, 0°C to r.t., overnight

6. Derivatization of the nitrile group

Derivatization of the nitrile functionality in cyanohydrin chemistry leads to a range of very useful intermediates in organic synthesis such as α-hydroxy-acids, α-hydroxy-esters and α-hydroxy-amides.[1-3,40,41]

The formation of α-hydroxy-*N*-*t*-butyl-amide from cyanohydrins *via* the Ritter reaction has been reported earlier.[42] The reaction is performed in a mixture of 60% H_2SO_4 and *t*-butanol as reagents/solvent. Removing the solvent from the enzymatic step was therefore necessary. Moreover, HCN incompatibility with strongly acidic media was a potential limitation. Using the general method for the preparation of (*R*)-mandelonitrile followed by the simple DSP described above, the reaction proceeded smoothly to afford the corresponding α-hydroxy-amide in good yield and very good *ee* (Scheme 7).

Scheme 7. Multistep synthesis of (*R*)-*N*-*t*-butyl-2-hydroxy-2-phenyl-acetamide (Ritter reaction); multistep synthesis of (*R*)-mandelic acid ethyl ester (Pinner reaction).



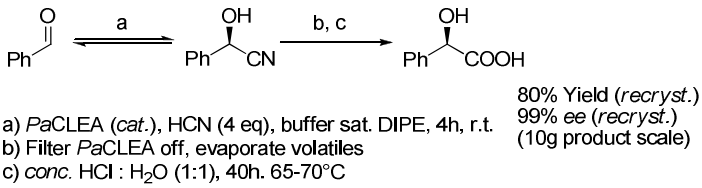
a) *Pa*CLEA (*cat.*), HCN (4 eq), buffer sat. DIPE, 4h, r.t.
b) Filter *Pa*CLEA off, evaporate volatiles
c) *t*-BuOH : 60% H_2SO_4 (1:1), 24h. r.t.
d) EtOH (*abs.*), HCl_g, 22h. r.t.

A similar reaction (the Pinner reaction) has been described for preparing α-hydroxy-esters from cyanohydrins and the corresponding alcohol using gaseous HCl.[43] The reaction needed to be performed in the alcohol of choice as

solvent and therefore DIPE had to be removed after the biocatalytic step. Moreover, the strongly acidic media required for the second step called for the removal of excess HCN as discussed for the Ritter reaction. The rate of addition of gaseous HCl was found to be critical for the exothermic reaction to proceed smoothly. Using abs. ethanol as reagent/solvent the corresponding mandelic acid ethyl ester could be isolated in good yield and excellent *ee* (Scheme 7).

As a final example of successful application of our HNL-CLEA based multistep strategy toward cyanohydrin derivatives we selected the synthesis of (*R*)-mandelic acid from benzaldehyde on a multigram scale. α-Hydroxy-acids are indeed prepared industrially on a multi-ton scale from the corresponding aldehyde using *Pa*HNL as catalyst in a biphasic medium followed by acid hydrolysis.[44] Moreover we reported in Chapter 3 the highly efficient synthesis of (*R*)-2-hydroxy-2-methyl-butyric acid from 2-butanone using a similar strategy based on CLEAs of the HNL from *Linum usitatissimum* (*Lu*HNL) as biocatalyst.[21] Using the *Pa*CLEA-catalyzed approach developed here, recrystallized (*R*)-mandelic acid was obtained in very good yields and excellent *ee* (Scheme 8).

Scheme 8. Gram scale multistep synthesis of (*R*)-mandelic acid.



Conclusion

Protecting groups are at the core of cyanohydrin chemistry and we focused on providing straightforward and robust two-step-one-pot or multistep

syntheses toward a range of enantioenriched *O*-protected cyanohydrins. This methodology can be further integrated into syntheses of complex compounds based on the nitrile reactivity of protected cyanohydrins as we demonstrated in the synthesis of (-)-ephedrine. Nitrile derivatization of unprotected cyanohydrins using our approach was also successful when the reaction required acidic catalyst/reagents.

These expedient procedures toward enantiopure cyanohydrin derivatives facilitate the recycling of the catalyst and enable high volumetric yields, which are all essential to the successful application in the laboratory and the implementation of cost-efficient industrial processes.

Experimental Section

General Remarks

CAUTION: *All procedures involving hydrogen cyanide were performed in a well-ventilated fume hood equipped with a HCN detector. HCN-containing wastes were neutralized using commercial bleach and stored independently over a large excess of bleach for disposal.*

Enzymes: Solutions of the Hydroxynitrile Lyases from *Prunus amygdalus* (*Pa*HNL, 690U/mL) and *Manihot esculenta* (*Me*HNL, 2.23kU/mL) were purchased from Codexis. The corresponding CLEAs were prepared from these solutions as previously reported.[17,22,23]

Chemicals: Chemicals and reagents were commercially available and used without further purification unless indicated otherwise. Benzaldehyde was distilled before use to remove traces of benzoic acid.

Analytical Methods: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Avance Ultrashield spectrometer. Gas chromatography (GC) was performed

using an Agilent Technologies 6890N chromatograph equipped with a HP-5 column or a β-Dex 225 chiral column and a FID detector. High performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1100 series chromatograph equipped a diode array detector. Specific conditions for chromatography and retention times are given in the experimental section for the respective compounds. Optical rotations were measured at 589nm in a 10cm sample tube.

Influence of the reaction medium on *Me*CLEA activity

The reaction was performed by suspending *Me*CLEA (10mg) in the 2M HCN stock solution (3mL, 6mmol) in the solvent of choice and an additional portion of the corresponding solvent (1mL) was added. The reaction was started by addition of benzaldehyde (200µL, 1.96mmol) and the conversion was determined after 1h of reaction by HPLC (Chiralcel OJ; Hex:iPA (90:10); 1.00mL/min; R_t(benzaldehyde)=3.76min, R_t(*R*)=12.18min, R_t(*S*)=15.62min). For the reactions performed in anhydrous conditions, anhydrous HCN was prepared according to literature procedure[29] and diluted into the anhydrous solvent of choice to a 2M stock solution. A 2M HCN stock solution in buffer saturated DIPE was prepared as reported previously[17] and used without further purification for reaction.

(*S*)-mandelonitrile pivaloate – Optimisation

*Me*CLEA (16mg) was suspended in a 2M stock solution of HCN in DIPE (3mL) and commercial DIPE was added (1mL). The suspension was stirred gently (150rpm) at r.t. and benzaldehyde (200µL, 1.96mmol) was added. After stirring for 3h at r.t., the *ee* of the cyanohydrin was measured as described above. The drying reagent of choice (100mg) was added and the mixture was stirred for 1h at r.t.. The *ee* of the cyanohydrin was again measured to detect

possible racemization and a catalytic amount of 4-DMAP was added followed by pivaloyl chloride (2, 3, 4 or 5eq depending on the experiment) and dry distilled pyridine (1.5, 2, 3 or 4eq respectively). After stirring for an additional 3h at r.t. analytical samples were taken to determine the conversion by GC (HP-5; 100°C for 2min then 50°C/min to 165°C; 25psi; R_t(benzaldehyde)=1.30min, R_t(pivaloate)=4.30min).

(*S*)-mandelonitrile pivaloate – Optimised conditions

*Me*CLEA (16mg) was suspended in a 2M stock solution of HCN in DIPE (3mL) and commercial DIPE was added (1mL). The suspension was stirred gently (150rpm) at r.t. and benzaldehyde (200µL, 1.96mmol) was added. After stirring for 3h at r.t., $MgSO_4$ (100mg) was added and the mixture was stirred for 30min at r.t.. A catalytic amount of 4-DMAP was added followed by pivaloyl chloride (1.23mL, 5eq) and dry distilled pyridine (653µL, 4eq). After stirring for an additional 3h at r.t. a conversion greater than 95% was observed and the enantiomeric excess (98% (*S*)) measured by chiral HPLC (Chiralpak AD; Hex:iPA (98:2); 1.00mL/min; R_t(*R*-ester)=7.26min, R_t(*S*-ester)=8.28min) could be maintained from the cyanohydrin without racemization. Purification of the ester was found to be very delicate due to the large amounts of pivaloic acid anhydride formed as a by-product. Typical yields of pure product after column chromatography (Hexane: EtOAc, 99:1) were slightly lower than 50% (from benzaldehyde). ^{1}H NMR[6] ($CDCl_3$, 400MHz): δ = 1.24 (s, 9H, *t*Bu), 6.41 (s, 1H, CH), 7.44-7.51 (m, 5H, Ph). ^{13}C NMR[6] ($CDCl_3$, 100.65MHz): δ = 26.8, 38.8, 62.6, 116.2, 127.5, 129.2, 130.2, 132.0, 176.4.

CLEA-catalyzed formation of (*R*)- and (*S*)-mandelonitrile – General method

The corresponding CLEA (*ca*. 15mg) was suspended in a 2M stock solution of HCN in DIPE (4mL, 8mmol) and benzaldehyde (200µL, 1.96mmol) was added. After stirring gently (150rpm) for 4h at r.t. conversion of benzaldehyde into mandelonitrile was greater than 95% and the *ee* of the corresponding cyanohydrin was greater than 97% (by HPLC as described above). ^{1}H NMR[45] ($CDCl_3$, 400MHz): δ = 3.46 (bs, 1H, OH), 5.54 (s, 1H, CH), 7.41-7.56 (m, 5H, Ph). ^{13}C NMR[45] ($CDCl_3$, 100.65MHz): δ = 63.3, 119.0, 126.8, 129.1, 129.8, 135.1.

THP-protected (*R*)-mandelonitrile

(*R*)-mandelonitrile was prepared from benzaldehyde (295.8mg, 2.79mmol) using *Pa*CLEA (14.0mg) as described in the general procedure. The CLEA was filtered off, rinsed with diethyl ether (5mL) and the combined ethereal phases were evaporated carefully. The crude cyanohydrin was cooled to 0°C and DHP (1.02mL, 4eq) was then added to the stirred residue at 0°C followed by a catalytic amount of *p*TsOH. The temperature was allowed to increase slowly and the mixture was kept stirring overnight at r.t.. The volatiles were evaporated and the crude product (82% conversion, 23% *de*, 98% *ee*) was purified by column chromatography (Hexane:EtOAc, 90:10) to give pure THP-protected (*R*)-mandelonitrile (347.9mg, 1.60mmol) as a white solid in 57% yield and 98% *ee*. ^{1}H NMR[6] ($CDCl_3$, 400MHz): *Major* δ = 1.50-1.93 (m, 6H), 3.65 (m, 1H), 3.83 (m, 1H), 5.11 (m, 1H), 5.60 (s, 1H), 7.42-7.55 (m, 5H). *Minor* δ = 1.50-1.93 (m, 6H), 3.65 (m, 1H), 4.00 (m, 1H), 4.74 (m, 1H), 5.42 (s, 1H), 7.42-7.55 (m, 5H). ^{13}C NMR[6] ($CDCl_3$, 100.65MHz): *Major* δ = 18.6, 25.1, 29.7, 62.4, 65.8, 96.7, 117.5, 127.4, 129.6, 129.6, 133.6. *Minor* δ = 19.7, 25.4, 30.6, 62.9, 66.5, 94.6, 118.3, 127.4, 129.2, 129.0, 133.6. *de* was determined by GC (HP-5; 160°C; 25psi; R_t(*major*)=4.44min, R_t(*minor*)=4.79min). All four

stereoisomers could be separated by HPLC in order to measure to *ee* of the cyanohydrin (Chiralcel OJ; Hex:iPA (90:10); 1.50mL/min; R_t(*S*-major)=6.61min, R_t(*R*-minor)=7.53min, R_t(*S*-minor)=9.35min, R_t(*R*-major)=11.77min).

(1*R*, 2*S*)-(-)-ephedrine

(*R*)-mandelonitrile was prepared from benzaldehyde (240.8mg, 2.27mmol) using *Pa*CLEA (15.8mg) as described in the general procedure. The CLEA was filtered off, rinsed with diethyl ether (5mL) and the combined ethereal phases were evaporated carefully. The crude cyanohydrin was cooled to 0°C and DHP (828µL, 4eq) was then added at 0°C followed by a catalytic amount of *p*-TsOH and the temperature was allowed to increase slowly to r.t.. After overnight stirring at r.t. the volatiles were evaporated under reduced pressure and the residue was taken up in absolute diethyl ether (2mL) under argon. Methyl magnesium iodide (freshly prepared and titrated according to standard procedures[46]) was added dropwise at r.t. as a 1.81M solution in diethyl ether (3.2mL, 2.5eq) and the reaction was allowed to proceed for 1h at r.t.. The mixture was then cooled to 0°C and methanol (8mL) was added dropwise at this temperature (exothermic reaction). A 2M solution of methyl amine in THF (10mL, 10eq) was then added at 0°C and the ice bath was removed and the reaction proceeded at r.t. for 1h. After 1h, the mixture was cooled to -78°C and $NaBH_4$ (459.7mg, 5.35eq) was added at this temperature. The temperature was allowed to increase slowly to r.t. and the mixture was kept stirring for 24h. After 24h, the reaction was cooled to -10°C (acetone/ice bath) and 45mL aqueous HCl 1M (45mL) was added carefully into the flask. When the addition was complete the bath was removed and the reaction was kept stirring for 2h at r.t. (pH was checked to be 1). Diethyl ether (50mL) was added to the reaction mixture and the phases were separated. The aqueous layer was extracted with diethyl ether (2x20mL) and the combined ethereal layers were

washed with aqueous HCl 1M (20mL). The combined aqueous layers were treated with 1M NaOH until pH=11 (ca. 40mL) and diethyl ether (100mL) was added. The phases were separated and the aqueous layer was extracted first with ethyl acetate (2x50mL) then with chloroform (2x50mL). The combined organic layers were dried with $MgSO_4$ and evaporated under reduced pressure. The crude product (301.3mg) was taken up in ethanol and HCl_g was bubbled into the solution. The volatiles were evaporated under reduced pressure and the solids were washed with diethyl ether then treated with 1M NaOH (20mL) and extracted with diethyl ether (3x30mL). The combined organic layers were washed with brine (5mL), dried and evaporated under reduced pressure to obtain a mixture of (1*R*, 2*R*)-pseudoephedrine and (1*R*, 2*S*)-ephedrine (163.2mg, 0.99mmol) in 44% yield and 78% *de* in favour of the (1*R*, 2*S*)-ephedrine. Enantiopure (1*R*, 2*S*)-(-)-ephedrine could be obtained by treating an ethereal solution of this mixture with HClg, washing the hydrochloric salt formed with cold (-20°C) acetone and regenerating the free base from this salt using NaOH and diethyl ether extraction as descried above. ^{1}H NMR[47] ($CDCl_3$, 400MHz): δ = 0.85 (d, *J* = 6.5Hz, 3H), 2.11 (bs, 2H), 2.51 (s, 3H), 2.83 (m, 1H), 4.80 (d, *J* = 3.9Hz, 1H), 7.23-7.34 (m, 5H). ^{13}C NMR[47] ($CDCl_3$, 100.65MHz): δ = 14.1, 33.9, 60.4, 72.8, 126.1, 127.1, 128.1, 141.3. The *ee* was determined by GC based on a modified version of the literature procedure[34] (Sample in 1.00mL DCM, 50µL trifluoroacetic acid anhydride, 50µL anhydrous pyridine; β-Dex 225; 120°C; 25psi; R_t(*Isomer1*)=24.39min, R_t(*Isomer2*)=24.76min, R_t(*Isomer3*)=32.60min, R_t(*Isomer4*)=36.96).

TMS-protected-(*R*)- and (*S*)-mandelonitrile

(*R*)-mandelonitrile was prepared from benzaldehyde (206.2mg, 1.94mmol) using *Pa*CLEA (15.3mg) and (*S*)-mandelonitrile from benzaldehyde (293.8mg, 2.79mmol) using *Me*CLEA (17.8mg) as described in the general procedure. After 4h at r.t. the CLEA was filtered off and rinsed with diethyl ether (5mL).

The volatiles were evaporated carefully and the residue was cooled to 0°C. TMSCl (635µL, 2.5eq) was added to the residue at 0°C under argon followed by a solution of pyridine (322µL, 2eq) in methylene chloride (2mL) dropwise. The mixture was stirred for 5min at 0°C and the ice water bath was removed. After 3h at r.t., the volatiles were evaporated carefully and the crude product was filtered through silica using hexane as eluent. The solvent was removed under reduced pressure to give (*R*)-TMS-mandelonitrile (339.3mg, 1.65mmol) in 85% yield and 99% *ee* and (*S*)-TMS-mandelonitrile (475.3mg, 2.31mmol) in 83% yield and 99% *ee* respectively, as clear liquids. ^{1}H NMR[48] ($CDCl_3$, 400MHz): δ = 0.24 (s, 9H, TMS), 5.50 (s, 1H, CH), 7.37-7.48 (m, 5H, Ph). ^{13}C NMR[48] ($CDCl_3$, 100.65MHz): δ = -0.3, 64.0, 119.4, 126.8, 129.7, 129.8, 135.9. The *ee* of TMS-mandelonitrile was determined by HPLC (Chiralpak AD; Hex:iPA (99.8:0.2); 1.00mL/min; R_t(*R*)=10.27min, R_t(*S*)=12.17min).

(1-(*S*)-phenyl-ethyl) carbamic acid (*R*)-cyano-phenyl-methyl ester

(*R*)-mandelonitrile was prepared from benzaldehyde (255.7mg, 2.41mmol) using *Pa*CLEA (14.9mg) as described in the general procedure. The CLEA was filtered off, rinsed with diethyl ether (5mL) and the combined ethereal layers were evaporated carefully. The crude cyanohydrin was cooled to 0°C and THF (5mL) was added. A solution of (*S*)-2-phenylethyl isocyanate[39] (534.2mg, 1.5eq) in THF (5mL) was added at this temperature followed by CuBr.Me_2S (736.2mg, 1.5eq) and the mixture was allowed to warm up slowly to r.t.. After overnight stirring, the solids were filtered off and rinsed with diethyl ether (20mL). The green solution was then concentrated *in vacuo* and the residue was stirred vigorously in diethyl ether (20mL). The insoluble materials were filtered off, rinsed with diethyl ether (20mL) and the volatiles were removed under reduced pressure to give the crude carbamate (722.4mg). The pure carbamate derivative (555.5mg, 1.98mmol) was obtained after recrystallisation from pentane/iPA as a white solid in 82% yield and 99.5% *ee*.

^{1}H NMR[49] ($CDCl_3$, 400MHz): δ = 1.48, (d, 3H), 4.84 (m_c, 1H), 5.18 (bs, 1H), 6.41 (s, 1H), 7.29-7.53 (m, 10H). ^{13}C NMR[49] ($CDCl_3$, 100.65MHz): δ = 22.1, 51.3, 63.6, 118.9, 125.6, 125.9, 127.7, 128.8, 129.1, 130.2, 132.1, 132.9, 154.4. *ee* determination by HPLC (Chiralpak AD; Hex:iPA (70:30); 1.50mL/min; R_t(*S*)=3.95min, R_t(*R*)=5.13min).

(*R*)-*N*-*t*-butyl-2-hydroxy-2-phenyl-acetamide

(*R*)-mandelonitrile was prepared from benzaldehyde (319.9mg, 3.01mmol) using *Pa*CLEA (15.4mg) as described in the general procedure. The CLEA was filtered off, rinsed with diethyl ether (5mL) and the combined ethereal layers were evaporated carefully. The crude cyanohydrin was dissolved in *t*-butanol (2mL) and aqueous 60% H_2SO_4 (2mL) was then added slowly to the mixture at r.t. and stirring was continued for 24h. The solution was diluted with 10mL of water and extracted with 3x50mL of methylene chloride. The combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure to give the crude product. Traces of *t*-butanol were removed by stirring the crude product under high vacuum and (*R*)-*N*-*t*-butyl-2-hydroxy-2-phenyl-acetamide (436.1mg, 2.10mmol) was obtained as a white solid in 70% yield and 96% *ee*. Enantiopure product (*ee*>99%) could be obtained by recrystallisation from a minimum amount of hexane. ^{1}H NMR[50] ($CDCl_3$, 400MHz): δ = 1.32 (s, 9H), 2.50 (bs, 1H), 4.90 (s, 1H), 5.83 (bs, 1H), 7.31-7.40 (m, 5H). ^{13}C NMR[50] ($CDCl_3$, 100.65MHz): δ = 28.6, 51.9, 73.8, 127.0, 128.6, 129.0, 139.1, 170.4. *ee* determination by HPLC (Chiralpak AD; Hex:iPA (90:10); 1.00mL/min; R_t(*R*)=6.35min, R_t(*S*)=7.06min).

(*R*)-mandelic acid ethyl ester

(*R*)-mandelonitrile was prepared from benzaldehyde (241.6mg, 2.28mmol) using *Pa*CLEA (16.5mg) as described in the general procedure. The CLEA was

filtered off, rinsed with diethyl ether (5mL) and the combined ethereal layers were evaporated carefully. The crude cyanohydrin was taken up in absolute ethanol (5mL) and gaseous HCl, generated by dropping conc. H_2SO_4 into aqueous HCl (37%), was bubbled into the alcoholic solution. The exothermic reaction was controlled by adding HCl_g repeatedly for short periods of time over 30min. After 30min, the addition of HCl was stopped and the reaction was allowed to proceed for 22h at r.t.. The volatiles were evaporated under reduced pressure and the residue was taken up in hexane (5mL). After heating the mixture gently the flask was allowed to cool to r.t. and stored overnight at -20°C. (*R*)-mandelic acid ethyl ester (338.6mg, 1.88mmol) separated from the solution and was obtained after filtration and drying in vacuum as a white solid in 82% yield and 98% *ee*. ^{1}H NMR[51] ($CDCl_3$, 400MHz): δ = 1.19 (t, *J* = 7.1Hz, 3H, CH_3), 4.18 (m_c, 2H, CH_2), 5.13 (s, 1H, CH), 7.28-7.43 (m. 5H, Ph). ^{13}C NMR[51] ($CDCl_3$, 100.65MHz): δ = 14.0, 62.2, 72.8, 126.5, 128.4, 128.5, 138.4, 173.7. *ee* determination by HPLC (Chiralpak AD; Hex:iPA (95:5); 0.80mL/min; R_t(*S*)=17.88min, R_t(*R*)=19.53min).

(*R*)-mandelic Acid – Gram Scale

*Pa*CLEA (677.8mg) was suspended in a 2M stock solution of HCN in DIPE (170mL, 0.34mol) and benzaldehyde (8.65mL, 85mmol) was added. The mixture was stirred for 4h at r.t. and the CLEA was filtered off, rinsed with diethyl ether (3x100mL) and the combined ethereal layers were evaporated carefully. A mixture (1:1) of conc. HCl: water (30mL) was added to the crude cyanohydrin, the solution was heated to 65-70°C and the reaction was allowed to proceed upon vigorous stirring at this temperature for 40h. The mixture was allowed to cool to r.t. and diethyl ether (200mL) was added and vigorous stirring was continued for 30min. The insoluble materials were filtered off and the phases were separated. The aqueous fraction was extracted with diethyl ether (2x200mL) and the combined ethereal layers were evaporated under

reduced pressure. The crude product (12.40g) was recrystallised from toluene (150mL) to give pure (*R*)-mandelic acid (10.34g, 68mmol) in 80% yield and 99% *ee*. ^{1}H NMR[52] ($CDCl_3$, 400MHz): δ = 5.12 (s, 1H), 7.27-7.36 (m, 3H), 7.46-7.49 (m, 2H) The *ee* was determined by comparison of the sample specific optical rotatory power to the literature data[53] $[\alpha]^{22}{}_D$ -151.9 (*c* 2.52, MeOH), (*lit.* $[\alpha]^{24}{}_D$ -152 (*c* 2.52, MeOH)).

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CHAPTER 5

Cyanohydrins from α,β-Unsaturated Ketones: Preparation and Rearrangement of Their Acetate Derivatives

Abstract: The preparation of racemic cyanohydrins from α,β-unsaturated ketones is exemplified with two model substrates. An acetylated derivative was also synthesized in a step-wise manner since no direct route from the corresponding ketone was found suitable. Regioselectivity in the rearrangement of this α,β-unsaturated cyanohydrin acetate proceeded in favor of the corresponding tetronic acid derivative while no Ireland-Claisen rearrangement product could be detected. Initial activity was detected for one α,β-unsaturated ketone in the HNL-catalyzed direct synthesis of an enantioenriched mixture. However, kinetic resolution proved to be a more practical approach for the preparation of this compound due to the unfavored equilibrium position.

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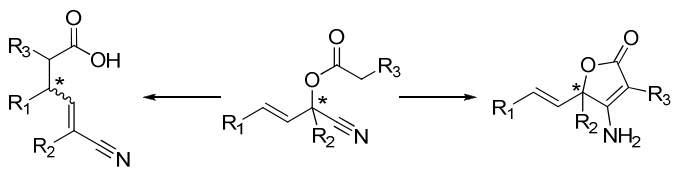
Introduction

Hydroxynitrile Lyases (HNLs) are known for their ability to accept a wide range of substrates.[1-7] Examples of cyanohydrins bearing new structural features are reported regularly.[8] For example, unsaturated cyanohydrins have been reported as even more versatile chiral intermediates.[9] The double bond indeed provides an additional level of possible derivatization via a wide range of reactions.[9] The HNL-catalyzed preparation of enantiopure cyanohydrins from α,β-unsaturated aldehydes has therefore been studied extensively.[10-14] However, the preparation of enantioenriched α,β-unsaturated cyanohydrins from prochiral ketones is limited to the example of methyl vinyl ketone.[15]

Further reaction of esters of the α,β-unsaturated cyanohydrins via deprotonation at the α position of the ester can potentially produce two products (Figure 1). On the one hand Ireland-Claisen rearrangement[16-18] of the enolate would result in a C-O to C-C transfer with possible retention of the stereochemistry from the starting cyanohydrin ester. On the other hand, intramolecular alkylation of the nitrile would lead to the formation of a cyclic compound analogous to tetronic acid.[19]

Figure 1. Possible products derived from esters of α,β-unsaturated cyanohydrin.



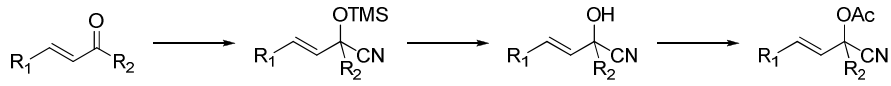
In this Chapter, we discuss the various challenges faced in the preparation of racemic and enantioenriched α,β-unsaturated cyanohydrins. The results of our investigations on the rearrangement of a racemic ester of these cyanohydrins will also be presented.

Results and Discussion

1. Racemic α,β-unsaturated cyanohydrins and their acetates

Two model compounds were selected to investigate the preparation of racemic α,β-unsaturated cyanohydrins and their corresponding acetates (Scheme 1)

Scheme 1. General approach toward α,β-unsaturated cyanohydrin acetates.



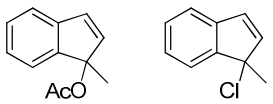
1a: R_1=Ph, R_2=Me
1b: R_1=Me, R_2=Et

2a, 2b **3a, 3b** **4a**

Although the preparation of the respective cyanohydrins and acetate derivatives had not been reported previously the direct conversion of ketone **1a** to a range of cyanohydrin esters in one step has been described.[20] The procedure could not be adapted to the preparation of the corresponding acetate **4a** using otherwise identical conditions. Optimization of the conditions was also unsuccessful. The preparation of cyanohydrin esters in one pot via the Francis reaction[21-23] was not attempted. Instead, we investigated the conversion of the TMS-protected cyanohydrin **2a** to the corresponding acetate **4a** in one step.

The TMS-protected cyanohydrin derivatives of compounds **1a** and **1b** were therefore prepared according to a literature procedure.[24] Although the synthesis was high yielding we noted that the procedure required TMSCN as the cyanide source, high loadings (30mol%) of 4-methylmorpholine *N*-oxide (NMO), dichloromethane as solvent and extended reaction time. The overall atom economy was therefore poor and the process could not be considered "green". An alternative to this procedure was later developed and is discussed in Chapter 6 of this thesis.[25] For the direct conversion of the TMS-protected cyanohydrin **2a** to the corresponding acetate **4a** various conditions were investigated (temperature, acetylating reagent, Lewis acid catalysis). In some instances the title compound **4a** was formed but always as a mixture. By-products were not characterized but we suggest that cyclisation products (Figure 2) were formed via Friedel Crafts alkylations upon Lewis acid catalysis in the presence of acetyl chloride. Our assumption was based on ^{1}H NMR results showing a range of compounds with "Z type" coupling constants of the protons at the sp^2 carbons similar to related structures reported in the literature.[26,27]

Figure 2. By-products in the direct conversion of **2a** to **4a**.



High yielding purification of **4a** could be performed neither by chromatography on silica gel nor distillation due to the relative instability of this acetate.[28] In consequence no direct route to acetate **4a** could be designed and the overall synthesis required a stepwise preparation of each intermediate as depicted in Scheme 1. Classical TMS-deprotection methods (HCl in THF) resulted in cyanohydrin **3a** decomposition to the corresponding ketone **1a** as pointed out in earlier reports.[29] The cyanohydrin could nonetheless be

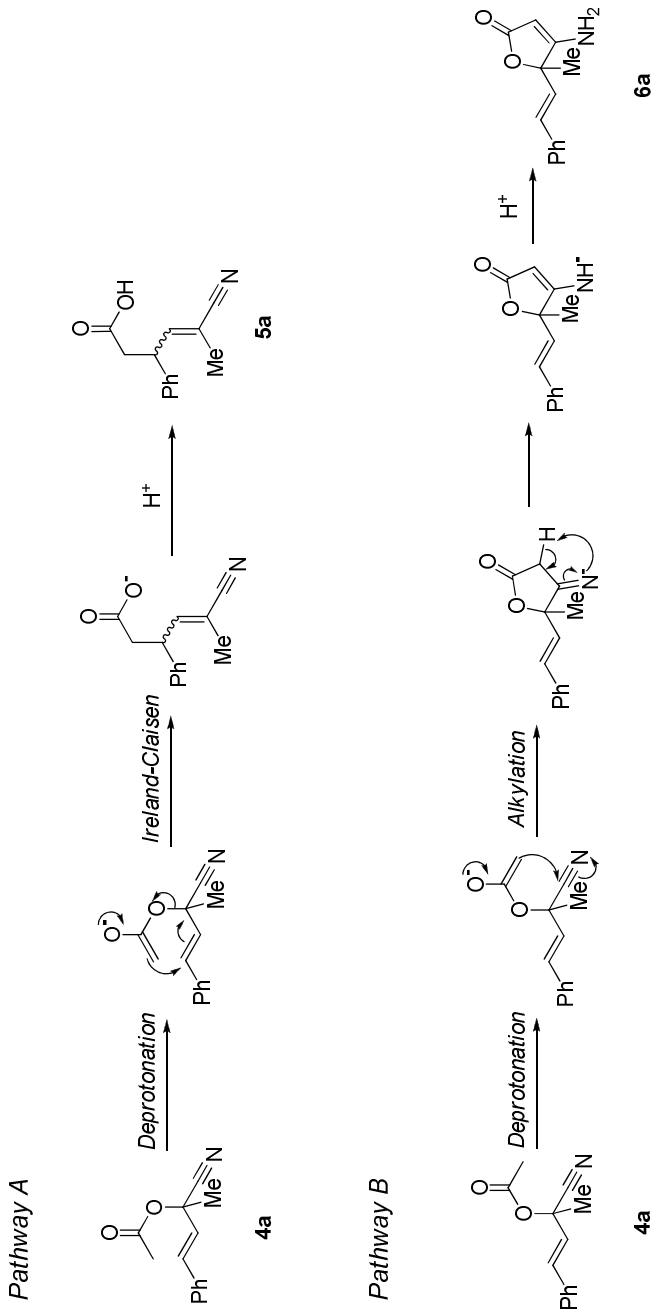
isolated in high yields using smoother conditions (HF in MeOH) as described earlier.[30] Following the protocol optimized for compound **3a**, cyanohydrin **3b** could be prepared accordingly in high yields and purity. Due to their relative instability, cyanohydrins **3a** and **3b** were prepared as needed from the corresponding TMS-derivatives (stable upon storage at -20°C) and used immediately after isolation. Acetylation of cyanohydrin **3a** required a low temperature to ensure the formation of the corresponding ester **4a** in high yields and good purity (see Experimental Section).

2. Rearrangement of α,β-unsaturated cyanohydrin acetates.

The metal-catalyzed [3,3] sigmatropic rearrangement of allylic esters[31] has been successfully applied to esters of α,β-unsaturated cyanohydrins as substrate using palladium[32] or titanium[33] as catalysts. Upon this type of catalysis the transfer of C-O bond to a new C-O bond is achieved since the ester's C=O bond is involved in the cyclic intermediate.

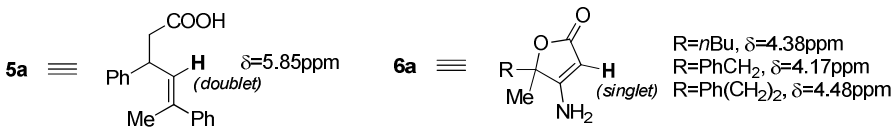
We were interested in the Chemistry of the enolate generated by deprotonation of the corresponding ester. In this context, two cyclic intermediates can be formed either on the α,β-unsaturation (Ireland-Claisen rearrangement) or via intramolecular alkylation at the nitrile as discussed earlier (Figure 1). The regioselectivity of the enol rearrangement was therefore investigated using racemic cyanohydrin acetate **4a** as a model substrate (Scheme 2).

Scheme 2. Possible pathways in the rearrangement of cyanohydrin acetate **4a**.



In order to detect the formation of either rearrangement products, ^{1}H NMR of crude mixtures after deprotonation and hydrolysis were analyzed. One particular proton was selected for each of the putative products in order to assess their formation. Although these compounds have not been reported previously, representative chemical shifts could be estimated based on literature data reported for similar compounds (Figure 3).

Figure 3. Reference compounds for chemical shift estimation in compounds **5a**[34] and **6a**[19].



As a result, compound **5a** can be identified by a doublet in the 5 – 6 ppm region of the spectra while the formation of compound **6a** can be detected via its singlet in the 4 – 4.5 ppm range (Figure 3). Due to the often complex nature of the spectra analyzed we estimated the detection method to be suitable when at least 10% of either compounds was formed.

A range of conditions was evaluated for the deprotonation reaction: the temperature (0°C to -100°C), the counter ion of the diisopropylamine base (lithium *vs.* potassium), and the sequence of addition of reagents. The formation of the enolate was evidenced by ^{1}H NMR after trapping the intermediate with TMSCl or TBDMSCl. However, only the formation of the cyclisation product **6a** was observed (Scheme 2, Pathway B) from the corresponding proton with δ = 4.47 ppm (Figure 3, and Experimental Section). In order to influence the rearrangement in favor of compound **5a** polar aprotic co-solvents (HMPA, DMPU) were added but to no avail. The thermal

rearrangement of the TMS and TBDMS enolates was also unsuccessful and resulted in fragmentation products with ketone **1a** as the major product.

The Ireland-Claisen reaction (concerted [3,3] sigmatropic rearrangement) toward compound **5a** was therefore not successful under the conditions we investigated here. Instead, alkylation proceeded onto the nitrile and the tetronic acid derivative **6a** could be prepared as a result. According to Baldwin's rules for ring closure[35,36] the formation of cyclic compound **6a** – a 5-exo-dig pattern – is indeed favored. Although this reaction has been described on saturated cyanohydrin esters derived from aldehydes with complete retention of selectivity,[19] the preparation of **6a** demonstrates that this synthetic route can also be applied to cyanohydrin esters derived from unsaturated ketone. Furthermore, high regioselectivity was achieved since the product of the Ireland-Claisen reaction (**5a**) could not be detected.

3. Enantioenriched α,β-unsaturated cyanohydrin

The commercially available Hydroxynitrile Lyases (HNLs) from *Prunus amygdalus*: (*R*)-*Pa*HNL, and *Manihot esculenta*: (*S*)-*Me*HNL were selected as potentially suitable biocatalysts for the preparation of enantioenriched α,β-unsaturated cyanohydrins derived from ketones **1a** and **1b**. Both enzymes have evolved from different parents and have different carbonyl compounds as natural substrates.

Crude HNL extracts were used in a biphasic medium buffer:diisopropylether (DIPE) throughout the study. The selection of this medium aimed at maintaining stability of the free enzyme while minimizing possible mass transfer limitations due to the poor solubility of the substrates in aqueous buffers.[9] We first investigated the *Pa*HNL and *Me*HNL-catalyzed

synthesis of cyanohydrins **3a** and **3b** from ketones **1a** and **1b** and HCN (Scheme 3, Table 1).

Scheme 3. HNL-catalyzed synthesis of cyanohydrins **3a** and **3b**.

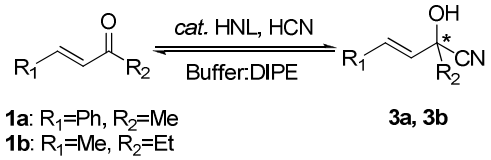


Table 1. Conversion and *ee* in the synthesis of cyanohydrins **3a** and **3b**.[a]

Substrate	Enzyme	Conversion (time)	*ee*
1a	*Pa*HNL	0% (8h)	-
	*Me*HNL	0% (8h)	-
1b	*Pa*HNL	2% (16h)	*nd*[b]
	*Me*HNL	12% (16h)	50%

[a] Reaction Conditions: 1.0 mmol of substrate in 5 mL DIPE, 30 U HNL in 1.0 mL of 50mM citrate buffer pH = 5.4. Reaction started by addition of HCN (4 mmol) from a 2M stock solution in DIPE and monitored while stirring at RT (see Experimental Section). [b] Conversion was too low for the reliable determination of the enantiomeric excess. *nd*: not determined.

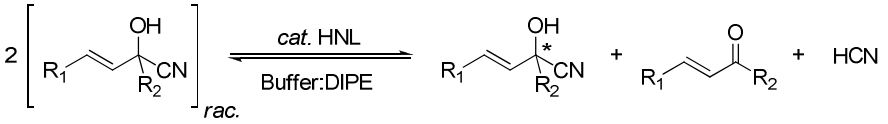
From the results presented in Table 1, the starting activity on substrate **1b** was established for both *Pa*HNL and *Me*HNL. *Me*HNL appeared to be more active toward this substrate than *Pa*HNL. Furthermore, the enantiomeric excess of cyanohydrin **3b** formed upon the catalysis of *Me*HNL was 50%. Although this value is only moderate, it indicates that *Me*HNL is selective in the preparation of **3b**. However, no activity was detected for either enzyme on substrate **1a**. This result does not necessarily indicate the absence of activity for this substrate since we suspect the thermodynamics of this system to be strongly in favor of the starting ketone. In consequence, the equilibrium of the reaction could have been reached at levels of cyanohydrins too low to detect.

Unfavorable equilibrium could also explain the low conversion obtained with substrate **1b** (12% at best).

The loss of conjugation in the preparation of cyanohydrin from α,β-unsaturated ketones accounts for the significant product instability observed for the preparation of racemic **3a** and **3b**. This result also indicates that the thermodynamics of such systems are likely to be in favor of the starting material and prompted us to investigate kinetic resolution of the cyanohydrin in parallel to the direct synthetic route to determine the most suitable approach for the preparation of enantioenriched **3a** and **3b**.

Considering that only 12% conversion could be achieved in the direct synthesis of cyanohydrin **3b**, the kinetic resolution of α,β-unsaturated cyanohydrins – which can theoretically yield up to 50% enantiopure product – was considered a viable alternative to the direct synthesis (Scheme 4, Table 2). This strategy has indeed been applied successfully for the preparation of enantiopure cyanohydrins from ketones.[37,38]

Scheme 4. HNL-catalyzed kinetic resolution of cyanohydrins **3a** and **3b**.



3a: R_1=Ph, R_2=Me
3b: R_1=Me, R_2=Et

3a, 3b **1a, 1b**

Table 2. Recovery[a] and *ee* in the kinetic resolution of cyanohydrins **3a** and **3b**.[b]

Substrate	Enzyme	Loading	Temp	Recovery[a] (time)	*ee*
3a	*Pa*HNL	30	RT	67% (2h)	0%
	*Me*HNL	30	RT	42% (2h)	0%
3b	*Pa*HNL	30	RT	73% (20h)	4%
	*Me*HNL	30	RT	62% (20h)	39%
	*Me*HNL	60	RT	44% (20h)	68%
	*Me*HNL	150	RT	12% (20h)	60%
	*Me*HNL	150	10°C	28% (27h)	90%

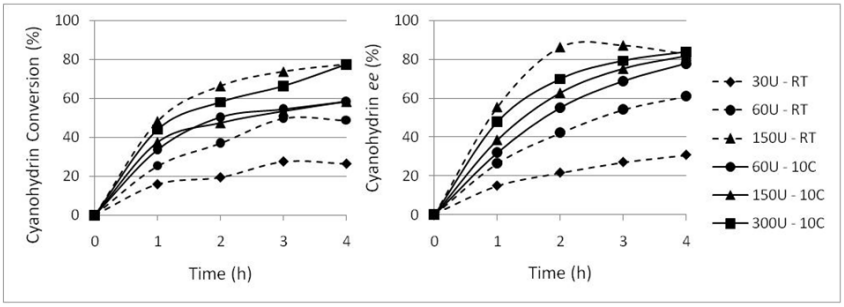
[a] Recovery refers to the amount of cyanohydrin left unreacted. [b] Reaction Conditions: 1.0 mmol of cyanohydrin in 5 mL DIPE, HNL (various amounts) diluted in 1.0 mL of 50 mM citrate buffer pH = 5.4. Samples were taken at various time points to monitor the reaction (see Experimental Section).

For the resolution of compound **3a** no selectivity was observed for either enzyme. The non-catalyzed decomposition of the cyanohydrin could possibly account for this result. Nevertheless we observed that the conversion obtained after 2h upon the catalysis of *Me*HNL was greater than with *Pa*HNL (Table 2). The determination of initial rates (over 1h reaction) for each enzyme on this substrate showed that in the presence of *Me*HNL, the decomposition of the cyanohydrin was more than twice as fast as for *Pa*HNL. It was therefore concluded that *Me*HNL was catalytically active but not selective for the resolution of **3a**. It remains unclear whether *Pa*HNL is active since no blank reaction (no enzyme added) was run in parallel to determine the non-catalyzed rate of decomposition of **3a** under these conditions. Nevertheless, the lack of selectivity for both enzymes indicated that the kinetic resolution approach was not suitable for **3a**.

When cyanohydrin **3b** was subjected to *Pa*HNL–catalyzed kinetic resolution, the *ee* of the cyanohydrin remained very poor (Table 2). Attempts to optimize the reaction conditions to improve the enantiopurity of the

cyanohydrin were not successful. This result is consistent with the low catalytic activity observed in the *Pa*HNL-catalyzed synthesis of **3b** from **1b** (Table 1). In the kinetic resolution this limited activity translates into poor selectivity due to the significant contribution of the unselective non-catalyzed decomposition of cyanohydrin **3b**. Encouraging results were nonetheless obtained when *Me*HNL was used for the kinetic resolution of **3b** (Table 2). We investigated the influence of enzyme loading and reaction temperature on the conversion and enantiomeric excess of the cyanohydrin (Figure 4).

Figure 4. Influence of *Me*HNL loading and temperature on the kinetic resolution of **3b**.



At room temperature the conversion and enantiomeric excess of the cyanohydrin increased consistently with higher enzyme loading. At 150 Units/mmol loading about 87% *ee* was obtained after 2h (Figure 4) but the *ee* of the cyanohydrin dropped to 60% after 20h (Table 2). Racemization of the cyanohydrin was therefore significant at room temperature. In order to limit this phenomenon the temperature was set at 10°C. At this temperature, the influence of the enzyme loading was less pronounced than at room temperature (Figure 4). Moreover, the enantiomeric excess obtained was improved significantly when compared to the corresponding reaction at room temperature (same enzyme loadings). The contribution of the non-catalyzed decomposition of **3b** and the racemization of the remaining cyanohydrin could

therefore be minimized by decreasing the reaction temperature. As a result we determined the most suitable reaction conditions to be: 150 U/mmol *Me*HNL loading, 10°C, and 27 hours in order to maximize the cyanohydrin enantiopurity (90%) and its recovery (28% of cyanohydrin left unreacted). The E value of the kinetic resolution under these conditions was 5.7. When compared to the direct synthesis route the kinetic resolution was very advantageous in terms of cyanohydrin formation (12% *vs.* 28%) and enantiopurity (50% *vs.* 90%).

An earlier report for the preparation of cyanohydrin **3b** via alkylation of a cyanohydrin phosphate (a pseudoephedrine derived chiral auxiliary) gave isolated yields of 10% (from crotonaldehyde) and 74% *ee*.[39] The enzymatic approach described here allows a theoretical yield of up to 24% from ketone **1b** with 90% *ee*. Furthermore, the reported chemical route is an overall 5 steps process from (+)-pseudoephedrine and crotonaldehyde with a relatively poor atom economy. In contrast, the synthesis of racemic cyanohydrin followed by kinetic resolution requires only three steps using readily available and inexpensive reagents.

Conclusion

A step-wise protocol was developed for the preparation of racemic α,β-unsaturated cyanohydrins in high yields. The rearrangement of α,β-unsaturated cyanohydrin esters was tested on a model substrate and afforded the corresponding tetronic acid derivative. The reaction was also regioselective since no competing Ireland-Claisen rearrangement was detected. The relative instability observed during the synthesis of the racemic cyanohydrins translated into an unfavorable equilibrium in the HNL-catalyzed preparation of enantioenriched mixtures. As a result, kinetic resolution proved to be a viable alternative to the direct synthesis approach.

Experimental Section

General Remarks

CAUTION: *Due to the toxic nature of cyanide derivatives such as hydrogen cyanide, and cyanohydrins all experiments were carried out in a well ventilated fume hood and a HCN detector was used for continuous monitoring. All cyanide wastes were neutralized with a technical grade solution of sodium hypochlorite (bleach) and stored separately over a large excess of bleach for disposal.*

Enzymes: Solutions in 50% glycerol of the Hydroxynitrile Lyases from *Prunus amygdalus* (*Pa*HNL, 589 U/mL), and *Manihot esculenta* (*Me*HNL, 1.84 kU/mL), were purchased from Codexis. The respective enzymatic activities were determined as reported.[40]

Chemicals: All compounds used in this study were commercially available chemicals of reagent grade purity and were used without further purification unless otherwise specified. A standard solution of HCN (2M in DIPE) was prepared as previously reported.[41] LDA was freshly prepared from diisopropylamine and titrated[42] butyl lithium commercial solutions (see representative example below). KDA was prepared in a similar manner from potassium *tert*-butoxide and diisopropylamine.

Analytical Methods: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Avance Ultrashield spectrometer. Chemical shifts in deuterated chloroform are reported in parts per million relative to tetramethylsilane for ^{1}H NMR spectra and the residual peak of the solvent for ^{13}C NMR spectra (δ = 77.0ppm). In DMSO-d_6 the chemical shifts are reported relative to the residual peak of the solvent (2.54ppm for ^{1}H and 40.45ppm for ^{13}C). Absorbance was measured using a Shimadzu Biospec-1601 UV spectrometer. Gas chromatography (GC) was performed using an Agilent Technologies 6890N chromatograph equipped

with a HP-5 (non-chiral) or a Chiraldex β-cyclodextrin permethyl (Chiral) column and a FID detector. Helium was used as carrier gas. High performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1100 series chromatograph equipped a diode array detector. Specific conditions for chromatography and retention times are given in the experimental section for the respective compounds.

TMS-protected cyanohydrins

4-methylmorpholine *N*-oxide (30 mol%) was dissolved in dichloromethane dried over CaH_2 (50 ml) under inert atmosphere. The respective ketone (50 mmol) was added at r.t. under argon to the stirred solution followed by trimethylsilylcyanide (8 ml, 64 mmol, 1.3 eq). The reaction flask was tightly closed and the reaction was allowed to proceed for 25h. The reaction mixture was then concentrated under reduced pressure and the residue was filtered through silica gel using hexane as eluant. The solvent was removed under reduced pressure to give pure TMS-protected cyanohydrin.

2a: 12.05 g of clear liquid (98% yield). ^{1}H NMR[25] (400 MHz; $CDCl_3$): δ = 0.24 (s, 9H), 1.75 (s, 3H), 6.13 (d, 1H, *J* = 15.9 Hz), 6.88 (d, 1H, J = 15.9 Hz), 7.29-7.43 (m, 5H). ^{13}C NMR[25] (100 MHz; $CDCl_3$): δ = 1.4 (3C), 30.9, 70.0, 120.7, 126.9, 128.6, 128.8, 129.6, 131.0, 135.2.
2b: 9.40 g of clear liquid (94% yield). ^{1}H NMR[25] (400 MHz; CDCl3): δ = 0.20 (s, 9H), 1.00 (t, 3H, *J* = 7.4 Hz), 1.68-1.89 (m, 2H), 1.77 (dd, 3H, *J* = 1.8 Hz & 6.7 Hz), 5.39 (dq, 1H, *J* = 1.8 Hz & 15.6 Hz), 6.04 (dq, 1H, *J* = 6.7 Hz & 15.6 Hz). ^{13}C NMR[25] (100 MHz; CDCl3): δ = 1.4 (3C), 8.4, 17.3, 36.2, 74.4, 120.2, 128.6, 131.0.

Cyanohydrin deprotection

The respective TMS-protected cyanohydrin (10 mmol) was dissolved in methanol (10 ml). HF (720 µl of 48 wt% aqueous solution, 20 mmol) was added to the vigorously stirred solution and the reaction was allowed to proceed at r.t. for 3h. The mixture was then poured over 40 ml of dichloromethane:water (3:1) and stirred vigorously. The phases were separated and the aqueous layer was extracted with dichloromethane (3x20 mL). The combined organic phases were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to give the corresponding cyanohydrin in satisfying purity. Cyanohydrins could be stored at -20°C without noticeable degradation over short period of time.

3a: 1.7386 g (99% yield) of slightly yellow liquid, which crystallizes upon storage at -20°C. ^{1}H NMR (400 MHz; $CDCl_3$): δ = 1.79 (s, 3H), 2.18 (broad s, 1H), 6.22 (d, 1H, *J* = 16.1 Hz), 6.97 (d, 1H, *J* = 16.1 Hz), 7.32-7.43 (m, 5H). ^{13}C NMR (100 MHz; $CDCl_3$): δ = 28.6, 68.9, 120.5, 127.0, 128.1, 128.8, 128.9, 132.3, 134.8. ***3b***: 1.1715 g of slightly yellow liquid (92% yield). ^{1}H NMR (400 MHz; $CDCl_3$): δ = 1.07 (t, 3H), 1.75-1.95 (m, 2H), 1.79 (dd, 3H, *J* = 1.8 Hz & 6.7 Hz), 5.50 (dq, 1H, *J* = 1.8 Hz & 15.4 Hz), 6.16 (dq, 1H, *J* = 6.7 Hz & 15.6 Hz). ^{13}C NMR (100 MHz; $CDCl_3$): δ = 8.4, 17.4, 34.1, 73.3, 120.0, 129.9, 130.3.

(*E*)-1-Phenyl-3-acetoxy-3-cyano-but-1-ene (4a)

Acetic anhydride (7.5 mL, 80 mmol) was cooled down to -78°C and pyridine (4.3mL, 53mmol) was added under argon at this temperature. A solution of cyanohydrin **3a** (1.3148g, 7.6mmol) in 2 mL of THF was added to the stirred solution at -78°C. The reaction mixture was allowed to warm up slowly to r.t. and stirring was prolonged for an overall of 18h. The mixture was diluted in dichloromethane (100 mL) and poured over water (100 mL). Phases were

separated and the aqueous layer was extracted with dichloromethane (2x100 mL). The combined organic phases were washed with 2x25 mL of water, dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. After drying in high vacuum for 24h, 1.3469g (6.3mmol, 83% yield) of **4a** was isolated as a slightly yellow oil. ^{1}H NMR (400 MHz; $CDCl_3$): δ = 1.91 (s, 3H), 2.12 (s, 3H), 6.19 (d, 1H, *J* = 16.1 Hz), 7.02 (d, 1H, *J* = 16.1Hz), 7.28-7.43 (m, 5H). ^{13}C NMR (100 MHz; $CDCl_3$): δ = 21.1, 26.7, 71.4, 117.6, 124.8, 127.1, 128.8, 129.0, 134.1, 134.7, 168.6.

(*E*)-4-Amino-5-methyl-5-styrylfuran-2(5*H*)-one (6a)

The synthesis was performed under argon atmosphere.

Anhydrous diisopropylamine (212 μL, 1.5 mmol) was dissolved in anhydrous THF (4 mL) and the solution was cooled to -78°C. Butyl lithium (1.58 M in hexane, 775 μL, 1.2 mmol) was added at this temperature and the mixture was allowed to warm up slowly to 0°C upon stirring. The volatiles were removed under reduced pressure and anhydrous THF (4 mL) was then added. The LDA solution obtained was cooled to -78°C. Cyanohydrin acetate **4a** (225.0 mg, 1.0 mmol) was dissolved in anhydrous THF (2 mL) and the stock solution was added dropwise to the LDA solution upon stirring at -78°C. The reaction was let to stir for 18h allowing the temperature to slowly warm up to r.t.. The mixture was then poured over 20 g of ice/water and the mixture was acidified using 85% solution of H_3PO_4 (*ca.* 1 mL). The mixture was stirred vigorously for 15 min and extracted with ether (3x20 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to give tetronic acid derivative **6a** (107.8 mg, 50% yield) as a white solid. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 1.62 (s, 3H), 3.38 (broad s, 2H), 4.47 (s, 1H), 6.46 (d, 1H, *J* = 16.3 Hz), 6.68 (d, 1H, *J* = 16.3 Hz), 7.30 – 7.48 (m, 5H). ^{13}C NMR (DMSO-d_6, 100.65 MHz): δ = 24.8, 79.9, 83.1, 127.5, 129.0, 129.7, 129.9, 130.3, 136.7, 173.7, 175.3. ^{13}C NMR-DEPT135 (DMSO-d_6, 100.65 MHz): δ

= 24.8, 79.9, 127.5, 129.0, 129.7, 129.9, 130.3. EA calcd for $C_{13}H_{13}NO_2$ C, 72.54; H, 6.09; N, 6.51; found C, 71.18; H, 6.46; N, 6.34.

HNL-catalyzed direct synthesis of 3a and 3b

The HNL stock solution was diluted to 30 U/mL in 50 mM citrate buffer pH = 5.4. A 200mM stock solution of the ketone in DIPE (5 mL, 1 mmol) was added to the dilute solution of enzyme (1.00 mL). The required internal standard for reaction monitoring was added to the biphasic system and the reaction was started with a 2M stock solution of HCN in DIPE (2mL, 4 mmol, 4 eq.). The reaction was allowed to proceed upon stirring at r.t.. Samples (20 µL) from the organic layer were taken periodically to monitor the course of the reaction.

*Synthesis of **3a***: Biphenyl (15 mg) was used as internal standard. The reaction samples were diluted in hexane (1.00 mL). The conversion and enantiomeric excess could be determined by chiral HPLC (Chiralcel OJ column; Mobile phase: Hex:iPA (90:10); Flow: 1.00 mL/min; Detection: UV at 254 nm R_t(Biphenyl) = 5.7 min , R_t(Ketone **1a**) = 9.3 min, R_t(**3a** enantiomers) = 15.5 min and 29.7 min. *Synthesis of **3b***: *n*-Dodecane (100 µL) was used as internal standard. The reaction samples were diluted in DIPE (1.00 mL). The conversion was determined by CG (HP-5 column; 135°C; 12 psi; R_t(Ketone **1b**) = 1.67 min, R_t(**3b**) = 2.03 min, R_t(*n*-dodecane) = 2.94 min) and the enantiomeric excess by chiral GC (Chiraldex β-cyclodextrin permethyl column; 120°C; 18 psi; R_t(**3b** enatiomers) = 7.86 min and 8.07 min.

HNL-catalyzed kinetic resolution of 3a and 3b

The HNL stock solution was diluted to the desired enzyme loading in 50 mM citrate buffer pH = 5.4. The required internal standard for reaction monitoring was added to a freshly prepared 200mM stock solution of racemic

cyanohydrin in DIPE. The reaction was started by adding 5 mL of the DIPE solution (1 mmol cyanohydrin) to the dilute solution of enzyme (1.00 mL). The reaction was allowed to proceed upon stirring at r.t. Samples (20 µL) from the organic layer were taken periodically to monitor the course of the reaction.

*Resolution of **3a***: Biphenyl (15 mg per 5 mL of DIPE stock solution) was used as internal standard. The reaction was monitored as described in the corresponding synthesis experiment

*Resolution of **3b***: *n*-Dodecane (100 µL per 5 mL of DIPE stock solution) was used as internal standard. The reaction was monitored using the chiral GC method described in the corresponding synthesis experiment.

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Chapter 6

Robust and Efficient, yet Uncatalyzed Synthesis of Trialkylsilyl-Protected Cyanohydrins from Ketones

Abstract: High-yielding cyanosilylation of ketones with NaCN and various chloro-trialkylsilanes in DMSO proceeds smoothly without catalysis to give silyl-protected ketone cyanohydrins. The unique role of DMSO consists in rendering naked cyanide anions that reversibly add to the C=O bond at the rate-determining step followed by fast trapping of the transient tertiary sodium cyanoalcoholates with chloro- or in situ generated cyano-trialkylsilanes. Preparatively, the reaction matches the best known catalytic cyanosilylation systems applying expensive Me_3SiCN and demonstrates unprecedented efficiency in the synthesis of sterically congested trialkylsilyl-protected cyanohydrins.

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Introduction

The direct synthesis of cyanohydrins from ketones and HCN proved difficult owing to inherent thermodynamic instability.[1,2] To circumvent this, cyanohydrins from ketones are generally prepared in *O*-protected form.[3,4] The cyanosilylation of ketones is particularly suitable since the silyl protecting groups can be removed under very mild reaction conditions. A fast, efficient, general, and cost-effective cyanosilylation of ketones is therefore required in order to develop the kinetic resolution of racemic cyanohydrins into a viable strategy.[5-8] Careful examination of the existing literature procedures revealed that nearly all existing methods for both racemic[3,9-19] and enantioselective[4,20-43] cyanosilylation of ketones employ expensive and potentially hazardous Me_3SiCN, with Lewis acid or base catalysts being required.[44] Syntheses of ketone cyanohydrins containing silyl protecting groups higher than trimethylsilyl are only scarcely described. Therefore, we aimed at developing a general and robust cyanosilylation method which would encompass a wide range of ketones and commercially available trialkylsilyl protecting groups.

Results and Discussion

Since chloro-trialkylsilanes are the most common and inexpensive trialkylsilyl protecting agents, we decided to use the respective chlorosilanes in combination with stable and inexpensive NaCN. However, the latter salt has negligible solubility in most aprotic solvents. For this reason, we turned our attention to DMSO which features the highest polarity among common dipolar aprotic solvents[45] combined with enhanced solvating ability towards hard metal cations. Conversely, anions in such media are poorly solvated and hence highly reactive. For this reason, inorganic salts with sodium or potassium cations soluble in DMSO are often referred to as “naked” anions[46-48] for their enhanced nucleophilicity. We first noticed that NaCN is appreciably soluble in

DMSO especially at 60°C (≥ 2 mmol/mL). Under these conditions, a cyanosilylation of the model substrate, acetophenone **1a** using TBDMSCl required only 5 min to attain conversions greater than 90% into the desired TBDMS-protected cyanohydrin **4a**.[49] Much to our satisfaction, high-yielding facile syntheses of a wide range of ketone cyanohydrins bearing various trialkysilyl protecting groups were possible (Scheme 1, Tables 1 and 2) under these conditions (Method A, Experimental Section).

Scheme 1. Synthesis of trialkylsilyl-protected cyanohydrins in DMSO.

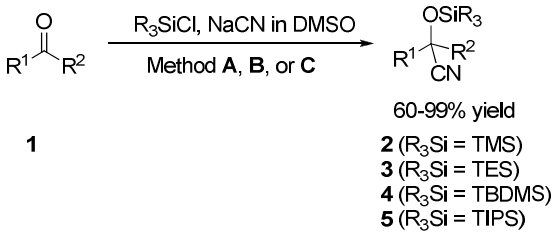
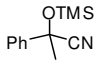
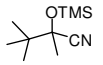
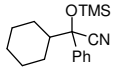
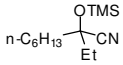
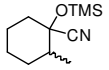
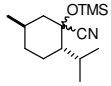
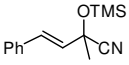
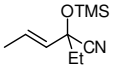
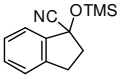
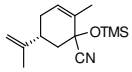
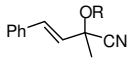


Table 1. Isolated Yields (%) for the preparation of TMS-derivatives **2**.

Entry	Substrate	Product		Isolated Yield (Method)[a]
1	Acetophenone, **1a**		**2a**	83 (**A**), 99 (**B**), 93 (**C**)
2	3,3-Dimethyl-butan-2-one, **1b**		**2b**	96 (**A**)
3	Cyclohexyl-phenyl ketone, **1c**		**2c**	96 (**A**)
4	Nonan-3-one, **1d**		**2d**	>99 (**A**)
5	*Rac*-2-methylcyclo-hexanone, **1e**		**2e**	97 (**A**) *[79:21]*[b]
6	(*L*)-Menthone[c], **1f**		**2f**	95 (**A**) *[5:81:2:12]*[b,d,e] >99 (**C**) *[38:59:0:3]*[b,d]
7	(*E*)-4-Phenylbut-3-en-2-one, **1g**		**2g**	71 (**B**), 79 (**C**)
8	(*E*)-Pent-3-en-2-one, **1h**		**2h**	84 (**B**), 94 (**C**)
9	1-Indanone, **1i**		**2i**	84[f] (**B**), 68[g] (**C**)
10	(*R*)-Carvone, **1j**		**2j**	>99[h] (**B**) *[79:21]*[b], 97[i] (**C**) *[81:19]*[b]

[a] Experimental procedure as given in the Experimental Section. [b] Distribution of diastereoisomers [% : %]. [c] Contains at least 3% of another diastereoisomer according to GC. [d] The ratio of peaks as they appear on GC. [e] Formation of another pair of diastereoisomers is presumably due to partial epimerization of the starting ketone **1f** at 60°C. [f] Contains 8% TMS-enol. [g] Contains 4% TMS-enol, [h] Contains 14% of 1-4 addition product. [i] Contains 5% of 1-4 addition product.

Table 2. Isolated Yields (%) for the preparation of trilakylsilyl-derivatives **3–5**[a].

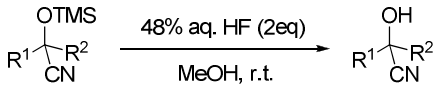
Structure	R=TES		R=TBDMS		R=TIPS	
	**3a**	83 (**A**), 92 (**B**)	**4a**	85 (**A**), 82 (**B**)	**5a**	82 (**A**)
	**3e**	>99 (**A**) *[88:12]*[b]	**4e**	82 (**A**) *[71:29]*[b]	**5e**	94 (**A**) *[81:19]*[b]
	**3g**	94 (**C**)	**4g**	60 (**C**)	**5g**	72 (**C**)[c]

[a] Experimental procedure as given in the Experimental Section. [b] Distribution of diastereoisomers [% : %]. [c] Reaction performed on 1mmol scale and purified by semi-preparative HPLC.

NaCN also acted as a base[47] leading to partial α-C epimerization of the ketone **1f** (Entry 6, Table 1) or conversion of the ketone **1i** to the corresponding silyl enol ether (Entry 9, Table 1). The formation of the latter side product could be minimized using a two-phase hexane–DMSO system (Method B, Experimental Section). Under these conditions, the yield of **2a** improved significantly (Entry 1, Table 1). However, with this protocol 1,4 addition of the cyanide ion to carvone **1j** was observed (Entry 10, Table 1).[50] When the reaction was performed at room temperature and greater dilution (Method C, Experimental section), the addition of the cyanide anion to conjugated enones proceeded in favor of the 1,2 cyanosilylation product with limited undesirable 1,4-addition to **1j** or polymerization (Table 1). The TMS-derivatives **2** were isolated in at least 98% purity (GC) by non-aqueous extraction of the products from DMSO with hexane followed by filtration through a short pad of silica gel. The trialkylsilyl-derivatives **3–5** were further purified by column chromatography or distillation to obtain this grade of purity.

The parent ketone cyanohydrins are smoothly liberated from the TMS-derivatives **2** using aqueous HF in MeOH (Scheme 2).

Scheme 2. Ketone cyanohydrins by deprotection of the TMS-derivatives **2**.



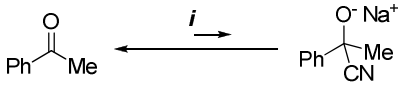
DMSO proved to be crucial for activating our Lewis acid / base free cyanosilylation system. The reaction was extremely slow when performed in another dipolar aprotic solvent, DMF.[51] To account for the unique role of DMSO and shed light on the reaction mechanism, we carried out a comparative kinetic study on the conversion of acetophenone **1a** into the TBDMS-derivative **4a**.

When TBDMSCN was used as a sole cyanide source (no NaCN added) no reaction could be observed, even in combination with TBDMSCl. This result demonstrates the crucial importance of free cyanide in inducing the observed cyanosilylation in DMSO.[52] TBDMSCl is found to react with NaCN to form TBDMSCN, the reaction rate being comparable with that of the cyanosilylation of the ketone **1a**. Parallel formation of TBDMSCN effectively reduces the concentration of free cyanide anions in the reaction medium. For this reason, a significant decrease in the reaction rate was observed when NaCN was used in equimolar amount with reference to TBDMSCl (1.2:1.2 ratio) when compared to the original ratio of TBDMSCl to NaCN of 1.2:2. The pivotal role of the free $CN^{\ominus}$ was further substantiated by the fact that the reaction rate increased when TBDMSCN was used as the trapping agent for the putative cyanoalcoholate (TBDMSCN to NaCN ratio of 1.2:2). Indeed, upon silylation with TBDMSCN, the concentration of free cyanide is maintained throughout the course of the reaction. To account for the above observations, we suggest a two-step reaction pathway including addition of highly nucleophilic $CN^{\ominus}$ in

DMSO across the C=O bond of the ketone at rate determining step followed by silylation of the intermediate tertiary sodium cyanoalcoholate with chloro-trialkylsilane or in situ formed cyano-trialkylsilane.

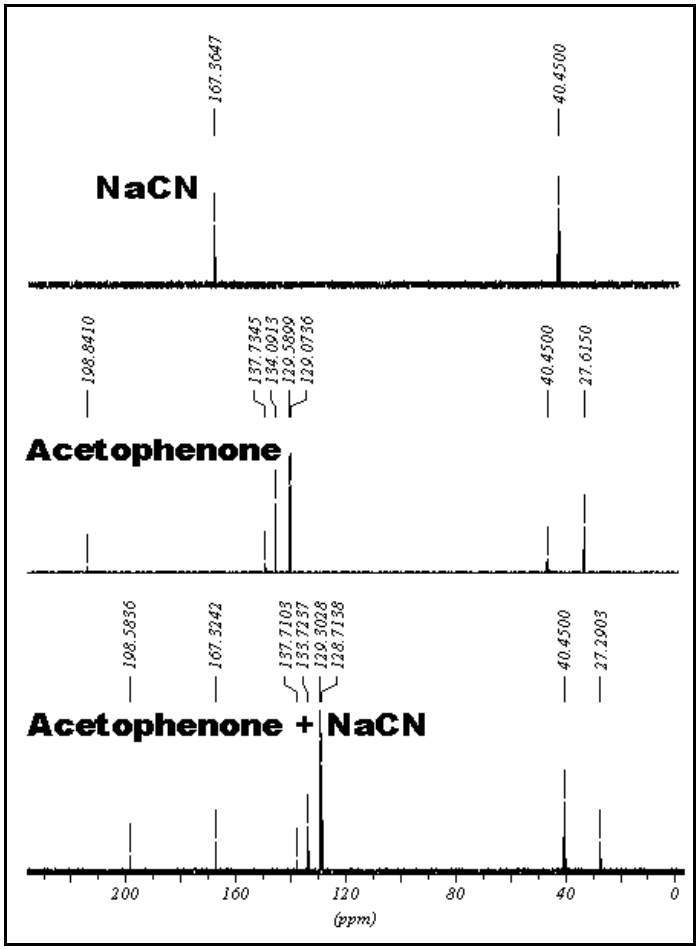
Despite the above kinetic evidence, we were unable to detect the putative tetrahedral intermediate, the cyanoalcoholate anion, in the mixture of acetophenone and NaCN by NMR in DMSO-d_6 at 60°C (Scheme 3, and Figure 1).

Scheme 3. Equilibrium between the parent carbonyl compound and the tetrahedral cyanoalcoholate in the presence of NaCN in DMSO-d_6.



i: NaCN, 60°C in DMSO-d_6

Figure 1. ^{13}C NMR spectra (100.6MHz, DMSO-d_6, 60°C) of the equilibrium mixtures.



This result confirms that despite significantly enhanced nucleophilicity of naked cyanide in DMSO, the equilibrium between ketones and the tertiary cyanoalcoholates anions remains essentially unaltered. It lies to such an extent on the side of the starting material (Scheme 3) that the intermediate cannot be detected by standard NMR techniques. However, although thermodynamically only minute quantities of alcoholate are formed, the kinetics of the uncatalyzed

equilibrium are such that they do not limit the synthesis of the protected cyanohydrins.

Conclusion

The cyanosilylation protocol described in this Chapter enables the most general synthesis of racemic silyl protected cyanohydrins known to date, applicable to a wide range of ketones and trialkylsilyl protecting groups. The method is simple, robust, cost-effective and safe since it requires neither catalysis nor expensive and potentially hazardous silyl cyanides.

Experimental Section

General Remarks

CAUTION: *Due to the toxic nature of cyanide derivatives such as hydrogen cyanide and trialkylsilyl cyanides all experiments were carried out in a well ventilated fume hood and a HCN detector was used for continuous monitoring. Although the toxicity of cyanide salt solutions in DMSO is not known we recommend the use of the appropriate type of gloves while handling these mixtures. All cyanide wastes were neutralized with a technical grade solution of sodium hypochlorite (bleach) and stored separately over a large excess of bleach for disposal.*

Chemicals: The carbonyl compounds, trialkylsilyl chlorides and cyanides used in this study were commercially available chemicals of reagent grade purity and were used without further purification. Commercial sodium cyanide was dried in a vacuum glass oven overnight (5mbar, 200°C). Dry DMSO ($H_2O \leq 0.005\%$) was purchased from commercial sources. Alternatively, commercial DMSO of a lower grade could be dried by refluxing it overnight under reduced

pressure (*ca.* 20 mbar) prior to distillation. After removing the head (about 1/3 of the overall volume), the core of the distillate could be used without further purification for the cyanosilation of ketones as described here. This procedure was also used to dry DMSO-d_6 for the kinetic study. Other solvents were dried according to standard laboratory procedures.

Analytical Methods: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Avance Ultrashield spectrometer. Chemical shifts in deuterated chloroform are reported in parts per million relative to tetramethylsilane for ^{1}H NMR spectra and the residual peak of the solvent for ^{13}C NMR spectra (δ = 77.0ppm). In DMSO-d_6 the chemical shifts are reported relative to the residual peak of the solvent (2.54ppm for ^{1}H and 40.45ppm for ^{13}C). Column chromatography was performed using Silica Gel 60 (0.040-0.063mm, 230-400 mesh) packed up to 300mm height in a 40mm diameter column. The mobile phase used was hexane:ethyl acetate (100:0 to 80:20) and the course of the elution was followed by gas chromatography. GC was performed using a HP-5 column and either a FID detector or a Mass Selective detector (for peak assignment and by-product identification). Semi-preparative scale HPLC was performed using a Zorbax RX-sil PrepHT normal phase column (21.5x250mm) and a diode array detector.

General method A

To a solution of sodium cyanide (20 mmol) in dry DMSO (10 mL) at 60°C under inert atmosphere was added the carbonyl compound (10 mmol). The mixture was stirred for 5 min at this temperature and the trialkylsilyl chloride (12 mmol) was added dropwise at 60°C. The reaction mixture was then stirred at 60°C under inert atmosphere for 5 min (TMS, TES, and TBDMS derivatives) or 10 min (TIPS derivatives). The DMSO phase was extracted with hexane (3×15 mL) and the combined hexane phases were cooled in an ice bath to separate residual DMSO. After evaporation of the solvent the residue was

filtered through a short pad of silica using hexane as eluent. The solvent was then removed under reduced pressure to give the TMS-derivatives in good purity (>98%). Other derivatives were further purified by column chromatography or distillation.

General method B

To a solution of sodium cyanide (20 mmol) in dry DMSO (10 mL) at 60°C under inert atmosphere was added dry hexane (10 mL). An initial amount of trialkylsilyl chloride (3 mmol) was added to the stirred mixture at 60°C under inert atmosphere followed immediately by dropwise addition of a solution of the carbonyl compound (10 mmol) and trialkylsilyl chloride (12 mmol) in hexane (5 mL). The reaction was allowed to proceed for 5 min at this temperature. The reaction mixture was then treated as described in general method A.

General method C

A solution of sodium cyanide (30 mmol) in dry DMSO (15 mL) under inert atmosphere was heated gently with a heat gun to ensure saturation and the mixture was allowed to cool to r.t.. The respective trialkylsilyl chloride (20 mmol) was added to the thick mixture at r.t. and stirring was continued for 1 min (TMS and TES derivatives) or 15 min (TBDMS and TIPS derivatives). The carbonyl compound (10 mmol) was then added slowly at r.t. under inert atmosphere and the mixture was stirred for 30 minutes under these conditions. The reaction mixture was then treated as described in general method A.

General method for the deprotection of TMS-derivatives 2

To a solution of TMS-protected cyanohydrin in methanol (1mmol/mL) was added commercial 48% aqueous solution of HF (2eq) and the reaction was stirred at ambient temperature. Toward the end of the reaction (NMR monitoring), the mixture was diluted with water and extracted with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure to give the corresponding cyanohydrin. TMS-acetophenone cyanohydrin **2a**, was deprotected according to this procedure within 2h and acetophenone cyanohydrin was obtained in 99% yield and excellent purity (NMR) without further purification.

Kinetic Study

Sodium cyanide (0.5 mmol) was weighed in an oven-dried NMR tube and dissolved in dry DMSO-d_6 (500 µL) by gentle heating. The solution was kept at 60°C and acetophenone (0.25 mmol) was added. After 15min at 60°C the ^{13}C NMR spectrum was recorded at 60°C (Figure 1). The spectra were recorded a second time after approximately 24h to ensure that the equilibrium was achieved. The reference spectra of acetophenone, acetophenone cyanohydrin, and sodium cyanide were recorded in DMSO-d_6 at 60°C under identical conditions.

Synthetic Procedures and Spectral Data

2-Phenyl-2-trimethylsilanyloxy-propionitrile (**2a**): The synthesis was carried out according to the general procedure (Method **B**) from **1a** (1.20mL, 10.3mmol), NaCN (1.086g, 22.1mmol), TMSCl (380µL, 3.0mmol followed by 1.50mL, 11.8mmol). After filtration through a pad of silica, the pure product **2a** was obtained as a clear liquid (2.232g, 99% yield). ^1H NMR[10] ($CDCl_3$, 400MHz): δ = 0.17 (s, 9H), 1.86 (s, 3H), 7.33-7.42 (m, 3H), 7.53-7.56 (m, 2H). ^{13}C NMR[10] ($CDCl_3$, 100.65MHz): δ = 1.0, 33.6, 71.6, 121.6, 124.6, 128.6, 142.0.

2-Phenyl-2-triethylsilanyloxy-propionitrile (**3a**): The synthesis was carried out according to the general procedure (Method **B**) from **1a** (1.20mL, 10.3mmol), NaCN (1.006g, 20.5mmol), TESCl (505µL, 3.0mmol followed by 2.00mL, 11.9mmol). After column chromatography, the pure product **3a** was obtained as a clear liquid (2.485g, 92% yield). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.69 (m_c, 6H), 0.94 (t, ^{3}J = 8.3 Hz, 9H), 1.86 (s, 3H), 7.32-7.41 (m, 3H), 7.54-7.57 (m, 2H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 5.4, 6.7, 33.7, 71.4, 121.8, 124.5, 128.6, 142.2. EA calcd for $C_{15}H_{23}NOSi$ C, 68.91; H, 8.87; N, 5.36; found C, 68.79; H, 8.83; N, 5.35. HRMS (EI) *m/z* calcd 261.1549, found 261.1550.

2-(tert-Butyl-dimethyl-silanyloxy)-2-phenyl-propionitrile (**4a**): The synthesis was carried out according to the general procedure (Method **A**) from **1a** (1.20mL, 10.3mmol), NaCN (1.135g, 23.1mmol), TBDMSCl (1.875g, 12.4mmol). After high vacuum treatment (120°C, 0.5mbar) to remove $TBDMS_2O$, the pure product **4a** was obtained as a clear liquid (2.294g, 85% yield). ^{1}H NMR[49] ($CDCl_3$, 400MHz): δ = 0.05 (s, 3H), 0.23 (s, 3H), 0.94 (s, 9H), 1.86 (s, 3H), 7.33-7.42 (m, 3H), 7.54-7.57 (m, 2H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = -3.9, -3.7, 18.2, 25.6, 33.4, 71.6, 121.7, 124.6, 128.6, 142.1.

2-Phenyl-2-triisopropylsilanyloxy-propionitrile (**5a**): The synthesis was carried out according to the general procedure (Method **A**) from **1a** (1.20mL, 10.3mmol), NaCN (1.035g, 21.1mmol), TIPSCl (2.60mL, 12.2mmol). After column chromatography, the pure product **5a** was obtained as a clear liquid (2.559g, 82% yield). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 1.05 (d, *J* = 7.3 Hz, 9H), 1.07 (d, *J* = 7.3 Hz, 9H), 1.26 (m_c, 3H), 1.89 (s, 3H), 7.32-7.42 (m, 3H), 7.58-7.61 (m, 2H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 12.7, 18.08, 18.12, 34.3, 71.6, 121.8, 124.5, 128.5, 142.5. EA calcd for $C_{18}H_{29}NOSi$ C, 71.23; H, 9.63; N, 4.61; found C, 71.49; H, 9.70; N, 4.67. HRMS (EI) *m/z* calcd 303.2018, found 303.2044.

2,3,3-Trimethyl-2-trimethylsilanyloxy-butyronitrile (**2b**): The synthesis was carried out according to the general procedure (Method **A**) from **1b** (1.20mL, 9.7mmol), NaCN (1.028g, 21.0mmol), TMSCl (1.50mL, 11.8mmol). After filtration through a pad of silica, the pure product **2b** was obtained as a clear liquid (1.859g, 96% yield). ^{1}H NMR[53] ($CDCl_3$, 400MHz): δ = 0.24 (s, 9H), 1.03 (s, 9H), 1.51 (s, 3H). ^{13}C NMR[53] ($CDCl_3$, 100.65MHz): δ =1.1, 23.7, 24.5, 38.8, 76.1, 121.8.

Cyclohexyl-phenyl-trimethylsilanyloxy-acetonitrile (**2c**): The synthesis was carried out according to the general procedure (Method **A**) from **1c** (1.895g, 10.1mmol), NaCN (1.075g, 21.9mmol), TMSCl (1.50mL, 11.8mmol). After filtration through a pad of silica, the pure product **2c** was obtained as a clear liquid (2.782g, 96% yield). ^{1}H NMR[14] ($CDCl_3$, 400MHz): δ = 0.09 (s, 9H), 1.01-1.21 (m, 5H), 1.36-1.38 (m, 1H), 1.57-1.81 (m, 4H), 2.00-2.02 (m, 1H), 7.31-7.40 (m, 3H), 7.45-7.48 (m, 2H). ^{13}C NMR[14] ($CDCl_3$, 100.65MHz): δ = 0.8, 25.87, 25.89, 25.94, 27.2, 27.3, 50.6, 79.5, 120.2, 125.8, 128.2, 128.4, 140.1.

2-Ethyl-2-trimethylsilanyloxy-octanenitrile (**2d**): The synthesis was carried out according to the general procedure (Method **A**) from **1d** (1.70mL, 9.8mmol), NaCN (1.092g, 22.3mmol), TMSCl (1.50mL, 11.8mmol). After filtration through a pad of silica, the pure product **2d** was obtained as a clear liquid (2.392g, >99% yield). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.23 (s, 9H), 0.90 (m, 3H), 1.03 (t, ^{3}J = 7.4 Hz, 3H), 1.27-1.54 (m, 8H), 1.65-1.81 (m, 4H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 1.3, 8.4, 14.0, 22.5, 23.9, 29.1, 31.6, 34.0, 40.5, 73.8, 121.6. EA calcd for $C_{13}H_{27}NOSi$ C, 64.67; H, 11.27; N, 5.80; found C, 63.12; H, 11.15; N, 5.78. HRMS (EI) *m/z* calcd 241.1862, found 241.1844.

2-Methyl-1-trimethylsilanyloxy-cyclohexanecarbonitrile (**2e**): The synthesis was carried out according to the general procedure (Method **A**) from **1e** (1.20mL, 9.9mmol), NaCN (1.115g, 22.8mmol), TMSCl (1.50mL, 11.8mmol). After

filtration through a pad of silica, the pure product **2e** was obtained as a clear liquid (2.030g, 97% yield), *de* = 58% (by GC: 150°C/30psi, Rt_{minor}=1.18min, Rt_{major}=1.22min). ^{1}H NMR[19] ($CDCl_3$, 400MHz): δ = 0.23 (s, 9H), 1.04 (d, ^3J = 6.4 Hz, 3H), 1.08 (d, ^3J = 6.4 Hz, 3H), 1.17-1.80 (m, 8H), 2.04-2.09 (m, 1H), 2.14-2.22 (m, 1H). ^{13}C NMR[19] ($CDCl_3$, 100.65MHz): Major: δ = 1.3, 16.3, 23.6, 24.8, 31.4, 39.6, 43.0, 75.9, 120.0. Minor: δ = 1.0, 16.3, 20.0, 24.4, 28.2, 38.0, 40.7, 71.5, 122.4.

2-Methyl-1-triethylsilanyloxy-cyclohexanecarbonitrile (**3e**): The synthesis was carried out according to the general procedure (Method **A**) from **1e** (1.20mL, 9.9mmol), NaCN (1.011g, 20.6mmol), TESCl (2.00mL, 11.9mmol). After column chromatography, the pure product **3e** was obtained as a clear liquid (2.535g, >99% yield), *de* = 76% (by GC: 150°C/20psi, Rt_{minor}=5.00min, Rt_{major}=5.13min). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.71 (m_c, 6H), 0.98 (t, ^3J = 7.8 Hz, 9H), 1.06 (d, ^3J = 6.4 Hz, 3H), 1.10 (d, ^3J = 6.4 Hz, 3H), 1.18-1.81 (m, 8H), 2.04-2.10 (m, 1H), 2.15-2.20 (m, 1H). ^{13}C NMR ($CDCl_3$, 100.65MHz): Major: δ = 5.7, 6.7, 16.3, 23.7, 24.7, 31.5, 39.5, 43.3, 75.8, 120.2. Minor: δ = 5.5, 6.8, 16.3, 20.1, 24.3, 28.2, 38.1, 41.0, 71.4, 122.5. EA calcd for $C_{14}H_{27}NOSi$ C, 66.34; H, 10.74; N, 5.53; found C, 65.96; H, 10.68; N, 5.53. HRMS (EI) *m/z* calcd 253.1862, found 253.1873.

1-(tert-Butyl-dimethyl-silanyloxy)-2-methyl-cyclohexanecarbonitrile (**4e**): The synthesis was carried out according to the general procedure (Method **A**) from **1e** (1.20mL, 9.9mmol), NaCN (1.162g, 23.7mmol), TBDMSCl (1.852g, 12.3mmol). After high vacuum treatment (120°C, 0.5mbar) to remove $TBDMS_2O$, the pure product **4e** was obtained as a clear liquid (2.061g, 82% yield), *de* = 42% (by GC: 160°C/15psi, Rt_{major}=3.72min, Rt_{minor}=3.77min). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.19 (s, 3H), 0.22 (s, 3H), 0.26 (s, 3H), 0.27 (s, 3H), 0.89 (s, 9H), 0.93 (s, 9H), 1.06 (d, ^3J = 6.7 Hz, 3H), 1.10 (d, ^3J = 6.7 Hz, 3H), 1.16-1.81 (m, 8H), 2.06-2.11 (m, 1H), 2.15-2.20 (m, 1H). ^{13}C NMR ($CDCl_3$, 100.65MHz): Major: δ = -4.0, -2.9, 16.4, 18.0, 23.6, 24.8, 25.4, 31.4, 39.6, 43.2,

75.9, 120.1. Minor: δ = -4.1, -3.5, 16.6, 18.4, 20.0, 24.6, 25.6, 28.3, 38.3, 41.1, 71.5, 122.4. EA calcd for $C_{14}H_{27}NOSi$ C, 66.34; H, 10.74; N, 5.53; found C, 65.35; H, 10.69; N, 5.39. HRMS (EI) *m/z* calcd 253.1862, found 253.1996.

2-Methyl-1-triisopropylsilanyloxy-cyclohexanecarbonitrile (**5e**): The synthesis was carried out according to the general procedure (Method **A**) from **1e** (1.20mL, 9.9mmol), NaCN (1.182g, 24.1mmol), TIPSCl (2.60mL, 12.2mmol). After column chromatography, the pure product **3e** was obtained as a clear liquid (2.753g, 94% yield), *de* = 62% (by GC: 160°C/30psi, Rt_{major}=6.41min, Rt_{minor}=6.64min). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 1.07-1.37 (m, 26H), 1.42-1.90 (m, 6H), 2.09-2.17 (m, 1H), 2.21-2.29 (m, 1H). ^{13}C NMR ($CDCl_3$, 100.65MHz): Major: δ = 12.9, 16.7, 18.10, 18.11, 23.7, 31.6, 39.3, 43.7, 76.3, 120.2. Minor: δ = 12.7, 18.15, 18.20, 20.5, 24.6, 28.5, 37.6, 41.5, 71.9, 122.6. EA calcd for $C_{17}H_{33}NOSi$ C, 69.09; H, 11.25; N, 4.74; found C, 69.90; H, 11.52; N, 4.90. HRMS (EI) *m/z* calcd 295.2331, found 295.2268.

Trimethylsilanyloxy-(L)-menthone-cyanohydrin (**2f**): The synthesis was carried out according to the general procedure (Method **C**) from **1f** (1.70mL, 9.8mmol), NaCN (1.562g, 31.9mmol), TMSCl (2.60mL, 20.5mmol). After filtration through a pad of silica, the pure product **2f** was obtained as a clear liquid (2.507g, >99% yield). Diastereoisomeric ratio of product via Method **C**: [38:59:0:3]. Diastereoisomeric ratio of product via Method **A**: [5:81:2:12] (by GC: 150°C/15psi, R$t_{(5\%)}$=3.61min, R$t_{(81\%)}$=3.70min, R$t_{(2\%)}$=3.92min, R$t_{(12\%)}$=4.19min with reference to the product ratio obtained with Method **A**). NMR spectral data are given for the major diastereoisomer (59%, Method **C**) and the minor diastereoisomer (38%, Method **C**). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.24 (s, 9H), 0.25 (s, 9H), 0.89 (d, ^3J = 6.6 Hz, 3H), 0.91 (d, ^3J = 6.9 Hz 3H), 0.96 (dd, ^3J = 7.0 Hz, ^4J = 5.4 Hz, 6H), 0.96 (dd, ^3J = 13.6 Hz, ^4J = 7.0 Hz, 6H), 1.22-1.53 (m, 4H), 1.62-1.84 (m, 3H), 2.07 (m_c, 1H), 2.13 (m_c, 1H), 2.23 (m_c, 1H). ^{13}C NMR ($CDCl_3$, 100.65MHz): Major: δ = 1.4, 17.1, 21.5, 23.0, 23.5, 25.7, 30.2, 34.0, 49.7,

53.2, 73.5, 121.5. Minor: δ = 1.1, 18.2, 20.1, 21.5, 23.4, 26.6, 29.5, 34.2, 47.8, 50.6, 73.8, 122.6. EA calcd for $C_{14}H_{27}NOSi$ C, 66.34; H, 10.74; N, 5.53; found C, 65.02; H, 10.58; N, 5.45. HRMS (EI) *m/z* calcd 253.1862, found 253.1992. All four diastereoisomers had identical GC-MS spectra with main fragments at *m/z* = 253, 238, 211 (100%), 183, 169 and 75. The *de* of the commercial (*L*)-menthone used as starting material was 94% (by GC: 120°C/15psi, Rt_{major}=2.81min, Rt_{minor}=2.92min). GC-MS spectra of these two peaks were identical with main fragments at *m/z* = 154, 139, 112 (100%), 97, 69. After deprotection of compound **2f** obtained via Method **C** (aqueous HF in methanol, 24h at r.t.), the ^{13}C NMR spectrum of the free cyanohydrin diastereoisomers was compared to literature data[54] and the major diastereoisomer of **2f** (59% Method **C**) was identified as (1*R*)-trimethylsilanyloxy-(*L*)-menthone-cyanohydrin while the minor diastereoisomer of **2f** described above (38% Method **C**) was identified as (1*S*)-trimethylsilanyloxy-(*L*)-menthone-cyanohydrin.

(E)-2-Methyl-4-phenyl-2-trimethylsilanyloxy-but-3-enenitrile (**2g**): The synthesis was carried out according to the general procedure (Method **C**) from **1g** (1.470g, 10.1mmol), NaCN (1.557g, 31.8mmol), TMSCl (2.60mL, 20.5mmol). After filtration through a pad of silica, the pure product **2g** was obtained as a clear liquid (1.962g, 79% yield). ^1H NMR[14, 19] ($CDCl_3$, 400MHz): δ = 0.24 (s, 9H), 1.75 (s, 3H), 6.13 (d, ^3J = 15.6 Hz, 1H), 6.88 (d, ^3J = 15.6 Hz, 1H), 7.28-7.43 (m, 5H). ^{13}C NMR[14, 19] ($CDCl_3$, 100.65MHz): δ = 1.4, 30.9, 70.0, 120.7, 126.9, 128.6, 128.8, 129.5, 131.0, 135.1.

(E)-2-Methyl-4-phenyl-2-triethylsilanyloxy-but-3-enenitrile (**3g**): The synthesis was carried out according to the general procedure (Method **C**) from **1g** (1.474g, 10.1mmol), NaCN (1.683g, 34.3mmol), TESCl (3.40mL, 20.3mmol). After column chromatography, the pure product **3g** was obtained as a clear liquid (2.723g, 94% yield). ^1H NMR ($CDCl_3$, 400MHz): δ = 0.65-0.79 (m_c, 6H),

0.98 (t, ^{3}J = 7.4 Hz, 9H), 1.75 (s, 3H), 6.13 (d, ^{3}J = 16.6 Hz, 1H), 6.89 (d, ^{3}J = 16.6 Hz, 1H), 7.29-7.42 (m, 5H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 5.6, 6.7, 31.0, 69.8, 120.8, 126.9, 128.6, 128.8, 129.6, 130.8, 135.2. EA calcd for $C_{17}H_{25}NOSi$ C, 71.03; H, 8.77; N, 4.87; found C, 70.75; H, 8.85; N, 4.95. HRMS (EI) *m/z* calcd 287.1705, found 287.1807.

(E)-2-(tert-Butyl-dimethyl-silanyloxy)-2-methyl-4-phenyl-but-3-enenitrile (**4g**): The synthesis was carried out according to the general procedure (Method **C**) from **1g** (1.493g, 10.2mmol), NaCN (1.536g, 31.3mmol), TBDMSCl (3.077g, 20.4mmol). After column chromatography, the pure product **4g** was obtained as a clear liquid (2.494g, 60% yield). ^{1}H NMR[49] ($CDCl_3$, 400MHz): δ = 0.19 (s, 3H), 0.25 (s, 3H), 0.92 (s, 9H), 1.74 (s, 3H), 6.13 (d, ^{3}J = 15.9 Hz, 1H), 6.89 (d, ^{3}J = 15.9 Hz, 1H), 7.28-7.42 (m, 5H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = -3.6, -3.2, 18.0, 25.5, 30.9, 69.9, 120.8, 126.9, 128.6, 128.8, 129.7, 130.8, 135.2.

(E)-2-Methyl-4-phenyl-2-triisopropylsilanyloxy-but-3-enenitrile (**5g**): The synthesis was carried out according to the general procedure (Method **C**) on 1mmol scale from **1g** (161.6mg, 1.11mmol), NaCN (154.9mg, 3.16mmol), TIPSCl (430μL, 2.01mmol). After purification by semi preparative HPLC (100% hexane, 5mL/min), the pure product **5g** was obtained as a clear liquid (263.6mg, 72% yield). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 1.09 (d, J = 7.4 Hz, 9H), 1.10 (d, J = 7.4 Hz, 9H), 1.14-1.28 (m, 3H), 1.79 (s, 3H), 6.17 (d, ^{3}J = 16.1 Hz, 1H), 6.91 (d, ^{3}J = 16.1 Hz, 1H), 7.28-7.41 (m, 5H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ =12.7, 18.1, 31.3, 69.9, 120.9, 126.9, 128.5, 128.8, 129.9, 130.6, 135.2. EA calcd for $C_{20}H_{31}NOSi$ C, 72.89; H, 9.48; N, 4.25; found C, 72.37; H, 9.70; N, 4.31. HRMS (EI) *m/z* calcd 329.2175, found 329.2216.

(E)-2-ethyl-2-trimethylsilanyloxy-pent-3-enenitrile (**2h**): The synthesis was carried out according to the general procedure (Method **C**) from **1h** (1.10mL, 9.6mmol), NaCN (1.531g, 31.2mmol), TMSCl (2.60mL, 20.5mmol). After

filtration through a pad of silica, the pure product **2h** was obtained as a clear liquid (1.780g, 94% yield). ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.20 (s, 9H), 1.00 (t, ^3J = 7.4 Hz, 3H), 1.68-1.90 (m_c, 2H), 1.77 (dd, ^3J = 6.7 Hz, ^4J = 1.6 Hz, 3H), 5.39 (dq, ^3J = 15.4 Hz, ^4J = 1.6 Hz, 1H), 6.04 (qd, ^3J = 15.4 Hz, ^3J = 6.7 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 1.3, 8.3, 17.3, 36.2, 74.4, 120.2, 128.6, 130.9. EA calcd for $C_{10}H_{19}NOSi$ C, 60.86; H, 9.70; N, 7.10; found C, 59.65; H, 9.73; N, 6.95. HRMS (EI) *m/z* calcd 197.1236, found 197.1247.

1-Trimethylsilanyloxy-indan-1-carbonitrile (**2i**): The synthesis was carried out according to the general procedure (Method **B**) from **1i** (1.310g, 9.9mmol), NaCN (1.156g, 23.6mmol), TMSCl (1.50mL, 11.8mmol). After filtration through a pad of silica, the product **2i** was obtained as a clear liquid containing 8% (by NMR) of the corresponding TMS-enol as by-product (1.896g, 84% yield). ^{1}H NMR[55] ($CDCl_3$, 400MHz): δ = 0.18 (s, 9H), 2.56 (m_c, 2H), 3.03 (m_c, 2H), 7.18-7.38 (m, 3H), 7.52-7.54 (m, 1H). ^{13}C NMR[55] ($CDCl_3$, 100.65MHz): δ =1.1, 29.4, 42.8, 76.5, 121.0, 124.1, 125.2, 127.3, 129.9, 142.1, 142.6. TMS-enol characteristic peaks: ^{1}H NMR[56] ($CDCl_3$, 400MHz): δ = 0.30 (s, 9H), 3.26 (d, 2H), 5.42 (t, 1H).

Trimethylsilanyloxy-(R)-carvone-cyanohydrin (**2j**): The synthesis was carried out according to the general procedure (Method **C**) from (*R*)-carvone **1j** (1.50mL, 9.6mmol), NaCN (1.580g, 32.2mmol), TMSCl (2.60mL, 20.5mmol). After filtration through a pad of silica, the product **2j** was obtained as a clear liquid containing 5% (by NMR) of the corresponding 1-4 addition product (2.332g, 97% yield), *de* = 62% (by GC: 150°C/20psi, Rt_{minor}=3.30min, Rt_{major}=3.40min). ^{1}H NMR[57] ($CDCl_3$, 400MHz): δ = 0.27 (s, 9H), 1.75 (m, 3H), 1.82 (m, 3H), 1.91-1.99 (m, 2H), 2.15-2.22 (m, 1H), 2.34-2.37 (m, 1H), 2.49-2.56 (m, 1H), 4.77 (d, 2H), 5.64 (d, 1H), 5.70 (d, 1H). ^{13}C NMR[57] ($CDCl_3$, 100.65MHz): Major: δ = 1.3, 17.4, 20.5, 30.6, 39.1, 42.3, 71.3, 110.0, 121.2,

127.0, 133.7, 146.9. Minor: δ = 1.2, 18.6, 20.6, 30.5, 35.0, 42.0, 69.8, 110.1, 121.5, 127.4, 131.3, 147.2.

1-4 addition product characteristic peaks: ^{1}H NMR ($CDCl_3$, 400MHz): δ = 0.20 (s, 9H), 1.72 (s, 3H), 1.76 (s, 3H), 1.97-2.19 (m, 4H), 2.51-2.63 (m, 1H), 3.19 (m, 1H), 4.78 (d, 2H). ^{13}C NMR ($CDCl_3$, 100.65MHz): δ = 206.8.

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SUMMARY

In this thesis I have addressed several issues related to the HNL-catalyzed preparation of cyanohydrins. I first demonstrated in Chapter 2 that immobilized HNL as sol-gels and as commercially available Cross Linked Enzyme Aggregates (CLEA®) improved several features of the biocatalyst such as solvent stability, and substrate or product inhibition/deactivation. In particular, *Me*CLEA was remarkably stable towards the deleterious effect of organic solvent and the enzymatic reaction could be carried out in organic media. The CLEA immobilization strategy is nonetheless enzyme-dependent and I successfully developed the biocatalyst *Lu*CLEA for optimum catalytic performances in organic media as described in Chapter 3. This enantioselective and recyclable biocatalyst appeared to be particularly effective for the preparation of 2-butanone cyanohydrin.

In Chapter 4, I used benzaldehyde as a model substrate to develop multistep strategies towards cyanohydrin derivatives based on HNL-CLEA catalysis in organic solvents. The reaction could be carried out in one pot or with limited downstream processing/purification of the cyanohydrin intermediate. In the case of ketones such as acetophenone where unfavourable thermodynamics limit the practical conversion, all attempts to derivatize the cyanohydrin *in situ* in order to shift the equilibrium were not successful. Cyanohydrins from ketones can indeed be considered as tertiary alcohols which require relatively reactive reagents for derivatization. Under these conditions the biocatalyst was rendered inactive.

Since no *in situ* derivatization method could de designed to enable complete conversion of unreactive ketones, kinetic resolution as a means to produce chiral cyanohydrin was explored in Chapter 5. I established enzymatic activity

for a previously unreported α,β-unsaturated ketone and showed that kinetic resolution was more suitable than the direct synthetic route for the preparation of the corresponding chiral cyanohydrin. As an extension of this work I also described the rearrangement of a similar α,β-unsatuared cyanohydrin acetate into the corresponding tetronic acid derivative. Chapter 6 concludes this thesis with straightforward synthetic procedures towards racemic cyanohydrins from unreactive ketones in order to improve the overall cost efficiency of the kinetic resolution approach.

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Singapore, August 2009

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CURRICULUM VITAE

Fabien L. Cabirol was born on the 14th of February 1978 in Mont-de-Marsan, France. In 1995, he completed his secondary education in Montauban, France and enrolled in the Classe Préparatoire Mathématiques Supérieures (PCSI) and Mathématiques Spéciales (PC*) at the Lycée Saliège in Toulouse, France. In 1998, he was accepted at the Ecole Nationale Supérieure de Chimie de Montpellier (ENSCM) in Montpellier, France.

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