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Synthesis of Aliphatic and α-Halogenated Ketone Cyanohydrins with the Hydroxynitrile Lyase from *Manihot esculenta*

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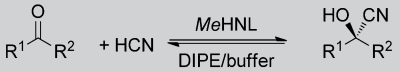
The potential of the hydroxynitrile lyase from Manihot esculenta towards ketone substrates was investigated. It was observed that the length of the aliphatic chain is a key parameter for the conversion of aliphatic, non-branched ketones. Smaller substrates are readily converted with high enantioselectivites, but the elongation of the chain length causes a significant loss in enzyme activity. For a number of halogenated, herein especially fluorinated, acetophenone derivatives the corresponding cyanohydrins have been synthesized with good to moderate enantioselectivities.

Hydroxynitrile lyases (HNLs) catalyze selectively the C–C coupling of hydrogen cyanide to carbonyl compounds to yield enantiopure cyanohydrins.[1–3] Owing to the formation of two new functional groups (one hydroxyl and one nitrile group), these enantiopure substances are considered as valuable intermediates for a variety of building blocks for pharmaceuticals, including α-hydroxy carboxylic acids, β-hydroxyamines, and α-aminonitriles.[4–8] Since the first experiments of Wöhler in 1837 and Rosenthaler in 1909, HNLs became extremely valuable biocatalysts for the synthesis of enantiopure cyanohydrins.[9–11] Important examples of highly enantioselective enzymes are the (*R*)-selective HNLs from *Prunus amygdalus* (*Pa*HNL, bitter almond) and *Arabidopsis thaliana* (*At*HNL, thale cress) and the (*S*)-selective HNLs from *Hevea brasiliensis* (*Hb*HNL, rubber tree) and *Manihot esculenta* (*Me*HNL, cassava).[12–15] Within the HNL-catalyzed synthesis of cyanohydrins, the natural defense mechanism (release of hydrogen cyanide) is simply reversed.[16–19] Furthermore, in comparison to other chemical cyanohydrin synthesis routes, the enzymatic method offers higher enantioselectivities, moderate reaction conditions, and higher applicability.[6] Thus, these biocatalysts were repeatedly applied in large-scale productions within the pharmaceutical and agrochemical industry.[20] Examples include (*S*)-3-phenoxybenzaldehyde cyanohydrin, which is a precursor of various synthetic pyrethroids, and (*R*)-2-hydroxy-4-phenylbutyronitrile for the production of angiotensin-converting enzyme inhibitors.[21–23]

The vast majority of successful HNL-catalyzed cyanohydrin formations include the synthesis of aldehyde cyanohydrins, which are typically obtained in high yield and enantiomeric purity. In contrast to this, ketone cyanohydrins are considered as less easily achievable owing to steric hindrance, lower stability, and the limitation of substrate acceptance of HNLs.[4,24–26] However, ketone cyanohydrins are considered as even more valuable chemicals, as they possess a tertiary alcohol group.[13,27–29] For example, the *Me*HNL-catalyzed synthesis of unsubstituted acetophenone cyanohydrin, a precursor for atrolactic acid, was found to be thermodynamically limited with an equilibrium cyanohydrin yield of 2%.[26] Medium engineering can be used to overcome, at least partially, the thermodynamic restrictions, but it is rather limited to liquid reactants and requires a significantly higher amount of biocatalyst. In contrast, several successful examples of enantioselective conversions of ketones into the respective cyanohydrins were reported in the past. Examples include the *At*HNL-catalyzed synthesis of 2-hexanone cyanohydrin with 48% conversion and 95% (*R*) enantiomeric excess (*ee*),[11] selected (branched) aliphatic ketone cyanohydrins with enantioselectivities up to 91% *ee* (*S*) (*Me*HNL),[24] and the synthesis of heterocyclic tertiary cyanohydrins with *Hb*HNL and *Pa*HNL.[30]

Table 1. HNL-catalyzed synthesis of aliphatic methyl ketone cyanohydrins and α-halogenated acetophenone cyanohydrins.



1a-1l **2a-2l**

1, 2	Aliphatic R¹	R²	1, 2	Aromatic R¹	R²
a	C_2H_5	CH_3	**h**	Ph	CH_3
b	*n*-C_3H_7	CH_3	**i**	Ph	CH_2F
c	*n*-C_4H_9	CH_3	**j**	Ph	CHF_2
d	*n*-C_5H_{11}	CH_3	**k**	Ph	CF_3
e	*n*-C_6H_{13}	CH_3	**l**	Ph	CF_2Cl
f	*n*-C_7H_{15}	CH_3			
g	*n*-C_8H_{17}	CH_3			

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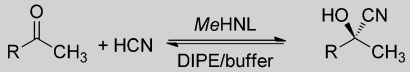
The aim of this study was to deepen the understanding of the synthesis of ketone cyanohydrins (Table 1). Herein, the biocatalyst *Me*HNL was chosen to study the conversion of a series of nonbranched aliphatic methyl ketones and α-halogen-substituted acetophenone cyanohydrins within industrially preferred two-phase systems consisting of a buffer and an organic solvent such as diisopropyl ether (DIPE).[31,32]

Corresponding cyanohydrins **2a**–**l** are known as highly valuable products.[4,33] In particular, fluorinated aromatic compounds are of great interest, as they facilitate a different lipophilicity in comparison to non-halogenated pharmaceuticals.[34,35] For example, enantiopure **2k** was used as a key intermediate in the synthesis of an anticonvulsant. Another application is its use as a precursor for potential inhibitors of a HIV protease and reverse transcriptase.[36] Within the related literature, Nguyen et al. presented an alternative approach to **2k**, which was obtained through esterase-catalyzed kinetic resolution from a racemic solution.[37] Unfortunately, this procedure required multiple reaction steps and yielded only a maximum conversion of 50% within the hydrolysis step, which resulted in a significance decrease in overall atom efficiency.[38] Therefore a direct HNL-catalyzed route to enantioenriched ketone cyanohydrins is preferred.

Aliphatic ketone cyanohydrins

The conversion of aliphatic methyl ketones into the corresponding aliphatic ketone cyanohydrins within a two-phase system revealed clearly that the length of the aliphatic chain is a key parameter (Table 2). Smaller substrates were readily converted by *Me*HNL at pH 4.0 in high yields with moderate to high enantioselectivities, for example, **1a** and **1b**. Unfortunately, racemization of the product occurred aside the non-enzymatic reaction (see also Figure S7, Supporting Information), which gradually decreased the enantiomeric excess of the product over time, especially for product **2b**. Lowering the pH value in the aqueous phase to pH 3.0 or even lower was not successful as a result of a significant loss of enzyme activity under the resulting reaction conditions.

Table 2. Synthesis of aliphatic ketone cyanohydrins.[a]



Substrate	pH	*t* [h]	Conversion [%]	*ee* (*S*) [%]
1a[b]		4[b]	91[b]	18[b]
1b	4	1	>99	78
		5	>99	19
1c	4	1	87	93
		5	94	87
1d	4	1	19	>90
		5	57	88
1e	4	1	6	n.d.[c]
		22.5	63	87
1f	4	1	<1	–
		22.5	19	n.d.
	5	1	59	>90
		22.5	88	75
1g	4	1	<1	–
		22.5	10	87
	5	1	7	85
		22.5	75	81

[a] Reaction conditions: Two-phase system consisting of a diisopropyl ether/50 mM citrate buffer pH 4/pH 5 (1:1), substrate (50 mM), hydrogen cyanide (250 mM), *Me*HNL (20 U mL^{-1}), 10 °C. [b] Reported by Förster et al.[39] (adsorbed *Me*HNL in monophasic diisopropyl ether). [c] n.d. = not determined.

Substrates with medium-sized chain lengths (i.e., **1c**–**e**) still allowed moderate to high yields at pH 4.0, but a significant loss of reactivity was observed. For example, **2c** was obtained with 94% conversion after 5 h, whereas the synthesis of **2e** required 22.5 h for a conversion of 63%. High enantioselectivities of approximately 90% *ee* (*S*) were obtained, which represent the perfect balance of reactivity and high enantioselectivity of the biocatalyst. Substrates with even longer side chains exhibited significantly reduced reactivity with *Me*HNL. At pH 4.0, **1f** was only converted with 19% and **1g** with 10% conversion even after prolonged reaction times. Fortunately, a further increase in the pH value to pH 5.0 enhanced the conversion of **1f** and **1g** to 88 and 75% with 87 and 81% *ee* (*S*), respectively.[12,40] The varying initial enantiomeric excess values of the products possibly depend on steric differences of both side chains flanking the carbonyl functionality. Thus, a better stereoselectivity is probably achieved for larger differences, as the substrate fits better into the active site.

The loss of reactivity of larger substrates may be related to an increase in hydrophobicity of the long-chained aliphatic substrates, which itself results in a decrease in substrate concentration within the aqueous phase. Thus, the apparent enzymatic activity of the biocatalyst, which is only located in the aqueous phase, eventually drops significantly [the substrate concentration in the aqueous phase is below the Michaelis-Menten constant (K_M) of the respective substrates]. In this case, the K_M value is required to be similar for all investigated substrates. Alternatively, the transformation of larger substrates may be hampered by steric effects in the active site of the enzyme. This may also increase the value of K_M for larger substrates, which would result in a decrease in the apparent activity of the enzyme. Eventually, higher substrate concentrations or enzyme loadings would be required.[41,42] Regardless, the observed effect is at least partially compensated with the shown increase in pH value from pH 4 to pH 5 in the aqueous phase. This effect is related to increased enzyme activity at higher pH values, as the pH optimum is found near pH 5.75.[43]

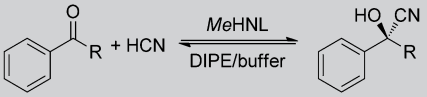
In summary, two-phase systems are feasible reaction systems for the conversion of aliphatic methyl ketones if the reaction conditions are carefully adjusted. Herein, the loss of enantiomeric excess throughout the reaction was almost completely suppressed and high substrate concentrations were available for the biocatalyst. Monophasic non-aqueous reaction systems, for example, biocompatible *tert*-butyl methyl ether in combination with immobilized biocatalysts, may also be useful alternatives for such substrates in the enzymatic synthesis of aliphatic methyl ketone cyanohydrins.

Aromatic α-halogenated acetophenone cyanohydrins

In contrast to the promising results of the HNL-catalyzed conversion of aliphatic methyl ketones **1a–g**, the use of the structurally similar substrate acetophenone (**1h**) resulted in a significant loss in equilibrium conversion. The conversion to **2h** dropped to an insufficient 2%, which prevented the efficient synthesis of the corresponding cyanohydrins; this reaction is also repeatedly given as a standard example of the limitations in HNL-catalyzed reactions.[31] As mentioned above, medium engineering can be used to overcome the restrictions, but it was only partially successful with 22% yield [96% *ee* (*S*)]. A further increase was only achievable by the additional introduction of electron-withdrawing groups at the aromatic ring of acetophenone, preferably fluoro and nitro substituents in the *ortho* position. Thereby, equilibrium concentrations were shifted towards the product side by electronic effects.[26]

In the present study, the introduction of halogen substituents into the α-standing methyl group was chosen to further tune the synthesis reaction towards high conversion and to broaden the understanding of the conversion of α-substituted ketones. A series of α-substituted acetophenone derivatives **1i–l** was converted in the two-phase system at pH 5.0 and compared to unsubstituted acetophenone **1h** (Table 3). The

Table 3. Synthesis of aromatic α-halogenated acetophenone derivatives.[a]



Substrate	*t* [h]	Conversion [%]	*ee* (*S*) [%]
1h[b]	24[b]	2[b]	–
1i	no enzymatic conversion		
1j	1.75	4	79
	31.5	74	61
1k	1.5	<1	–
	44.25	76	60
1l	1	17.5	n.d.[c]
	30	68	58

[a] Reaction conditions: two-phase system consisting of diisopropyl ether/50 mM citrate phosphate buffer pH 5 (1:1), substrate (50 mM), hydrogen cyanide (250 mM), *Me*HNL (133 U mL^{-1}), 10 °C. [b] Obtained from von Langermann et al.[26] [c] n.d. = not determined.

HNL-catalyzed synthesis of substituted products **2i–l** has not yet been described in the literature to the best of our knowledge.

The results show clearly that in comparison to unsubstituted acetophenone, significantly higher conversions (≈75%) were achievable by using a two-phase system. An exception was α-fluoroacetophenone (**1i**), which was not converted by *Me*HNL. Possibly, the substrate did not fit correctly into the active site or a microkinetic step was significantly slowed down. Further studies investigating the main kinetic parameters (reaction velocity and K_M values) would be required (for **1i** and similar substrates) to explain the observed effect.

In comparison to aliphatic substrates **1a–g** even at the chosen acidity of pH 5.0, a strong increase in the enzyme concentration was necessary to achieve the conversion of these acetophenone derivatives. A further adjustment to pH 6.0 within the aqueous phase to compensate this effect was not possible, as a parallel increase in the non-enzymatic addition reaction and the racemization of the products occurred (see also Figure 1). Prolonged reaction times were also observed

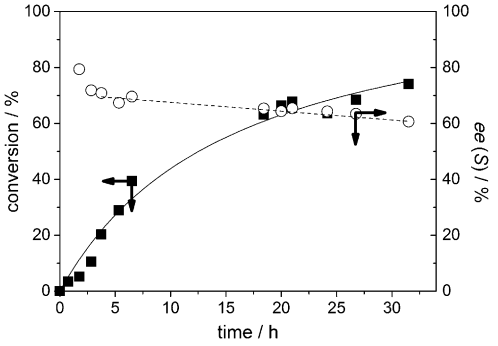


Figure 1. Conversion and enantiomeric excess values of the enzymatic synthesis of α,α-difluoroacetophenone cyanohydrin. Reagents and conditions: two-phase system consisting of diisopropyl ether/50 mM citrate phosphate buffer pH 5 (1:1), substrate (50 mM), hydrogen cyanide (250 mM), *Me*HNL (133 U mL^{-1}), 10 °C.

with an increasing degree of substitution at the α position. Whereas α,α-difluoroacetophenone (**1j**) was converted with 74% after 31.5 h, α,α,α,-trifluoroacetophenone (**1k**) required an additional 12 h (≈44 h total) for comparable conversion (76%). A further increase in size by exchange of one fluoro group by a chloro substituent resulted in slightly higher reactivity and required a total of 30 h for a conversion of 68%, which represents equilibrium conversion. The loss of reactivity for all derivatives can be explained by the nonoptimal fit within the active site pocket as a result of the additional size requirement from the introduced halogen atoms (as was deduced from examination of the active site of the enzyme, crystal structure: PDB code: 1EB9).

The obtained enantioselectivities of α-substituted cyanohydrins **2j** and **2l** were in general significantly lower than the enantioselectivity of unsubstituted acetophenone cyanohydrin **2h**. However, similar values were obtained for all investigated halogen-substituted derivatives, which indicates again a general limitation for substituents at the α-standing methyl group. The tendency of the loss of enantiomeric excess was investigated in detail for **2j** (Figure 1). The enantiomeric excess of the product steadily decreased with increasing product concentration over time, and this highlights the initial relatively high enantioselectivity of 79% (after 2 h). This loss is mainly caused by the parallel non-enzymatic reaction and only to an insignificant degree by product racemization. As shown in Figure 1, after 31.5 h the enantiomeric excess dropped to 61%.

In conclusion, a series of aliphatic methyl ketone and α-substituted acetophenone derivatives were successfully converted into the corresponding cyanohydrins with the hydroxynitrile

lyase from *Manihot esculenta*. Aliphatic ketone substrates **1a–g** were easily transformed into the corresponding cyanohydrins, and the length of the aliphatic chain seemed to be of minor importance. In contrast, introduction of substitutions at the α-standing methyl group of acetophenone cyanohydrins caused a strong drop in reactivity. This indicates a size limitation within the active site at this position. The presented results help to evaluate and understand the *Me*HNL-catalyzed synthesis of ketone cyanohydrins.

Experimental Section

Chemicals

All required chemicals were obtained from Sigma–Aldrich and were used as received. Mandelonitrile was aliquoted and stored at −18 °C to reduce spontaneous decomposition to benzaldehyde and hydrogen cyanide. The enzyme (*S*)-hydroxynitrile lyase from *Manihot esculenta*, *Me*HNL, was a gift from Julich Chiral Solutions, GmbH (now Codexis).

Hydrogen cyanide

All reactions involving the use of hydrogen cyanide or its formation should be executed within a well-ventilated fume hood. The use of an electrochemical hydrogen cyanide detector is strongly recommended. The desired amount of HCN was freshly distilled before use. In a general approach, 5 M H_2SO_4 (5 mL) was added dropwise to a solution of NaCN (1.6 g) in H_2O (5 mL), and subsequently, the mixture was heated to 70 °C. The formed free HCN was condensed, cooled, dried with anhydrous Na_2SO_4, and stored below 5 °C. An electrochemical HCN detector (Micro III G203 by GfG-Gesellschaft für Gerätebau, Dortmund, Germany) was placed in the fume hood for continuous HCN monitoring.

Enzyme assay

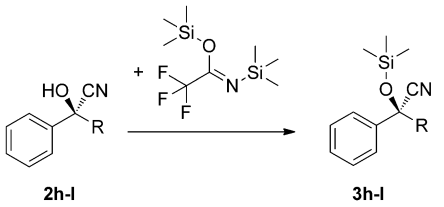
Enzymatic activity was determined photometrically with a UV/Vis spectrometer SPECORD 200 (Analytik Jena, Jena, Germany) by monitoring the occurrence of benzaldehyde at 280 nm from the consumption of racemic mandelonitrile. Therein, 50 mM citrate-phosphate buffer (pH 5, 700 μL) and the enzyme solution (100 μL) were combined in a quartz cuvette, which was tempered at 25 °C. The measurement started by the injection of 67 mM mandelonitrile (200 μL) in 50 mM citrate buffer (pH 2). The enzyme activity was eventually corrected by subtraction of the non-enzymatic mandelonitrile cleavage reaction, which was monitored in parallel without the addition of enzyme solution (100 μL citrate phosphate buffer pH 5 was used instead). One unit (1 U) of enzyme activity was defined as the formation of 1 μmol benzaldehyde per minute under standard assay conditions.

Synthesis reaction

The enzymatic synthesis of the cyanohydrins was performed in an aqueous two-phase system (ATPS) consisting of diisopropyl ether and 50 mM citrate (phosphate) buffer at 10 °C in a ratio of 1:1 v/v. The pH of the buffer was adjusted to the apparent reactivity of the enzyme towards the corresponding substrate. After the addition of the substrate (50 mM) and the enzyme (400 U), the reaction mixture was shaken horizontally and started by adding 250 mM HCN. The non-enzymatic reaction was monitored by mixing the components without the enzyme. Samples were drawn from the organic phase and derivatized as mentioned below.

Derivatization

Owing to the thermal instability of ketone cyanohydrins, derivatization prior to gas chromatography analysis was required. Samples of aliphatic ketone cyanohydrins (or a 50 μL sample) were derivatized in dichloromethane (700 μL), trifluoroacetic acid anhydride (50 μL), and pyridine (50 μL) at room temperature. Derivatization of α-substituted acetophenone cyanohydrins (or a 50 μL sample) required O-silylation with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (50 μL) in dichloromethane (800 μL) and a reaction time of 12–20 h at room temperature (Scheme 1).



2h-l **3h-l**

Scheme 1. Derivatization of the acetophenone cyanohydrins with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (50 μL) to form *O*-silylated cyanohydrins.

For the identification of silylated cyanohydrins enantiomers **3h–k** with the applied GC method, silylated racemic mixtures were synthesized according to Cabriol et al.[44] NaCN (20 mmol) was dissolved in dry DMSO in a three-necked, round-bottomed flask equipped with a magnetic stirrer under an argon atmosphere. After the addition of an initial amount of trimethylsilyl chloride (TMSCl, 3 mmol), a mixture of the substrate (10 mmol) and TMSCl (12 mmol) in 5 mL dry *n*-hexane was added dropwise, and the resulting mixture was heated to 60 °C. After 15 min, the reaction was stopped, and the mixture was extracted with *n*-hexane. The organic layer was washed with DMSO, and the residual amount of DMSO was removed by cooling with ice, filtering, and eluting over a silica plug. The synthesis of **2l** (and **3l**) was not successful.

GC analysis

The enzyme-catalyzed conversion of the ketones into the corresponding cyanohydrins and the resulting enantiomeric excess values were determined by gas chromatographic analysis by using a CP CHIRASIL-Dex CB capillary column equipped with a flame ionization detector (FID) in a VARIAN CP 3800 gas chromatograph. *n*-Decane was used as the internal standard. Carrier gas = 2 mL min^{-1} (He), oven temperature = 80 °C (isothermal), injector temperature = 200 °C, detector temperature = 250 °C.

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Keywords: biocatalysis · chirality · enzymes · hydrogen cyanide · oxynitrilase

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