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One-pot chemoenzymatic synthesis of protected cyanohydrins

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Abstract—In a chemoenzymatic one-pot reaction of ethyl cyanoformate with benzaldehyde catalyzed by the hydroxynitrile lyase from *Prunus amygdalus* ethoxycarbonylated (*R*)-mandelonitrile is formed in a highly enatioselective manner. The reaction was performed both in aqueous and organic media. ^{1}H NMR investigations revealed a two-step procedure consisting of an enzyme-catalyzed addition of HCN, generated by hydrolysis of ethyl cyanoformate, to the aldehyde followed by ethoxycarbonylation of the free cyanohydrin in a second step

1. Introduction

Enantiopure cyanohydrins and their derivatives, such as α-hydroxy acids, α-hydroxy ketones and aldehydes and β-hydroxy amines, serve as important synthetic intermediates for the production of pharmaceuticals and agrochemicals.[1] These compounds can be synthesized by asymmetric addition of HCN to prochiral carbonyl compounds under the catalytic action of hydroxynitrile lyases.[2] These biocatalysts show a broad substrate acceptance including aromatic, heteroaromatic and saturated and unsaturated aliphatic aldehydes and ketones.[3]

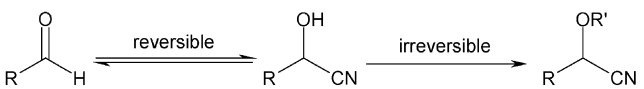
However, the reversibility of the cyanohydrin formation constitutes a major problem since the corresponding back reaction regenerates the substrate unless an excess of HCN or another cyanide donor is used. Carrying out the process in aqueous or biphasic aqueous-organic emulsion systems requires low pH values in order to stabilize the products which potentially leads to decreased biocatalyst activity. Furthermore, working at low pH suppresses the spontaneous unselective non-enzymatic HCN addition to the carbonyl substrates and products with excellent optical purity are obtained. Nevertheless, the equilibrium of the reaction often is not in favour of the desired cyanohydrins.

A potential solution of this problem would be to couple the cyanohydrin synthesis with an irreversible derivatization step in a one-pot procedure. This would furnish protected and hence more stable products. At the same time the reaction equilibrium will be shifted towards the product side resulting in higher yields (Scheme 1).

Recently, Hanefeld et al. attempted to combine two biocatalytic steps, cyanohydrin formation catalyzed by hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) and acetylation mediated by *Candida antarctica* lipase B. In this case, problems occurred due to hydrolysis of the acyl donor and subsequent deactivation of the *Hb*HNL.[4] A chemoenzymatic approach has been developed by Oda[5] and Kanerva.[6] In a dynamic kinetic resolution procedure enantiomerically enriched cyanohydrins are formed by lipase catalyzed acetylation being coupled with preliminary chemical in situ formation and racemization of the latter compounds.

In addition to cyanohydrin formation chemoenzymatic protocols for the synthesis of optically pure alcohols and amines combining chemically catalyzed racemization and lipase-catalyzed acylation or deacylation of the substrates have been developed.[7]

Recently, several groups reported that cyanoformate esters react in the presence of a chiral chemical catalyst (either cinchona alkaloids or BINOL–metal complexes) with carbonyl compounds to give the protected cyanohydrins.[8] Herein we wish to report our studies on the application of ethyl cyanoformate (**2**) as HCN donor and protecting



Scheme 1.

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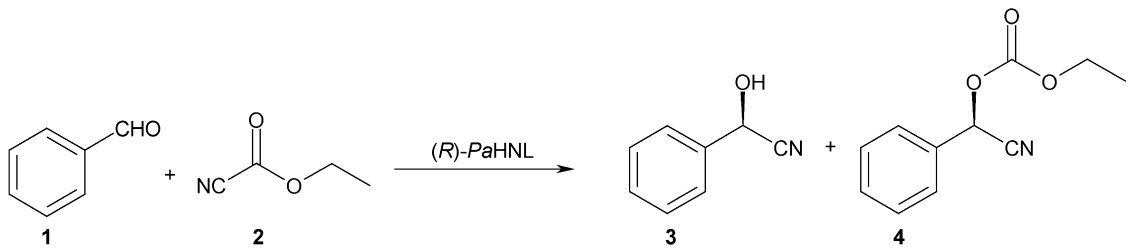

Scheme 2.

reagent in the enzymatic cyanohydrin synthesis catalyzed by the *R*-selective hydroxynitrile lyase from *Prunus amygdalus* (*Pa*HNL, from almonds). For model studies we chose benzaldehyde (**1**), the natural substrate of the enzyme, as starting material.

2. Results and discussion

Benzaldehyde (**1**) was subjected to the reaction with three equivalents of ethyl cyanoformate (**2**) in a weakly acidic aqueous solution (pH 3.3, potassium phosphate/citrate buffer) of recombinant *Pa*HNL[9] at room temperature (Scheme 2). The course of the reaction was followed by GC after extraction with *tert*-butyl methyl ether and acetylation (Ac_2O/pyridine) of a sample of the reaction mixture. After 3 h mandelonitrile (**3**) was found to be the major product whereas only a small amount of 2-ethoxycarbonyloxy-2-phenyl acetonitrile (**4**) was detected (Table 1, entry 1). Both compounds were formed with excellent enantiopurity. No other products were found. As the reaction proceeded further, **4** was formed with consumption of **3**, and only traces of **1** were left after 23 h. At this stage the ee of **3** dropped slightly probably due to slow racemization of the free cyanohydrin **3**. The yield of **4** could be increased to 66% after the addition of a further 3 equiv. of **2**.

These results and especially the formation of mandelonitrile (**3**) indicated a two step procedure which prompted us to carry out NMR studies to obtain mechanistic insights.[10] Initially the stability of **2** in D_2O was examined at 22 °C. Under aqueous conditions **2** is decomposed into HCN, CO_2 and ethanol and its half-life is approximately 4.5 h. In order to confirm the assumption of a two-step procedure the enzymatic reaction was carried out in the NMR tube using the conditions described above. Figure 1 clearly indicates that mandelonitrile (**3**) is formed prior to the acylation step furnishing **4**.

As can be seen from Figure 2, enantiomerically enriched **3** is formed by asymmetric HCN addition to benzaldehyde (**1**) catalyzed by *Pa*HNL, as in the first step HCN is generated in the preceding hydrolysis of ethyl cyanoformate (**2**). In the second step **2** acts as an activated acyl donor which leads to the conversion of **3** into **4** as the final product regenerating HCN. In a comparative experiment, the reaction of racemic **3** with **2** in an aqueous acidic solution (pH 3.3) of recombinant *Pa*HNL was examined. Apparently, since only racemic **4** was formed, only the first step in the procedure described above, namely the formation of **3**, exhibits stereoselectivity.

In order to enhance the lifetime of **2** during the reaction, further experiments were performed in organic solvents. For this purpose, the biocatalyst was immobilized on Celite® by lyophilization of a heterogeneous mixture of carrier and the buffered enzyme solution (50 mM phosphate buffer, pH 7.0). During our studies with *Pa*HNL immobilized on Celite® we found that toluene and dichloromethane are excellent solvents for carrying out cyanohydrin syntheses with this particular biocatalyst. Furthermore, studies with immobilized hydroxynitrile lyases revealed that it is necessary to have certain amounts of buffer on the carrier to achieve maximum enzyme activity.[11] For this reason either 1 or 2% (v/v) of buffer solution (50 mM phosphate buffer, pH 7.0) was added to the suspension of the immobilized enzyme in the respective solvent. All

Table 1. Yield and ee of **3** and **4** in aqueous or organic media according to the reaction in Scheme 2

Entry	Solvent	pH	(*R*)-*Pa*HNL (U/mmol)	HCN (equiv.)	**2** (equiv.)	*t* (h)	**3**[a] Yield% (%ee)	**4** Yield% (%ee)
1	H_2O	3.3	750	0	3	3	77 (>99)	8 (>99)
						23	65 (94)	33 (>99)
						44.5[b]	34 (89)	66 (95)
2	Toluene 1%	7.0	300	0	2.5	24	11 (94)	3 (94)
3	Toluene 1%	7.0	300	1	2.5	24	49 (95)	37 (98)
4	Toluene 2%	7.0	300	0	2.5	24	17 (69)	4 (58)
5	Toluene 2%	7.0	300	1	2.5	24	47 (84)	47 (99)
6	CH_2Cl_2 1%	7.0	300	0	2.5	24	5 (95)	6 (98)
7	CH_2Cl_2 1%	7.0	300	1	2.5	24	52 (97)	31 (97)
8	CH_2Cl_2 2%	7.0	300	0	2.5	24	6 (96)	4 (98)
9	CH_2Cl_2 2%	7.0	300	1	2.5	24	39 (97)	43 (98)

[a] Determined by GC after acetylation
[b] After 26 h a further 3 equiv. of **2** were added (double column).

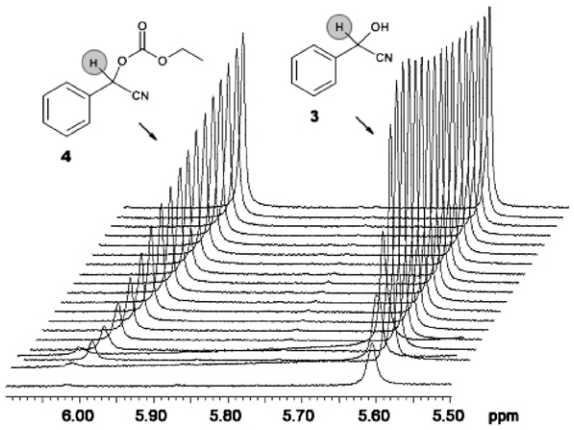

Figure 1. NMR-monitoring of the *Pa*HNL catalyzed formation of **3** and **4**. Spectra were recorded every 15 min.

transformations were performed at room temperature. The progress of the reaction was analyzed after 24 h by GC after acetylation (Ac_2O/pyridine, Table 1).

When the reaction mixture contained 1% (v/v) of buffer in toluene only 11% of **3** and 3% of **4** could be detected (Table 1, entry 2). As a plausible reason for this low product yield, the lack of free HCN owing to the enhanced stability of **2** in toluene compared to water was assumed. As a consequence the reaction was triggered by the addition of 1 equiv. of HCN at the start, yielding 49% of **3** and 37% of **4** (entry 3). Both products were formed with satisfying optical purity. This is a promising result because less than half of the catalyst was applied compared to the aqueous procedure even though enzymes are generally less active when used as immobilized formulations in organic solvents. On the other hand the solubility and stability of the reagents is increased. Adding 2% (v/v) of buffer to the system did not change the yields significantly but the optical purity of the products dropped probably due to the enhanced racemization rate of **3** (entries 4 and 5). When the reaction was carried out in dichloromethane under the same conditions as described above for toluene, the yields of **4** were slightly lower. Even when the system contained 2% (v/v) of buffer the ee values were good to excellent (entries 6–9).

In order to deepen the mechanistic understanding, the following investigations concerning the acylation step were undertaken. Reacting racemic **3** with **2** in either dry toluene or after the addition of 1% (v/v) of buffer furnished only traces of **4** after 24 h. In contrast, when the same reaction was carried out in toluene (2% buffer) in the presence of the immobilized enzyme 59% of **4** was formed after 25 h. Using Celite® as the sole additive (the enzyme solution was displaced by water prior to lyophilization) 54% of **4** was detected after 25 h. This indicates a catalytic activity of the carrier whereas the slightly increased yield of **4** in the presence of the enzyme does not seem to be significant.

In conclusion, a chemoenzymatic method for the synthesis of protected cyanohydrins in a one-pot manner was developed. This work is aiming towards an improvement of biocatalytic cyanohydrin reactions with unfavorable equilibrium conditions, whereas it is well known that benzaldehyde, our model substrate, is converted to mandelonitrile in almost quantitative yield in biphasic media (see Ref. 3a).

3. Experimental

3.1. Materials and methods

All solvents and materials were commercially available and were appropriately purified, if necessary. ^1H and ^{13}C NMR spectra were recorded either on a Varian INOVA 500 (^1H 499.82 MHz, ^{13}C 125.69 MHz) or a Varian GEMINI 200 (^1H 199.92 MHz, ^{13}C 50.25 MHz). GC analyses were performed using a Hewlett Packard 6890 instrument and either a Chirasil-DEX CB or a HP-5 column. The CHN analysis was carried out with a Perkin Elmer 2400 CHN Elemental Analyzer at the Institute of Physical Chemistry, University of Vienna.

3.2. HCN formation—caution

All reaction equipment in which cyanides were used or produced were placed in a well ventilated hood. The

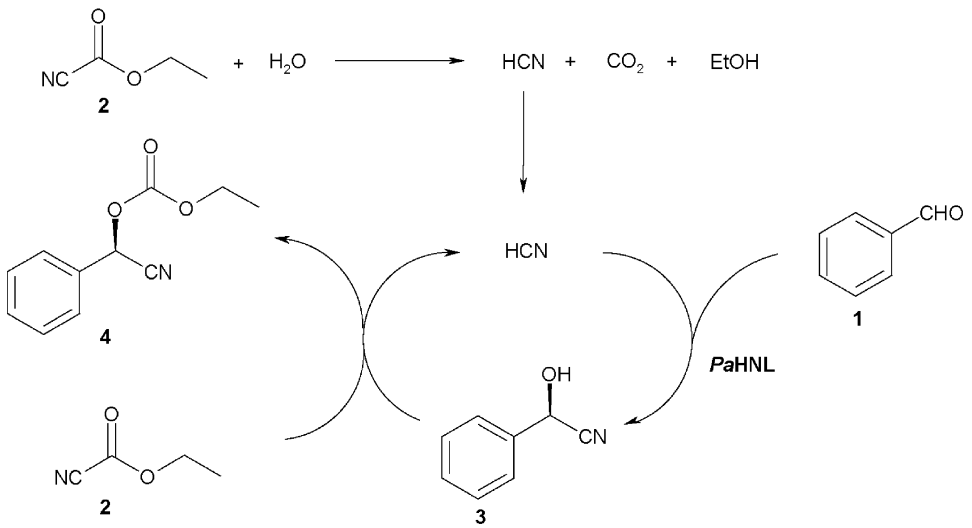

Figure 2. Tentative mechanism of the *Pa*HNL catalyzed formation of **3** and **4**.

required amount of HCN was freshly formed by dropping a saturated NaCN solution into aqueous sulfuric acid (60%) at 80 °C and trapping HCN at −12 °C in a cooling trap. For continuous warning, an electrochemical sensor for HCN detection was used. Waste solutions containing cyanides were treated with aqueous sodium hypochlorite (10%). Subsequently the pH was adjusted to 7.0 with aqueous sulfuric acid.

3.2.1. Racemic 2-acetoxy-2-phenyl acetonitrile (acetylated racemic 3). To a stirred solution of **3** (2.00 g, 15.02 mmol) and pyridine (2.90 mL, 36.04 mmol) in 15 mL dry CH_2Cl_2, acetyl chloride (1.30 mL, 18.30 mmol) was added dropwise at 0 °C under an argon atmosphere. Stirring was continued for 1 h. Subsequently, the solution was poured onto ice water. The organic phase was extracted with $NaHSO_4$ 5% and sat. $NaHCO_3$, dried (Na_2SO_4) and concentrated under reduced pressure. Purification using column chromatography (cyclohexane/EtOAc=50:1–20:1) yielded acetylated **3** (1.81 g, 69%) as a colourless oil. ^{1}H NMR ($CDCl_3$): δ (ppm)=2.17 (s, 3H), 6.42 (s, 1H), 7.46–7.51 (m, 5H); ^{13}C NMR ($CDCl_3$): δ (ppm)=20.71, 63.09, 116.37, 128.12, 129.50, 130.66, 131.99, 169.18. The NMR data were consistent with those previously reported.[12]

3.2.2. Racemic 2-ethoxycarbonyloxy-2-phenyl acetonitrile (racemic 4). To a stirred solution of **1** (1.90 mL, 18.69 mmol) in 10 mL dry THF under an argon atmosphere were added **2** (3.70 mL, 37.45 mmol) and DABCO (210 mg, 1.87 mmol) at 0 °C. The ice bath was removed and after 1.5 h the reaction was diluted with 30 mL of ether. The single phase was washed with sat. $NaHCO_3$ (2×20 mL). The aqueous phase was re-extracted with ether (2×20 mL). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. Purification using column chromatography (cyclohexane/EtOAc=15:1) yielded **4** (3.22 g, 84%) as a colourless oil. ^{1}H NMR ($CDCl_3$): δ (ppm)=1.34 (t, J=7 Hz, 3H), 4.29 (q, J=7 Hz, 2H), 6.27 (s, 1H), 7.44–7.57 (m, 5H); ^{13}C NMR ($CDCl_3$): δ (ppm)= 14.34, 65.86, 66.60, 116.02, 128.12, 129.51, 130.87, 131.49, 153.67. Anal. calcd for $C_{11}H_{11}NO_3$: C, 64.38; H, 5.40; N, 6.83; Found: C, 64.60; H, 5.41; N, 6.74.

3.2.3. NMR measurements. For stability studies 40 μL of **2** were dissolved in 0.5 mL of D_2O containing 1% (v/v) of DMSO-d_6. Spectra were recorded every 15 min over a period of 16.5 h at 22 °C. For the biocatalytic reaction, to 0.6 mL of a solution of recombinant *Pa*HNL (80 U/mL after dilution with D_2O and pH adjustment to 3.3 with an aqueous citric acid solution) containing 1% (v/v) of DMSO-d_6 were added 30 μL of **1** (0.30 mmol) and 90 μL of **2** (0.91 mmol). Spectra were recorded every 15 min over a period of 5 h at 22 °C. The residual water signal was suppressed by the transmitter presaturation method.

3.2.4. Reaction in water. 2.5 mL of a solution of recombinant *Pa*HNL (300 U/mL) were adjusted to pH 3.3 with an aqueous solution of citric acid and diluted with 2.5 mL of 50 mM potassium phosphate/citrate buffer (pH 3.3). **1** (106 mg, 1 mmol) and **2** (297 μL, 3 mmol) were added and the mixture was stirred at room temperature. The course of the reaction was monitored by GC after acetylation.

3.2.5. Reaction in organic solvents. 0.5 g of Celite® were swollen in 4 mL of 50 mM phosphate buffer (pH 7.0) for 2 h. After filtration, 4.2 mL of a solution of recombinant *Pa*HNL (175 U/mL) and 2 mL of phosphate buffer (pH 7.0) were added and the mixture was stirred for 10 min at room temperature. After freezing in liquid nitrogen the preparation was lyophilized. To a suspension of the dried enzyme formulation and **1** (250 μL, 2.46 mmol) in 6 mL of dry solvent (either toluene or CH_2Cl_2) were added either 60 or 120 μL of phosphate buffer [pH 7.0, 1 or 2% (v/v)] and **2** (610 μL, 6.17 mmol) and the mixture was stirred at room temperature. The course of the reaction was followed by GC after acetylation. In the case of HCN-triggering, HCN (95 μL, 2.46 mmol) was added prior to the addition of **2**.

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