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Enzymatic Hydrocyanation of a Sterically Hindered Aldehyde. Optimization of a Chemoenzymatic Procedure for (*R*)-2-Chloromandelic Acid

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Abstract:

The asymmetric synthesis of (*R*)-*o*-chloromandelic acid, a key intermediate for the anti-thrombotic agent clopidogrel, via the almond meal catalyzed hydrocyanation of 2-chlorobenzaldehyde and subsequent acidic hydrolysis was developed into an industrially viable procedure. The use of a minimum amount of water consistent with enzyme activity and a slow feed of the reactants were the keys to obtaining (*R*)-2-chloromandelonitrile in a high (98%) yield and satisfactory (90%) enantiomeric excess. Acidic hydrolysis of the nitrile followed by crystallization from toluene afforded enantiopure (ee > 99%) (*R*)-2-chloromandelic acid.

Introduction

Enantiopure 2-hydroxycarboxyxlic acids are useful chiral intermediates and building blocks. Thus, (*R*)-2-(2-chlorophenyl)-hydroxyacetic acid ((*R*)-*o*-chloromandelic acid) is emerging as the preferred chiral intermediate for the industrial synthesis of the anti-thrombotic agent clopidogrel.[1] Resolution procedures for (*R*)-*o*-chloromandelic acid include diastereomeric crystallization[2−4] and enantioselective enzymatic hydrolyis of the corresponding nitrile.[5] Such resolution procedures generally require the racemization of the undesired enantiomer. In the case of *o*-chloromandelic acid, this can be accomplished in aqueous alkaline DMSO at 100 °C. (*R*)-*o*-Chloromandelic acid has also been synthesized via asymmetric transformations, which do not suffer from this latter drawback. These include a microbial reduction of 2-(2-chlorophenyl)-glyoxylic acid[6] and the asymmetric addition of trimethylsilyl cyanide to 2-chlorobenzaldehyde in the presence of a chiral transition metal complex.[7]

The (*R*)-specific oxynitrilase from almonds (hydroxynitrile lyase from *Prunus amygdalus*, E.C. 4.1.2.10), which has considerable scope in the synthesis of enantiopure 2-hydroxynitriles,[8−11] acted with only a modest enantioselectivity (ee 83%) in the hydrocyanation of 2-chlorobenzaldehyde.[12]

This latter reaction was carried out in an aqueous−organic two-phase solvent system, which is a proven concept for the enzymatic synthesis of cyanohydrins. In such a reaction system, the enzyme resides in the aqueous (working) phase and the reactants and products reside in the organic (extractive) phase. The resulting low reactant concentration in the aqueous phase suppresses the competing uncatalyzed background reaction,[13] which otherwise would erode the enantioselectivity. Additionally, the pH is maintained in the acidic range to reduce the rate of the nonenzymatic reaction.[14] Such systems perform quite satisfactorily with a wide range of aldehydes, and a computer model that predicts the final conversion and enantiomeric excess has been constructed.[15,16]

Alternatively, the reaction can be conducted in a microaqueous organic phase, in combination with almond meal,[17−19] which is the cheapest possible immobilisate of almond oxynitrilase. We decided to investigate the scope of such systems in the enzymatic hydrocyanation of 2-chlorobenzaldehyde. We now report the development of an efficient chemoenzymatic route to (*R*)-*o*-chloromandelic acid.

Results and Discussion

A baseline case was established by comparing the enzymatic hydrocyanation of benzaldehyde (**1**) and 2-chlorobenzaldehyde (**2**) in a diisopropyl ether−water (50:50, v/v) biphasic medium. Whereas **1** was rapidly converted into (*R*)-mandelonitrile (**3**, ee > 99%), **2** reacted quite sluggishly at

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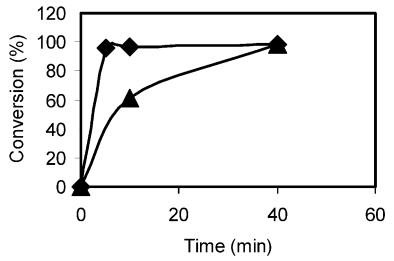
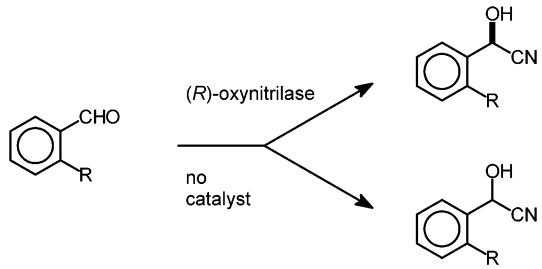


Figure 1. **Enzymatic hydrocyanation of benzaldehyde and 2-chlorobenzaldehyde. (*R*)-Mandelonitrile (ee > 99%): ◆. (*R*)-2-Chloromandelonitrile (ee 75%): ▲.**



1 (R = H)
2 (R = Cl)
3 (R = H)
4 (R = Cl)

Figure 2. **Enzymatic and uncatalyzed hydrocyanation of benzaldehyde and 2-chlorobenzaldehyde.**

a rate that was approximately 4 times slower than that of **1** (Figure 1). The ee of the 2-(2-chlorophenyl)-2-hydroxyacetonitrile (**4**) formed also was disappointingly low (ee 52% (*R*)) and only modestly improved to 77% upon a reduction of the temperature to 0 °C, which is close to the reported value of 83%. These results explain why the enzymatic hydrocyanation of 2-substitued benzaldehydes is conspicuous by its absence in the literature.[8−10] We reasoned that the enzymatic reaction of the severely hindered **2** is too slow to compete adequately with the uncatalyzed background reaction (see Figure 2). The obvious remedy was a drastic reduction of the aqueous phase.

Exploratory experiments with defatted almond meal from Sigma (see Table 1) revealed that an aqueous phase of ~0.4 mL/g of biocatalyst, which corresponds with 4% (v/v) of the reaction volume, was required for optimum activity. This amount of buffer was easily absorbed by the biocatalyst, which remained freely dispersed in the organic solvent. Larger amounts of buffer increased the tendency of the biocatalyst to coagulate, at a considerable penalty in rate and ee. In contrast with the enzymatic hydrocyanation of **1**, which results in enantiopure (*R*)-**3**, **4** was formed with a much more modest ee. Lowering the reaction temperature to 0 °C improved the ee to 91%, at the expense of a 5-fold reduction in reaction rate.

Similar experiments were carried out with almond meals from various commerical sources. The one from California Flavors also required 0.4 mL of buffer per g of biocatalyst; hence, this amount was adopted as a standard. Other almond meal preparations performed similarly to the Sigma preparation. We noted with some surprise that there was no systematic difference between biocatalysts prepared from bitter and sweet almonds. Hence, it would seem that the latter express the enzyme at the same high level, although it has no biological function. Meals prepared from blanched almonds (i.e., almonds from which the skin had been removed) generally performed somewhat poorer. The blanching process involves a brief submersion in boiling water, which presumably causes partial inactivation of the *R*-oxynitrilase, resulting, in turn, in a reduced ee.

We surmised that the diverging activities and enantioselectivities observed with the various almond meals could be caused by uncontrolled heating during the grinding of the almonds. Hence, we decided to prepare almond meal under carefully controlled conditions. A preparation with a very high activity was obtained, but the ee of the product did not increase beyond 91%. We note that 2-fluorobenzaldehyde also reacted with reduced enantioselectivity (84% ee);[20] possibly the effect is inherent in a 2-halo substituent.

The described concept was subsequently expanded to a 0.1−1 L scale (see Table 2). A good result as regards reaction time and ee was obtained at a relatively low (0.2 M) aldehyde concentration, using only 10−20% excess HCN. When the concentration of **2** was increased to 0.4 M, however, a product with only 82% ee was obtained, even though the reactant/catalyst ratio was kept at the same value. An experiment at 5 °C gave a very similar result. We ascribe this result to increased competition by the second-order−uncatalysed background reaction. A 4-fold rate increase of the latter is to be expected upon a doubling of the reactant concentration, whereas the rate of the enzymatic reaction increased only 1.75 times.

Competition by the nonenzymatic background reaction has in the past been addressed through the optimization of the aqueous−organic phase ratio and the catalyst loading and by introducing reactant transport limitations.[15,16,21] We surmised that a slow feed of the reactants, to keep their concentrations low, could adequately suppress the background reaction. This indeed proved to be the case; we even could increase the reaction temperature to 5−15 °C with a considerable reduction in the reaction time while maintaining the ee at approximately 90%. Only at a 20 °C reaction temperature the ee dropped to 86%.

A further improvement could be achieved by performing the reaction at 10 °C while feeding either the aldehyde or HCN. The latter procedure, which was much faster, resulted in a 96% yield and 91% ee of **4** at a space-time yield of 14 g L^{-1} h^{-1} (336 g L^{-1} d^{-1}). The productivity of the biocatalyst was 1.3 g g^{-1}.

Further experiments revealed that a major fraction of the biocatalyst rapidly settled when the stirrer was stopped at the end of the reaction. A fraction of fine particles remained suspended in the reaction medium, however, and had to be separated by centrifugation. Alternatively, these could be removed from the supernatant by stirring the latter with a small amount (5%, *v/v*) of acidified water containing sodium dodecyl sulfate. All fines migrated to the aqueous phase and could easily be discarded.

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Table 1. **Performance of Various Almond Meal Preparations in the Hydrocyanation of 2-Chlorobenzaldehyde**

source	pretreatment	catalyst (g L^{-1})	buffer mL (g cat)$^{-1}$	temp (°C)	initial rate (mmol min^{-1} (gcat)$^{-1}$)	time (h)	conversion (%)	ee (% *R*)
Sigma	none	100	0.1	20	0.014	1.5	50	53
Sigma	none	100	0.2	20	0.014	3	97	78
Sigma	none	100	0.4	20	0.08	1.5	90	82
Sigma	none	100	0.4	0	0.07	5	94	91
CF	none	200	0.2	0	0.008	3	98	86
CF	defatted	200	0.2	0	0.008	1.5	56	80
CF	defatted	200	0.4	0	0.020	1.8	98	90
Alldrin	none	200	0.4	0	0.012	4	88	90
Alldrin	blanched	200	0.4	0	0.012	4	89	90
Nuts4U	none	200	0.4	0	0.010	2	93	91
Nuts4U	blanched	200	0.4	0	0.014	3	76	86
Sictia	none	200	0.4	0	0.010	4	98	77
Sictia	defatted	200	0.4	0	0.008	1.5	50	73
Local	defatted	200	0.4	0	0.036	1	96	91
Local	blanched, defatted	200	0.4	0	0.009	2	58	88

Table 2. **Preparative-Scale Hydrocyanation of 2-Chlorobenzaldehyde (2)**[a]

2 (M)	catalyst (g L^{-1})	temp (°C)	procedure	time (h)	conversion (%)	ee (%)	STY g L^{-1} h^{-1}
0.2	50	0	batch	14	97	90	2.4
0.4	100	0	batch	8	98	82	8.2
0.4	50	5	batch	8	97	84	8.1
0.4	100	0	fed batch	12	94	88	5.2
0.4	50	5	fed batch	10	98	90	6.5
0.4	50	10	fed batch	9	97	91	7.2
0.6	50	15	fed batch	8	98	89	12.2
0.4	50	20	fed batch	5	98	86	13.1
0.4	50	10	HCN fed	4.5	96	91	14.2
0.4	50	10	**2** fed	6	95	90	10.5

[a] Reaction conditions: 1.1−1.2-fold excess of HCN (dissolved in diisopropyl ether), biocatalyst pretreated with 0.5 mL g^{-1} of 20 mM citrate buffer pH 5.5; reactant feeding time (where appropriate) 5−6 h.

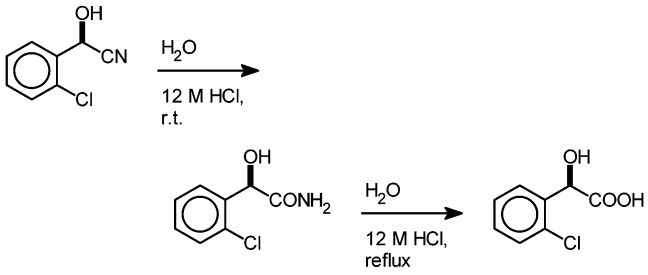

Figure 3. **Acid-catalyzed stereoretentive hydrolysis of (*R*)-2-chloromandelonitrile into (*R*)-2-chloromandelic acid.**

After the separation of the biocatalyst, the diisopropyl ether was removed under reduced pressure, yielding (*R*)-**4** as a yellow oil that rapidly crystallized. Treatment with concentrated hydrochloric acid afforded (*R*)-2-chloromandelic acid without any racemization (Figure 3). Under the applied conditions, a red color developed which was not observed when hydrolyzing the chemically prepared cyanohydrin. Most likely this contaminant originates from the almond meal. Extracting the acid after the hydrolysis procedure with an organic solvent, for example, diisopropyl ether, effectively separated the contaminant from the acid product. The resulting crude acid obtained was enantiomerically enriched to 99.9% ee by recrystallization from toluene, as already has been described in the literature.[22]

Conclusion

An industrially viable synthesis of (*R*)-2-chloromandelic acid of high enantiomeric purity was developed, based on the almond meal catalyzed asymmetric hydrocyanation of 2-chlorobenzaldehyde and subsequent chemical hydrolysis of the obtained cyanohydrin. The major improvement compared with existing procedures using almond meal was the gradual addition of the substrates to the reaction mixture. In the case of the hydrocyanation of 2-chlorobenzaldehyde, this improved the space-time yield of the process while the enantiopurity of the product did not suffer.

Experimental Section

Chemicals. 2-Chlorobenzaldehyde was obtained from Tessenderlo Chemie, Belgium as a gift. Diisopropyl ether and sodium cyanide were from Acros; citric acid monohydrate and hydrochloric acid were procured from Baker. Whole sweet almonds were purchased at a local grocery shop; food grade almond meals were obtained from California Flavors (Yuba City, California, USA), Alldrin Brothers Almond Company (Turlock, California, USA), Nuts4U (Sugar Land, Texas, USA), and Sictia (France); and defatted almond meal was obtained from Sigma.

Preparation of Almond Meal. Almonds (230 g) were cooled to 4 °C, milled using a kitchen device, and extracted 6 times with 160 mL of diisopropyl ether. The solids were separated by centrifugation, resulting in 300 g of "wet" meal (as a slurry of meal in diisopropyl ether), which was stored at 4 °C. This wet meal contained approximately 38% (w/v) of dry almond meal. The commonly used drying step was found to be superfluous and was, hence, omitted.

Preparation of the Reactant Solutions. *WARNING.* Sodium cyanide and hydrocyanic acid are highly poisonous. They should only be handled in a fume cupboard with a good draught. It is strongly advised to keep an HCN alarm switched on.

Sodium cyanide (20 g, 0.4 mol) and citric acid (0.2 g) were dissolved in 200 mL of water. The solution was cooled in an ice−water bath and stirred with 100 mL of diisopropyl ether, while the pH was brought to 5.5 by the slow addition

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of 33% (w/v) hydrochloric acid. The aqueous layer with the suspended sodium chloride was extracted 2 times with 50 mL of diisopropyl ether. The combined organic layers were stored on a small volume of citrate buffer pH 5.5 in a dark bottle.

The HCN concentration in the resulting solution was 2 M as established using the following procedure: exactly 5 mL of HCN solution was added to 25 mL of 2 M NaOH, and the mixture was stirred for 2 min. A small amount of potassium chromate was added as indicator, and the solution was titrated with 0.1 M silver nitrate.

A 2 M solution of 2-chlorobenzaldehyde in diisopropyl ether was prepared and stored on a saturated sodium bicarbonate solution. Diisopropyl ether used in the enzymatic hydrocyanation reactions was presaturated with 20 mM citrate buffer pH 5.5.

Enzymatic Hydrocyanation. To a suspension of the appropriate amount of almond meal in diisopropyl ether in a polypropylene reaction vessel equipped with a magnetic stirrer or turbine stirrer was added the appropriate volume of 20 mM citrate buffer pH 5.5. The suspension was stirred while cooling to the preferred temperature. Depending on the procedure, the substrate solutions were added directly to the reaction mixture or fed over a 5−6 h period.

Samples were taken from the reaction mixture and centrifuged. The supernatant was diluted with hexane−trifluoroacetic acid (95:5, v/v) and analyzed by HPLC. Samples for enantiomeric analysis were, after evaporation of the solvent, derivatized in dioxane−acetic anhydride−pyridine (25:3:0.5, v/v).

The enzymatic hydrocyanation rates of **1** and **2** were compared as follows: a 0.5 g amount of almond meal (Sigma) was dispersed in 5.5 mL of a 20 mM citrate buffer pH 5.5 and 3 mL of diisopropyl ether. A 0.5 mL aliquot of a 2 M solution of aldehyde and 2 mL of a 2 M HCN solution (both in diisopropyl ether) were added. Samples (10 μL) were withdrawn at regular intervals and analyzed by HPLC.

Downstream Processing of the Hydrocyanation Product. At the end point of a typical enzymatic hydrocyanation reaction (1 L reaction volume, 200 mM aldehyde), the stirrer was stopped and the almond meal was allowed to settle for 5 min. The supernatant was discharged, and the remaining almond meal was washed with two portions of 250 mL of diisopropyl ether at 0 °C. The combined organic layers were centrifuged for 10 min at 1000 rpm and 0 °C; the resulting clear solution was concentrated using a rotary evaporator under reduced pressure.

The resulting solid yellowish cyanohydrin was added to 50 mL of hydrochloric acid (33%, w/v), and the suspension was stirred at room temperature until dissolution was complete (approximately 1 h). The solution was refluxed for 3 h until all amide was converted into acid. The progress of the reaction was monitored by HPLC. The ammonium chloride precipitate was filtered off, and the solution was extracted 6 times with 50 mL of diisopropyl ether. The combined extracts were concentrated in vacuo; the resulting crude product was recrystallized from 100 mL of toluene to yield 25 g (134 mmol, 67%) of (*R*)-2-chloromandelic acid with ee 99.9%; mp 119 °C, α_D −78.4° (*c* 0.5, ethanol) (literature:[23] mp 119−120 °C, α_D −78.0° (*c* 0.5, ethanol)). ^{1}H NMR (DMSO-d_6) δ 5.34 (s, 1H), 7.32−7.44 (m, 4H).

HPLC Analysis. Samples were dissolved in hexane−isopropyl alcohol (90:10, v/v), containing 1% trifluoroacetic acid, and dried over sodium sulfate. The enantiomeric excess of 2-chloromandelonitrile was measured after acetylation with acetic anhydride. The hydrocyanation products were analyzed by HPLC using a Chiralcel OB-H column, eluant hexane−2-propanol (90:10, v/v). Retention times (in min): 2-chlorobenzaldehyde, 4; 2-chloromandelonitrile, 5; *O*-acetyl-(*S*)-2-chloromandelonitrile, 7; *O*-acetyl-(*R*)-2-chloromandelonitrile, 10.

The hydrolysis products of 2-chloromandelonitrile were analyzed using a Nucleosil C-18 column, eluant methanol−water (40:60, v/v) acidified with phosphoric acid to pH 3.5.

The optical purity of 2-chloromandelic acid was measured as follows: samples were esterified using boron trifluoride in methanol and subjected to chiral HPLC using a Chiralcel OD column, eluant hexane−2-propanol (80:20, v/v). Retention times (in min): (*S*)-2-chloromandelic acid methyl ester, 9.6; (*R*)-2-chloromandelic acid methyl ester, 10.8.

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