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Asymmetric synthesis of cyanohydrin derived from pyridine aldehyde with cross-linked aggregates of hydroxynitrile lyases

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Abstract—Enantiocomplementary *R*- and *S*-cyanohydrins with >93% ee were synthesized through the hydrocyanation of 3-pyridinecarboxaldehyde in >65% yield. Hydroxynitrile lyases (HNLs) from cassava and almond were used in commercially available cross-linked enzyme aggregate (CLEA) forms to catalyze the reactions and produce material with optical purities significantly greater than has been reported previously. The use of a dichloromethane reaction system with enzyme aggregates and free hydrogen cyanide was crucial in improving cyanohydrin stereoselectivity through minimizing background racemic cyanide addition and enzyme-catalyzed racemization of the product.

1. Introduction

The formation of enantiomerically enriched cyanohydrins from aldehydes and ketones is an increasingly important tool in industrial synthesis design.[1] Cyanohydrin synthons have significant utility due to the ability of both the nitrile and alcohol functional groups of these compounds to be effectively derivatized without a resultant loss in optical purity.[2] Such transformations lead to a wide variety of products including α-hydroxyketones, β-hydroxyamines, α-aminonitriles, α-hydroxyesters, α-sulfonyloxynitriles, and α-fluoronitriles.[1] Accordingly, the study of chemical and biological catalysts useful for asymmetric cyanohydrin production has been an area of intense research activity for the past decade. The ligands and enzymes that have been developed and characterized have been successfully applied to the stereoselective conversion of a wide variety of aliphatic, aromatic, and oxygen- and sulfur-containing heteroaromatic aldehydes and ketones.[3–6] Nitrogen-containing heteroaryl carboxaldehydes, though, have been shown as a class to be poor substrates for both chemical catalysts and hydroxynitrile lyase (HNL) enzymes, producing cyanohydrins with enantiomeric purity insufficient for most synthetic applications. For example, transformations of 3-pyridinecarboxaldehyde **1** with monometallic bifunctional BINOLAM catalysts have produced *S*-cyanohydrin with poor ee of <50%,[7–9] and with HNL have produced *R*-cyanohydrin with only moderate ee of no more than 80%.[10–12]

A key cause of these low selectivities is the presence of a base-catalyzed aqueous background reaction involving the racemic addition of cyanide to the substrate. For many HNL-catalyzed processes, the rate of this background reaction can be sufficiently slowed by reducing pH and operating in biphasic systems in which the starting material preferentially partitions into an organic solvent layer. The very high aqueous solubility of the pyridyl aldehydes, however, necessitates operating in a nearly water-free environment in which the enzyme must be immobilized into a rigid configuration that is not denatured by high organic solvent concentration. A recently developed technique for such enzyme immobilization involves the formation of cross-linked enzyme aggregates (CLEAs) in which individual precipitated protein molecules are chemically bonded to one another through the formation of imines from, for example, the amine functional groups of the protein and a polyaldehyde cross-linker.[13] In this Letter, we describe the application of two different HNL–CLEAs to the first production of highly chiral and enantiocomplementary cyanohydrins from 3-pyridinecarboxaldehyde (Scheme 1).

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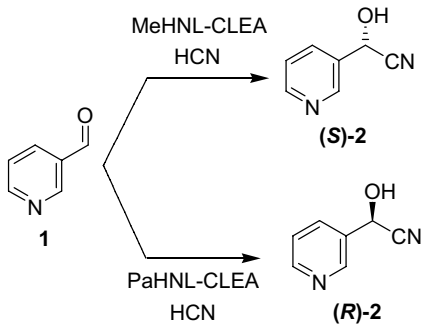

Scheme 1. Synthesis of chiral cyanohydrins from 3-pyridinecarboxaldehyde.

2. Results and discussion

The relative rates of the uncatalyzed chemical conversion of aldehyde **1** to racemic cyanohydrin **2** in aqueous solutions at different pH values were determined and are shown in Figure 1. For reactions at pH greater than 3.5, the compound was shown to convert quite readily, with a maximum conversion of 80% achieved with overnight aging at pH >5.5. In contrast, when a mixture of the aldehyde and acetone cyanohydrin was allowed to react in toluene at identical temperatures and times, this background reaction was suppressed, with only 1% conversion taking place.

A screening test was then performed to examine the different commercially available preparations of HNLs isolated from almond (*Prunus amygdalus*, PaHNL),[14] flax (*Linum usitatissimum*, LuHNL),[15] and cassava (*Manihot esculenta*, MeHNL).[16] All three enzymes were tested as liquid preparations stabilized in glycerol. In addition, immobilized preparations of MeHNL and PaHNL in which the enzymes were either covalently bound to or non-covalently absorbed onto inert carrier resins were also examined. Finally, dry CLEA forms of MeHNL and an aqueous suspension of PaHNL–CLEA in phosphate buffer were also used. Two distinct lots of MeHNL–CLEA were tested as they were produced using significantly different protocols.[17]

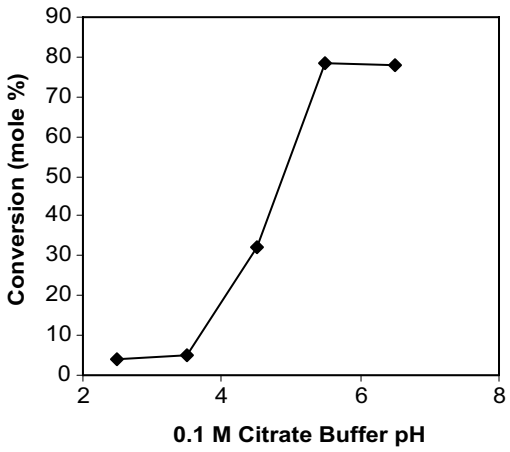

Figure 1. 8.8 μl Aldehyde **1** and 14.9 μl acetone cyanohydrin were incubated in 1 ml of 0.1 M citrate buffers at the indicated pH values for 17 h at room temperature.

From the results presented in Table 1, as a result of PaHNL biocatalyst deactivation[18] no enzymatic conversion was seen when preparations of this enzyme were tested in the aqueous system (entries 1 and 2). In contrast, the MeHNL and LuHNL preparations that were tested in aqueous systems (entries 3–6) showed good activity towards aldehyde **1**, giving yields of 55–90%, but poor enantioselectivity. When the solid immobilized preparations of PaHNL and MeHNL were tested in systems of 100% toluene (entries 7–12), both enzymes were found capable of generating cyanohydrin **2**, although the preparations absorbed onto a resin (entries 7–10) also showed no enantioselectivity. Of all cases tested, only the CLEA forms of MeHNL applied in toluene (entries 11 and 12) were found to give cyanohydrin with substantial ee.

Based on the promising results seen with MeHNL in toluene, a panel of nine water–immiscible organic solvents were then tested to determine their effects on the reaction rates and selectivities. From the results given in Table 2, three solvents—toluene, ethyl acetate, and dichloromethane—were found to support reactions initially yielding ***S*-2** with ee values between approximately 50% and 60%. Allowing these three reactions to continue for 1 day revealed that the yields of cyanohydrin increased, but at the expense of chiral purity. The degree to which this decrease occurred varied depending on the solvent medium, with dichloromethane providing the best balance between yields and chiral stability.

To further study the causes of this observed drop in cyanohydrin ee, the stabilities of both ***S*-2a** and (*R*)-mandelonitrile were determined in systems both with and without an excess of biocatalyst. In each case, the chiral cyanohydrin was aged in dichloromethane for 17 h in the presence of threefold more MeHNL–CLEA than was used in the solvent screen. In these tests, which did not contain acetone cyanohydrin, the optical purity of the compounds decreased from initial values of 85–90% ee to approximately 25% ee. In contrast, no decreases in optical purity were observed for the cyanohydrins in dichloromethane in the absence of MeHNL–CLEA. Thus, enzyme-catalyzed cyanohydrin racemization is at least a major component of the drop in product ee noted in Table 2. Because acetone cyanohydrin was not present in these racemization tests, acetone was not a factor in the ee drops. Also, that the degree of racemization was extremely similar for both of the cyanohydrins tested indicates that the racemization reaction is not exclusive to pyridinealdehydes. It was not possible to determine if the mechanism of this racemization was specific to the CLEA formulation of the enzyme because a direct comparison with the alternative free enzyme preparation was not feasible. This is because in an organic solvent the free enzyme is rapidly deactivated, while in an aqueous system the cyanide

Table 1. Biocatalyst screen results[a]

Entry	Biocatalyst	Medium	Yield[b] (%)	ee (%)
1	PaHNL[c]	0.1 M Citrate pH 2.5	0	N/A
2	PaHNL–CLEA, aq[c]	0.1 M Citrate pH 2.5	0	N/A
3	MeHNL[c]	0.1 M Citrate pH 2.5	75	13
4	MeHNL–CLEA (lot H40441.02)[d]	0.1 M Citrate pH 2.5	79	5
5	MeHNL–CLEA (lot H50442.01)[d]	0.1 M Citrate pH 2.5	90	8
6	LuHNL[c]	0.1 M Citrate pH 2.5	55	0
7	PaHNL, cov. immob.[d]	Toluene	15	0
8	PaHNL, non-cov. immob.[d]	Toluene	8	0
9	MeHNL, cov. immob.[d]	Toluene	39	1
10	MeHNL, non-cov. immob.[d]	Toluene	38	1
11	MeHNL–CLEA (lot H40441.02)[d]	Toluene	40	48
12	MeHNL–CLEA (lot H50442.01)[d]	Toluene	60	51

[a] The reactions initially contained 8.8 μl aldehyde **1** and 14.9 μl acetone cyanohydrin in 1 ml total volume and were incubated for 17 h at room temperature.
[b] Yields were calculated by subtracting % background conversion in negative controls with identical reaction conditions.
[c] Liquid enzyme preparation charge was 100 μl.
[d] Solid enzyme preparation charge was 30 mg.

Table 2. Solvent screen results[a]

Solvent	Time (h)	Yield[b] (%)	ee (%)
Toluene	4	12	49
Toluene	29	60	13
Ethyl acetate	4	4	61
Ethyl acetate	29	20	35
Dichloromethane	4	11	49
Dichloromethane	29	85	24
Acetone	4	4	36
Tetrahydrofuran	4	5	34
Heptane	4	9	27
Methyl *tert*-butyl ether	4	4	22
Hexane	4	12	22
Methanol	4	2	−8
Toluene[b]	17	78	30
Dichloromethane[b]	17	67	29

[a] The reactions initially contained 8.8 μl aldehyde **1**, 10 mg MeHNL–CLEA lot H40441.02, 14.9 μl acetone cyanohydrin and 970 μl solvent.
[b] The reactions were catalyzed by MeHNL–CLEA lot H50442.01.

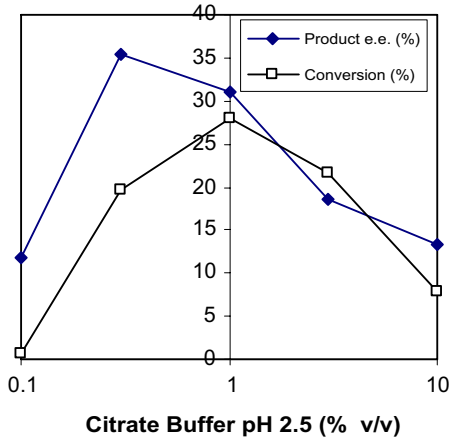

Figure 2. 26.5 μl Aldehyde **1**, 10 mg MeHNL–CLEA lot H40441.02 and 44.5 μl acetone cyanohydrin were incubated in 950 μl dichloromethane and the indicated amount of 0.1 M, pH 2.5, citrate buffer for 30 min at room temperature.

liberated from the cyanohydrin in the reverse reaction will be then added back racemically through the non-enzymatic background reaction.

Having identified dichloromethane as the optimal solvent in part due to its minimizing of the rate of this racemization, we next investigated the effects of varying the water content of the reaction mixture. From the data shown in Figure 2, it can be seen that an optimal water concentration exists for both the productivity and chiral selectivity of the reaction. In organic solvent reaction systems, a higher aqueous content will increase the biocatalytic reaction rate and resulting overall enantioselectivity because of an increase in the interfacial area where catalysis is believed to predominantly or exclusively occur.[19] In contrast, the reduction of product yield and chiral purity seen above the optimum aqueous concentration is the result of a higher rate for the background reaction and may also be due to greater enzyme degradation associated with larger interfacial areas.[20] Subsequent further experimentation identified as 0.18 vol. % the optimum water amount for the process.

Following the selection of solvent choice and water concentrations, we studied the impacts of cyanide equivalents and cyanide source on the reaction process. A proven technique for driving the equilibrium of the reactions catalyzed by HNL enzymes towards cyanohydrin formation is to increase the available concentration of cyanide. This then may reduce the degree to which back conversion and cyanohydrin racemization take place. However, in our reaction system when the concentration of the acetone cyanohydrin cyanide donor was raised, a decrease in the optical purity of ***S*-2** was seen, as is shown in Figure 3. Also from the data presented in this graph, it was discovered that by using the optimized water concentration and lower acetone cyanohydrin levels along with the alternate MeHNL–CLEA lot H50442.01, cyanohydrin could be produced with a significantly higher ee of 80%. This was the first evidence

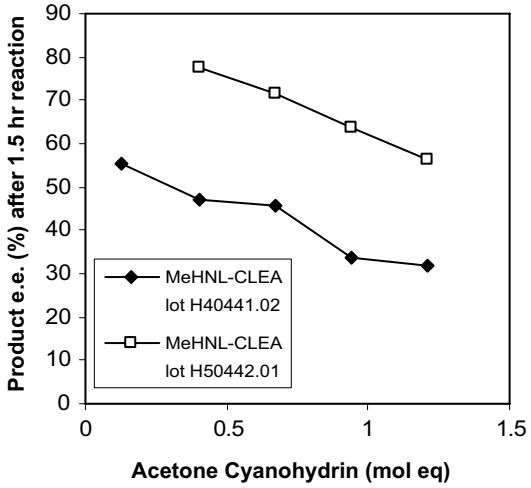

Figure 3. 132 μl Aldehyde **1**, 10 mg MeHNL–CLEA, 1.8 μl of 0.1 M, pH 2.5, citrate buffer and the indicated amount of acetone cyanohydrin were incubated in dichloromethane to give a 1 ml total volume for 90 min at room temperature.

of differing enantiospecific behavior between the two CLEA biocatalyst preparations toward this substrate, and as a result this biocatalyst was used in all subsequent process development work. To eliminate the negative effects associated with the presence of acetone cyanohydrin in the reaction system, the use of free HCN as an alternate cyanide donor was explored. From the data in the graph of Figure 4a, an aldehyde charge of 30 g/l is 80% converted to cyanohydrin **2** in 15 min when using HCN and 10 g/l MeHNL–CLEA, instead of 2 h when using acetone cyanohydrin. Also, the enzyme concentration can be reduced to 2 g/l in the case with HCN while still matching the reaction rate of the control case with acetone cyanohydrin and 10 g/l CLEA. From the product ee trends of Figure 4b, the advantages of using HCN as the cyanide donor extend to the overall enantioselectivity of the reaction as well, with the cyanohydrin optical purity increasing from 80% in the control reaction to 88% in the improved process.

Further improvements were realized by reducing the temperature to 5 °C, slowing the background racemic chemical hydrocyanation and the enzyme-catalyzed cyanohydrin racemization. At that temperature, 4 g/l MeHNL–CLEA converts 85% of 30 g/l aldehyde **1** to 94% ee ***S*-2** in 2 h using HCN as the cyanide donor. It is apparent that the reduction in temperature affects the kinetics of the chemical reaction and/or the racemization reaction more significantly than those of the forward enzymatic reaction, resulting in an increase in overall enantioselectivity. Also, the chiral cyanohydrin is very stable at the lower temperature, with minimal loss of optical purity occurring through racemization during an additional 24-h hold. Further decreasing the process temperature to −10 °C resulted in a slower reaction rate with no additional increase in the product's optical purity.

In testing the applicability of the alternate biocatalyst PaHNL–CLEA to the described reaction process, a

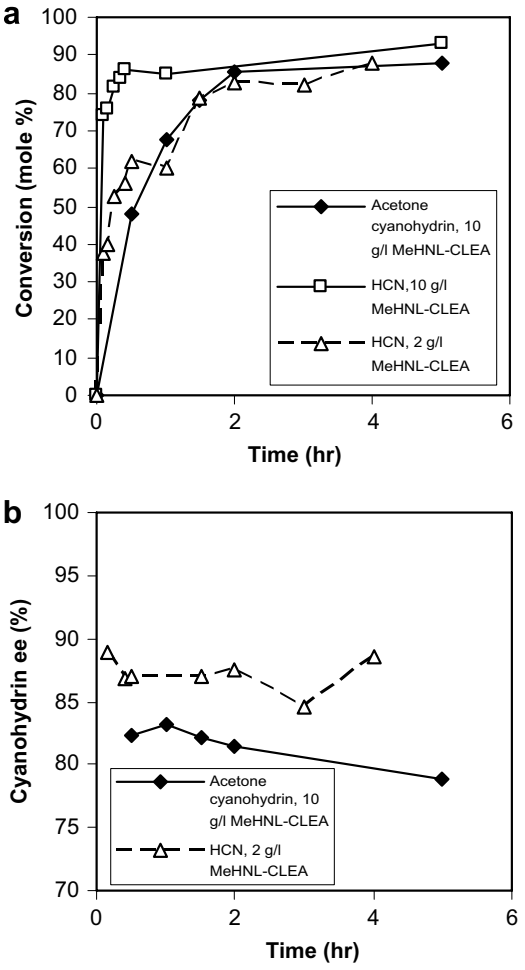

Figure 4. 26.5 μl Aldehyde **1** and 1.8 μl of 0.1 M, pH 2.5, citrate buffer were added to 950 μl dichloromethane, 10 g/l MeHNL–CLEA and 2.6 μl acetone cyanohydrin fed every 30 min (◆), 1 ml dichloromethane containing 0.25 M HCN and 10 g/l MeHNL–CLEA (□), or 1 ml dichloromethane containing 0.25 M HCN and 2 g/l MeHNL–CLEA (△). Graphs give (a) yield of **2** on **1** and (b) ee of **2**.

dry CLEA form was used to successfully convert aldehyde **1** to 93% ee ***R*-2**. The yield of the reaction over a 20-h period was 65%.

In summary, enantiocomplementary reaction processes have been developed for the generation of *R*- and *S*-cyanohydrin from the heteroaryl aldehyde 3-pyridinecarboxaldehyde. By using commercially available CLEA forms of HNLs from cassava and almond, high ee material has been produced from this substrate which has proven to be otherwise difficult to convert with adequate enantioselectivity using chemical catalysis techniques. Key to increasing the optical purity of the resulting cyanohydrin was the minimizing of side reactions—namely non-specific chemical hydrocyanation and enzymatic cyanohydrin racemization—that degraded the overall selectivity of the process. The kinetics of these undesired reactions were reduced by choosing operating conditions that included a low reaction temperature, a microaqueous environment containing the proper solvent and optimized water concentration, and a CLEA formulation and cyanide donor that

were most compatible with the reaction being studied. With the application of these techniques, and in particular the testing of a larger biocatalyst library of HNL–CLEAs, the effective substrate ranges of the already very useful HNLs are likely to be expanded even further.

3. Experimental

3.1. Enzymes and chemicals

All aldehyde compounds and organic solvents were purchased from Sigma–Aldrich (St. Louis, MO). Both the original lot (H40441.02) and revised lot (H50442.01) of MeHNL–CLEA as well as PaHNL–CLEA and liquid preparations of MeHNL, PaHNL, and LuHNL were purchased from Julich Chiral Solutions (Julich, Germany). Other immobilized preparations of MeHNL and PaHNL were purchased from Biocatalytics (Pasadena, CA).

3.2. Preparation of HCN solution in organic solvent

A mixture of 2.5 ml H_2O, 1.6 g citrate and 10 ml dichloromethane was stirred at 0 °C until the citrate dissolved completely. To the stirred mixture, a solution of 520 mg KCN in 1 ml H_2O was slowly added. The resultant mixture was stirred at 0 °C for an additional 15 min. The dichloromethane layer containing HCN was then separated for use as the reaction medium.

The cyanide concentration in the dichloromethane was determined by titration according to a modified version of the Mohr method.[21] A cyanide salt solution was formed by combining 1 ml of the dichloromethane/HCN solution and 5 ml of 2 M NaOH and stirring for 2 min at room temperature. To the stirred mixture, 120 μl of 5% aqueous potassium chromate was introduced and the solution was titrated with 0.1 M aqueous silver nitrate to the first permanent appearance of red silver chromate precipitant.

3.3. General procedure for the enzymatic reaction

To 970 μl of the dichloromethane/HCN solution described above were added 2 μl of 0.1 M, pH 2.5, aqueous citrate, 30 mg aldehyde substrate and 4 mg CLEA biocatalyst. This reaction was then stirred at 5 °C for 2–20 h.

3.4. Assay of the reaction mixture

A 50 μl sample was removed from the reaction and the dichloromethane component was evaporated under nitrogen for 20 s. The sample was then resuspended in 600 μl isopropanol and assayed by chiral HPLC. A 250 mm × 4.6 mm Chiralpak AD-H column was used with an eluant of 85:15 heptane/ethanol, a flow rate of 3 ml/min, a temperature of 10 °C, a detection wavelength of 245 nm and a sample injection amount of 2 μl.

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