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Synthesis of enantiomerically pure (*S*)-mandelic acid using an oxynitrilase–nitrilase bienzymatic cascade: a nitrilase surprisingly shows nitrile hydratase activity

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Abstract—Benzaldehyde was converted into enantiomerically pure (*S*)-mandelic acid by sequential HCN addition and hydrolysis in the presence of a cross-linked enzyme aggregate composed of the (*S*)-selective oxynitrilase from *Manihot esculenta* and the non-selective recombinant nitrilase from *Pseudomonas fluorescens* EBC 191. Surprisingly, (*S*)-mandelic amide was formed in large amounts. It was shown, in separate experiments, that the nitrilase hydrolyses (*S*)-mandelonitrile into an approx. equimolar mixture of acid and amide, whereas with the (*R*)-enantiomer only 10% of amide was formed.

1. Introduction

In recent years, there has been increasing interest in the synthesis of enantiopure 2-hydroxy acids via asymmetric enzymatic transformations; two pathways have risen into prominence. The first is based on the enantioselective hydrocyanation of an appropriate aldehyde in the presence of an oxynitrilase (hydroxynitrile lyase, HnL, E.C. 4.1.2.10), which gives rise to the corresponding enantiomerically pure cyanohydrin, followed by chemical hydrolysis in the presence of a strong acid (Fig. 1A). This latter step generates copious quantities of salt and is not compatible with sensitive functional groups, which is a serious drawback. Alternatively, enantiopure 2-hydroxy acids can be obtained via a dynamic kinetic resolution of the (chemically synthesised) cyanohydrin in the presence of an enantioselective nitrilase (NLase, E.C. 3.5.5.1, see Fig. 1B). This latter methodology, which is industrially applied for the multiton-scale synthesis of (*R*)-mandelic acid,[1] is restricted to (*R*)-2-hydroxy acids, because no (*S*)-selective nitrilases for cyanohydrin substrates have yet been identified.

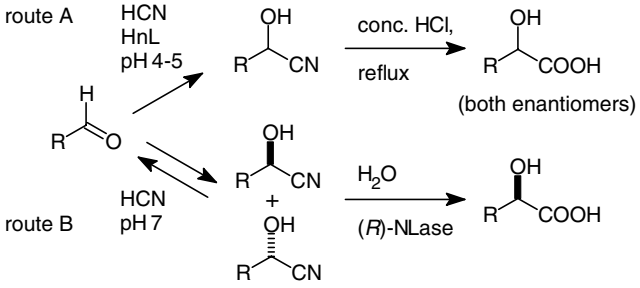

Figure 1. Synthetic routes to enantiomerically pure 2-hydroxy acids, via oxynitrilase (hydroxynitrile lyase, HnL) catalysed enantioselective hydrocyanation (route A) and (*R*)-nitrilase (NLase) mediated dynamic kinetic resolution (route B).

We surmised that a fully enzymatic route to the (*S*)-acids could be possible by combining an (*S*)-selective oxynitrilase and a non-selective nitrilase in a bienzymatic cascade (see Fig. 2). In addition to being more environmentally acceptable, the mild reaction conditions of the combined enzymatic reaction are compatible with a wide range of hydrolisable groups.

Potential incompatibilities that must be resolved are the pH and reaction medium. Oxynitrilase mediated hydrocyanations are preferably carried out at pH < 5 to

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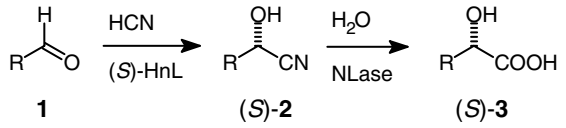

Figure 2. Bienzymatic procedure for the synthesis of (*S*)-2-hydroxy-acids, using an (*S*)-specific HnL and a nonspecific NLase in tandem.

suppress the competing uncatalysed hydrocyanation.[2] The background reaction can be further reduced by the use of a biphasic aqueous–organic[2] or micro-aqueous reaction medium.[3] Such reaction conditions are tolerated rather well by the readily available (*S*)-selective HnL from *Manihot esculenta* (MeHnL).[2,4] Nitrilases, in contrast, are preferentially used at a pH close to 7 and are readily deactivated by organic solvents. Rendering the nitrilase compatible with the conditions of enzymatic hydrocyanation is obviously an issue on which the success of the methodology depends. We reasoned that immobilisation as a cross-linked enzyme aggregate (CLEA)[5] would present a solution to this latter problem. We herein report the application of our bienzymatic methodology to the synthesis of enantiomerically pure (*S*)-mandelic acid.

2. Bienzymatic synthesis of (*S*)-mandelic acid

As noted above, enzymatic hydrocyanations are preferably performed at pH < 5, to suppress the non-enzymatic background reaction, whereas the optimum pH of the common NLases is 7. A compromise in pH is obviously required and we accordingly assessed the effects of the pH on the MeHnL-mediated hydrocyanation of benzaldehyde **1a** (Fig. 2) in a biphasic aqueous-diisopropyl ether (DIPE) medium. We found that enantioselectivity was maintained at pH 5.5, provided that the aqueous buffer phase accounted for <10% of the reaction volume.[4] In experiments at pH 6.0 and 6.5, we surprisingly found a progressively faster reaction, at the cost of a significant loss in enantioselectivity (Table 1).

Table 1. The effect of pH on the hydrocyanation of **1a** in the presence of MeHNL[a]

pH	Time (min)	Conv (%)	ee (% *S*)
5.5	120	90	95.4
	180	94	94.7
	240	95	94.3
6.0	60	90	92.9
	120	94	90.9
	180	94	88.4
6.5	60	91	89.5
	90	93	87.8
	120	94	85.8

[a] Reaction conditions: **1a** (0.1 mmol), HCN (0.5 mmol), MeHnL CLEA (0.25 mg, 19.6 U) in 0.1 mL, 0.1 M citrate buffer and 0.9 mL DIPE at rt.

The enzymatic hydrolysis of mandelonitrile **2a** was investigated in some detail, as this is evidently the critical step. It should be fast, moreover, to avoid accumulation—and possible racemisation—of (*S*)-**2a**. Few nitrilases displayed a useful activity at pH 5.5–6; most of these were strongly (>90% ee) biased towards the (*R*)-enantiomer and only converted (*S*)-mandelonitrile sluggishly, if at all. A recombinantly expressed[6] nitrilase from *P. fluorescens* EBC 191 (PfNLase),[7] was an exception as it converted (*S*)- and (*R*)-**2a** at comparable rates pH 5.5,[6] which we adopted as a compromise pH for the bienzymatic reactions.

Accordingly, we conducted experiments with CLEAs of MeHnL and PfNLase in tandem in a 90:10 DIPE buffer pH 5.5 medium. The reaction proceeded to nearly full conversion (Fig. 3a) and the product ee was 94%. Combining both enzymes in a bienzymatic catalyst (combi-CLEA, Fig. 3b) resulted in further improvement and 98% enantiomerically pure (*S*)-**3a** was obtained.[8] It would seem that the nitrile intermediate is immediately hydrolysed in the combi-CLEA particles, which suppresses diffusion into the water phase and possible

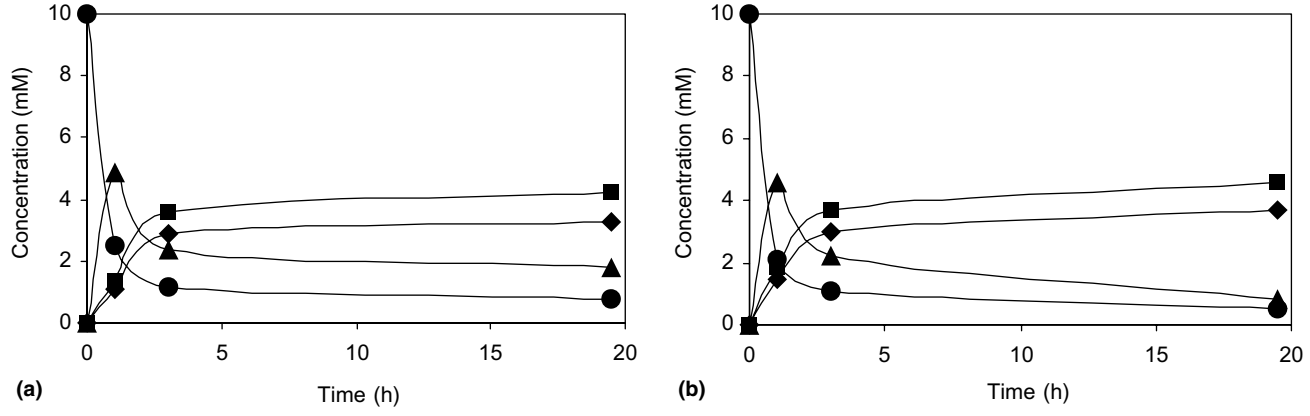

Figure 3. Time course of the bienzymatic synthesis of (*S*)-mandelic acid from benzaldehyde and HCN in the presence of a MeHnL and PfNLase in 90:10 DIPE buffer pH 5.5, rt,[8] (a) two CLEAs, (b) combi-CLEA; legend: **1a**: (●), **2a**: (▲), **3a**: (■), **4a**: (◆).

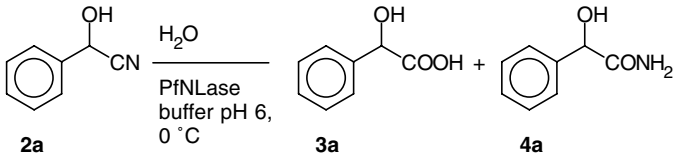

Figure 4. Hydrolysis of **2a** in the presence of *P. fluorescens* NLase.

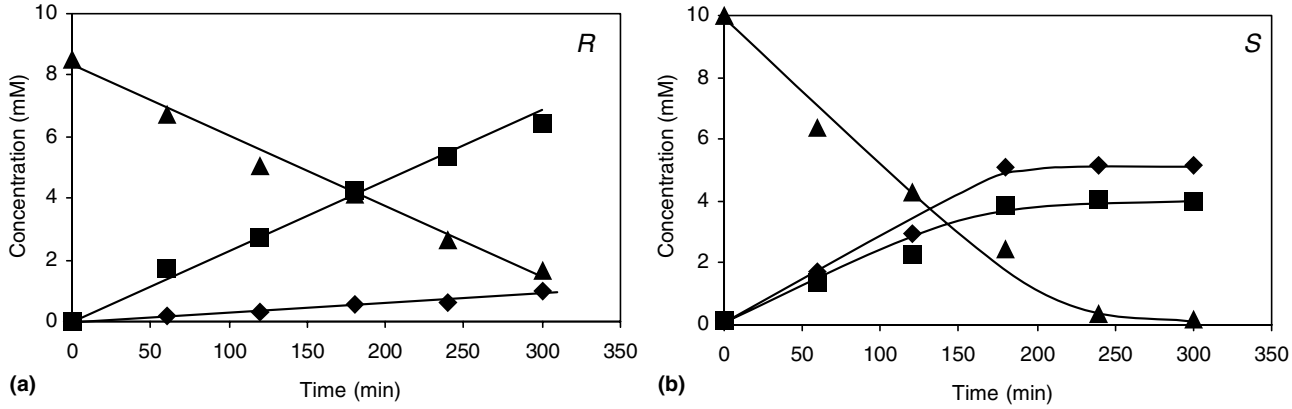

Figure 5. Time course of the hydrolysis of (*R*)- and (*S*)-mandelonitrile in the presence of *P. fluorescens* NLase at pH 6, 0 °C; legend: **2a**: (▲), **3a**: (■), **4a**: (◆).

racemisation. A full analysis of the reaction products showed, however, that very appreciable amounts of (*S*)-mandelic amide ((S)-**4a**, see Fig. 3, approx. 40%) accompanied the formation of (*S*)-**3a** (see later).

We finally attempted to improve the reaction rate by increasing the pH to 6, expecting that the in situ hydrolysis of (*S*)-**2a** would prevent its racemisation. The increase in rate was quite modest, however, and came at the expense of a slight decrease in product ee (96%).

In conclusion, the formation of nearly enantiomerically pure (*S*)-**3a** proves that the bienzymatic methodology is sound and that racemisation of the intermediate can be avoided. The unexpected formation of large amounts of (*S*)-mandelic amide somewhat reduces the immediate practical value of our procedure, but we are confident that amide formation can be obviated by proper genetic engineering of the NLase.

3. Amide formation

The formation of minor amounts of amides over the course of NLase-catalyses nitrile hydrolysis has been noted[9] and the nitrilase from *Arabidopsis thaliana* promiscuously produced amides as the major product in the hydrolysis of α-fluoronitriles[10] as well as other electron-deficient nitriles.[11] One would suppose that minor amounts of amide formation have often gone unnoticed in the past, due to the presence of contaminating amidases (Fig. 4).

We investigated the amide formation from **2a** more closely, by subjecting enantiomerically pure (*R*)- and (*S*)-**2a** to hydrolysis in the presence of PfNLase (see Figs. 4 and 5). To avoid racemisation of the nitrile, the reactions were performed at 0 °C.[†] It became clear that only a minor amount of amide was formed from the (*R*)-enantiomer (acid/amide ~8), whereas the amide was a major product formed from the (*S*)-enantiomer under otherwise identical conditions (acid/amide <1, see Fig. 5). Stereochemical integrity was fully maintained under the reaction conditions and **3a** and **4a** were formed with complete retention of configuration, as would be expected. It should be noted that (*S*)-**4a** is a reaction product, and not an intermediate in the formation of (*S*)-**3a**, as the acid/amide ratio stays constant throughout the reaction. Neither does (*S*)-**4a** result from a reverse reaction of (*S*)-**3a** and ammonia, since such a reaction did not take place in a separate experiment.

We wished to exclude any possibility that the near-absence of the amide in the hydrolysis product of (*R*)-**2a** was caused by spurious amidase activity of PfNLase, rather than by the reactant stereochemistry. Accordingly, when we incubated (*R*)-**4a** with PfNLase at either pH 5.5 or 7, no reaction was observed. One more question remained as to whether the formation of (*S*)-amide could be ascribed to the presence of a contaminating (*S*)-selective nitrile hydratase, but SDS-PAGE analysis showed that no contaminating proteins were present.[6]

4. Conclusion

We can conclude that the amides are products of the NLase and that the stereochemical configuration of

[†] These experiments were carried out at somewhat different conditions as they are part of a larger investigation.

the nitrile exerts a major influence on their formation, in addition to the nature of the NLase and the electron density at the α-C atom in the nitrile.

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References

1. (a) Ress-Löschke, M.; Friedrich, T.; Hauer, B.; Mattes, R.; Engels, D. (BASF AG), DE 19848129, 2000 [*Chem. Abstr.* **2000**, *132*, 292813]; (b) Yamaguchi, Y.; Ushigome, M.; Kato, T. (Nitto Chem. Ind.), EP 773297, 1997 [*Chem. Abstr.* **1997**, *127*, 4190]; (c) Endo, T.; Tamura, K. (Nitto Chem. Ind.), EP 449648, 1991 [*Chem. Abstr.* **1992**, *116*, 5338].
2. Schmidt, M.; Griengl, H. *Top. Curr. Chem.* **1999**, *200*, 193–226.
3. (a) Han, S.; Lin, G.; Li, Z. *Tetrahedron: Asymmetry* **1998**, *9*, 1835–1838; (b) Van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Org. Proc. Res. Dev.* **2003**, *7*, 828–831.
4. Chmura, A.; Mateo, C.; Van der Kraan, G. M.; Celik, A.; Van Langen, L. M.; Van Rantwijk, F.; Sheldon, R. A., in preparation.
5. (a) Cao, L.; van Rantwijk, F.; Sheldon, R. A. *Org. Lett.* **2000**, *2*, 1361–1364; (b) Mateo, C.; Palomo, J. M.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. *Biotechnol. Bioeng.* **2004**, *86*, 273–276; (c) Van Langen, L. M.; Selassa, R. P.; Van Rantwijk, F.; Sheldon, R. A. *Org. Lett.* **2005**, *7*, 327–329.
6. Kiziak, C.; Conradt, D.; Stolz, A.; Mattes, R.; Klein, J. *Microbiology* **2005**, *151*, 3639–3648.
7. Layh, N.; Parratt, J.; Willets, A. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 467–474.
8. *Combi-CLEA preparation:* MeHnL (3 kU, from Jülich Fine Chemicals) and semi-purified PfNLase (85 U, see below) were mixed with dextranpolyaldehyde solution (4 mL, cross-linking agent),[5b] 0.5 M phosphate buffer pH 7.5 (2 mL) and 1,2-dimethoxyethane (8 mL, aggregation agent). The mixture was stirred overnight at 40 °C and centrifuged. The pellet was resuspended in 0.1 M sodium bicarbonate solution containing 1 g L^{-1} of sodium borohydride (40 mL) to reduce the Schiff's bases, stirred for 45 min at 40 °C, washed twice with Milli-Q water (40 mL), resuspended in 5 mL 0.5 M citrate buffer pH 5.5 and stored at 4 °C.
   One unit (U) of PfNLase will convert 1 μmol of racemic mandelonitrile per min at rt and pH 7.
   One pot synthesis of (*S*)-mandelic acid: Combi-CLEA suspension (0.5 mL), DIPE, HCN solution in DIPE (0.25 mmol)—CAUTION: HCN is highly poisonous; use a well-ventilated hood and keep a HCN detector switched on—were combined and DIPE was added to a total volume of 5 mL. Benzaldehyde (0.05 mmol) was added and the mixture stirred vigorously. Samples were withdrawn periodically.
   Analysis: the reaction progress was monitored by reversed-phase HPLC (Merck 4.6 × 50 mm Chromolith™ Speed-ROD RP18e, ACN–H_2O (5:95), 0.03% heptaflurobutyric acid, 30 mM ammonium formate pH 2.3 at 1 mL min^{-1}). The enantiomeric purity was measured using chiral HPLC (Daicel 4.6 × 250 mm 10μ Chiralcel OD, hexane–isopropyl alcohol (80:20), 0.1% TFA at 0.5 mL min^{-1}).
9. (a) Hook, R. H.; Robinson, W. G. *J. Biol. Chem.* **1964**, *239*, 4263–4267; (b) Stevenson, D. E.; Dumas, F.; Groleau, D.; Mihoc, A.; Storer, A. C. *Biotechnol. Appl. Biochem.* **1992**, *15*, 283–302.
10. Effenberger, F.; Oßwald, S. *Tetrahedron: Asymmetry* **2001**, *12*, 279–285.
11. Osswald, S.; Wajant, H.; Effenberger, F. *Eur. J. Biochem.* **2002**, *269*, 680–687.