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Asymmetric Synthesis of an (*R*)-Cyanohydrin Using Enzymes Entrapped in Lens-Shaped Gels

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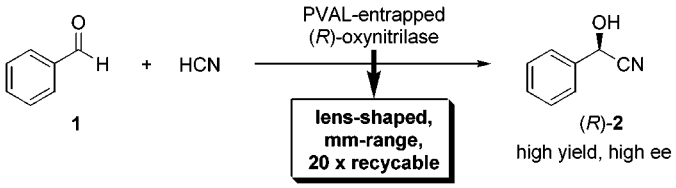
ABSTRACT



A novel synthesis of (*R*)-cyanohydrins is described which is based on the use of cross-linked and subsequently poly(vinyl alcohol)-entrapped (*R*)-oxynitrilases. These immobilized lens-shaped biocatalysts have a well-defined macroscopic size in the mm range, show no catalyst leaching, and can be recycled efficiently. Furthermore, this immobilization method is cheap and the entrapped (*R*)-oxynitrilases gave similar good results compared with those of free enzymes. The (*R*)-cyanohydrin was obtained in good yields and with high enantioselectivities of up to >99% ee.

Among industrial production of chiral building blocks, cyanohydrins play an important role, since these molecules find a wide range of pharmaceutical and agrochemical applications.[1] Highly efficient asymmetric methods to these molecules[2] have been realized by Shibasaki[3a−e] et al., Inoue[3f,g] et al., and other groups[3h] using metal-based complexes or organic catalysts, respectively. The formation of (*R*)-cyanohydrins can be also realized by means of a biocatalytic hydrocyanation using (*R*)-oxynitrilases. This enzymatic approach has been developed by Effenberger, Kula, and other groups.[2,4a−d] Immobilization was used to try to prevent the disadvantages associated with "free" (*R*)-oxynitrilases (e.g., rapid deactivation).[2,4a−d,5] However, many known immobilization methods with oxynitrilases have practical problems on a large scale. Among the main problems are the

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(1) Important industrial applications of chiral cyanohydrins are the manufacture of pyrethroids (insecticides), e.g., deltamethrin, as well as the production of α-hydroxy carboxylic acids as intermediates for pharmaceuticals and resolving agents. For example, (*R*)-mandelic acid (a derivative of (*R*)-mandelonitrile) represents a fine chemical which is produced on multihundred ton scale.

(2) For excellent reviews about the asymmetric synthesis of cyanohydrins, see: (a) Effenberger, F. *Angew. Chem.* **1994**, *106*, 1609−1619; *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1555−1564. (b) Gregory, R. J. H. *Chem. Rev.* **1999**, *99*, 3649−3682.

(3) (a) Hamashima, Y.; Sawada, D.; Kanai, M.; Shibasaki, M. *J. Am. Chem. Soc.* **1999**, *121*, 2641−2642. (b) Sawada, D.; Shibasaki, M. *Angew. Chem.* **2000**, *112*, 215−218; *Angew. Chem., Int. Ed.* **2000**, *39*, 209−213. (c) Kanai, M.; Hamashima, Y.; Shibasaki, M. *Tetrahedron Lett.* **2000**, *41*, 2405−2409. (d) Hamashima, Y.; Kanai, M.; Shibasaki, M. *J. Am. Chem. Soc.* **2000**, *122*, 7412−7413. (e) Hamashima, Y.; Kanai, M.; Shibasaki, M. *Tetrahedron Lett.* **2001**, *42*, 691−694. (f) Tanaka, K.; Mori, A.; Inoue, S. *J. Org. Chem.* **1990**, *55*, 181−185. (g) Mori, A.; Nitta, H.; Kudo, M.; Inoue, S.; *Tetrahedron Lett.* **1991**, *32*, 4333−4336. (h) For reviews, see ref 2.

(4) (a) Effenberger, F.; Ziegler, T.; Förster, S. *Angew. Chem.* **1987**, *99*, 491−492; *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 458−460. (b) Wehtje, E.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. Bioeng.* **1990**, *36*, 39−46. (c) Wehtje, E.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. Bioeng.* **1993**, *41*, 171−178. (d) Gill, I.; Ballesteros, A. *J. Am. Chem. Soc.* **1999**, *120*, 8587−8598. (e) At pH 5.5, a low ee of 77% was obtained.

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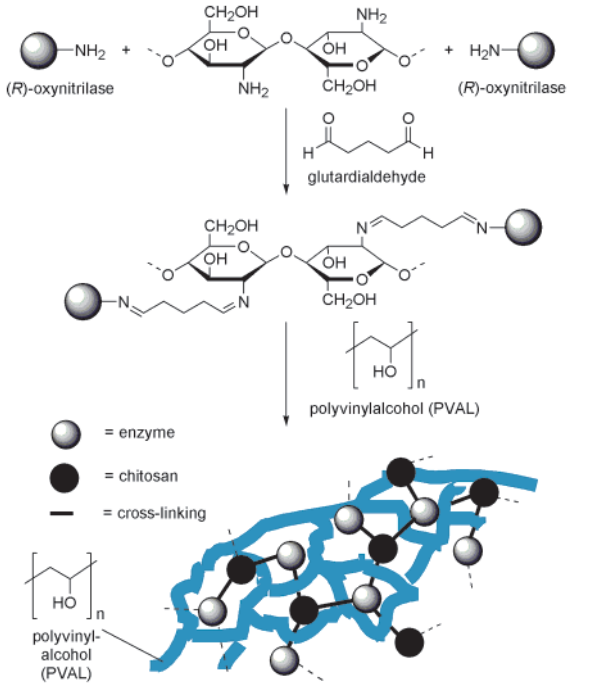
nonsatisfied long-term stability, catalyst leaching, abrasion of the immobilizate, and a wide particle distribution (instead of a well-defined macroscopic size) in the case of powdered immobilizates.[5,6]

On the other hand, Vorlop et al. has recently developed a highly efficient immobilization method for cells by entrapping them in a hydrogel matrix mainly based on poly(vinyl alcohol).[7] This method represents a cheap, efficient, and industrially feasible immobilization technique, which not only shows negligible catalyst leaching but also gives macroscopically well-defined and highly flexible lens-shaped particles with a high activity (type, LentiKats; diameter, 3−5 mm; thickness, 300−400 μm).[7a] Several successful applications of this concept have been previously reported by Vorlop and co-workers, e.g., for the synthesis of itaconic acid and 1,3-propanediol.[7c,d] It is noteworthy from an industrial point of view that these lens-shaped hydrogels can easily be separated (due to a diameter of 3−5 mm), are suitable for stirred tank reactors, and show no abrasion as well as minimized diffusion limitations (due to low thickness of <0.5 mm). This technology has been already commercialized by geniaLab.[8] We envisioned that this concept could be extended to a suitable immobilization method for oxynitrilases (and moreover for enzymes in general).

In the following we report the first application of LentiKat-entrapped enzymes (here: oxynitrilases) in organic catalysis, namely in the synthesis of (*R*)-mandelonitrile. For our studies we used a nonpurified (*R*)-oxynitrilase for economical reasons.[9] To prepare an efficient entrapped oxynitrilase, a two-step procedure has been chosen. In a first step, a cross-linking process was carried out which led to an increased molecular weight of the (cross-linked) enzymes. This cross-linking procedure is necessary since enzymes with a molecular weight of 50000 maximum (as in the case of oxynitrilases) would not be restrained in the hydrogels. At first the cross-linking process was carried out only with glutaraldehyde, but the resulting cross-linked enzymes showed a drastically decreased activity (probably due to deactivation of the enzyme). However, a combination of glutaraldehyde and chitosan led to an improved cross-linked enzyme which showed 89% of its original activity.

In a subsequent step, this cross-linked enzyme was entrapped in a hydrogel matrix which is (mainly) based on poly(vinyl alcohol). In this step a lens-shaped catalyst with a well-defined particle diameter of 3 or 5 mm and a thickness of 0.3−0.4 mm is produced. These lens-shaped hydrogels are highly elastic and flexible toward mechanical treatment.[10] The concept of this two-step immobilization method is shown in Scheme 1, and a corresponding synthetic protocol is given in the Supporting Information.

Scheme 1. Two-Step Procedure for Entrapping (*R*)-Oxynitrilases



Regarding the synthetic potential of the "catalytic capsules", investigations have been carried out using "free" and entrapped oxynitrilases. In the presence of the free, nonpurified oxynitrilase with an activity of 15 U/mmol a satisfactory result (95% ee, 85% yield) was obtained at pH 4.5 under biphasic conditions (Table 1, entry 1).[4e]

When applying the entrapped catalyst (8.16 U/g)[11] in the model reaction, we were pleased to find that—despite modifying the enzyme by cross-linking and entrapping—a comparable result was obtained regarding enantioselectivity as well as yield. The desired (*R*)-mandelonitrile was obtained with 94% ee and 93% yield (Table 1, entry 2) which

(5) One of the rare exceptions of a very practical immobilization method represents the production of (*R*)-mandelonitrile with the continuous membrane reactor technique which is mainly applied for continuous processes, see: (a) Vasic-Racki, D.; Jonas, M.; Wandrey, C.; Hummel, W.; Kula, M.-R. *Appl. Microbiol. Biotechnol.* **1989**, *31*, 215−222. (b) Bommarius, A. S.; Drauz, K.; Groeger, U.; Wandrey, C. In *Chirality in Industry*; Collins, A. N., Sheldrake, G. N., Crosby, J., Eds.; John Wiley & Sons Ltd: New York, 1992; Chapter 20, pp 371−397.

(6) (a) Despite the high potential of immobilized biocatalysts, in a United Nations publication from 1989, only eight industrial processes have been reported which are based on immobilized enzymes or microbial cells, see: Klyosov Report, A. A. UNIDO/IPTC. 93, V-89-61316. Order No. PB90-210360, 111 pp. (b) The manufacture of 7-aminocephalosporanic acid represents (probably) the most important industrial process which is based on the use of an immobilized enzyme.

(7) (a) Jekel, M.; Buhr, A.; Willke, T.; Vorlop, K.-D. *Chem. Eng. Technol.* **1998**, *21*, 275−278. (b) Wittlich, P.; Schlieker, M.; Jahnz, U.; Willke, T.; Vorlop, K.-D. *Proc. 9th Eur. Congr. Biotechnol.* **1999**, No. P2762, ISBN 805215-1-5. (c) Welter, K.; Willke, T.; Vorlop, K.-D. *SchrR Nachwachsende Rohstoffe* **1999**, *14*, 520−521. (d) Wittlich, P.; Schlieker, M.; Lutz, J.; Reimann, C.; Willke, T.; Vorlop, K.-D. *SchrR Nachwachsende Rohstoffe* **1999**, *14*, 524−532. (e) Durieux, A.; Nicolay, X.; Simon, J.-P. *Biotechnol. Lett.* **2000**, *22*, 1679−1684.

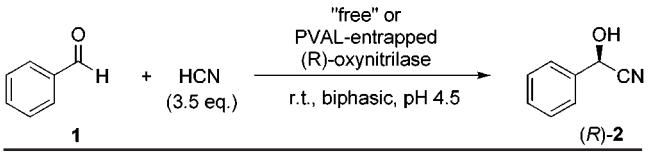
(8) For information about commercial applications of this type of immobilization technology as well as commercial products from geniaLab, see: http://www.geniaLab.de.

(9) The experiments described herein have been carried out using a nonpurified oxynitrilase purchased from ASA Spezialenzyme GmbH, Braunschweig (specific activity, 13.6 U/mg proteine; amount of proteine, 7.6 mg/mL). Although purity and activity are somewhat lower compared with those of other commercially available, highly purified oxynitrilases, this enzyme was chosen on basis of cost.

(10) The physical properties of such type of lens-shaped hydrogels in general have been described earlier; for details, see ref 7.

(11) The reaction conditions for the preparation of the lens-shaped hydrogels can be varied widely, e.g., resulting in a different catalyst loading.

Table 1. Entrapped Biocatalysts in Asymmetric Hydrocyanation



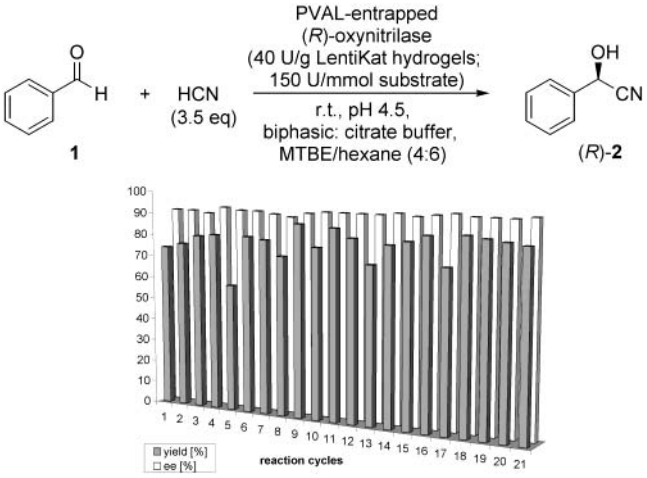
entry	type of oxynitrilase[a]	U per mmol[b]	organic solvent[c]	yield [%][d]	ee [%][e]
1	free	15	MTBE/hex.	85	95
2	entrapped (8)	15	MTBE/hex.	93	94
3	entrapped (40)	150	MTBE/hex.	74	91
4[f]	entrapped (40)	75	EtOAc	62	93
5[f]	entrapped (40)	75	MTBE	70	92
6[g]	entrapped (40)	100	*i*-Pr_2O	84	99

[a] The catalyst loading is given in U per g of capsules in parentheses; a general procedure is as follows. To a solution of the PVAL-entrapped (*R*)-oxynitrilases (lens-shaped capsules; g of lenses per mmol of substrate: entries 2 and 4−5, 1.84 g; entry 3, 3.67 g; entry 6, 2.47 g) in 4 mL of a citric buffer, subsequently 4 mL of the organic solvent, 1 mmol of benzaldehyde, and 3.5 mmol of HCN (as an 20% aqueous solution) are added. After stirring of the reaction mixture for 2 h, the organic layer is separated, and the aqueous layer is washed with 2 × 20 mL MTBE (subsequently, the aqueous layer is treated with NaOCl solution in order to decompose HCN which has been used in excess amount). The organic phases are dried over magnesium sulfate, and after filtration the volatile materials are removed in vacuo. The product (*R*)-mandelonitrile is obtained with a purity of >90−95% (in some cases of up to 99%). CAUTION: HCN is very toxic and must be handled with high caution. Safety instructions are given in the material safety data sheet (MSDS) of HCN. For further safety information, see the international chemical safety card of HCN (ICSC0492) which is available from the Internet: http://www.cdc.gov/niosh/ipcsneng/neng0492.html. [b] The catalytic amount is defined as U per mmol of substrate (benzaldehyde). [c] MTBE/hex. = mixture of MTBE and hexane in a ratio of 4:6. [d] The yield was determined from proton NMR spectroscopic data of the crude product (after the evaporation step) which contains (*R*)-mandelonitrile as the main product with a purity of >90−95% (in some cases of up to 99%). [e] The enantiomeric excess (ee) was determined by HPLC or by proton NMR spectroscopic data after derivatization with a chiral carboxylic acid chloride. [f] This experiment has been carried out at pH 4.25. [g] The reaction time was 3 h.

corresponds to a volumetric productivity of 1.4 mol/(d·L). It is noteworthy that all reaction parameters remained unchanged when compared with the standard reaction using the free enzyme. Using entrapped oxynitrilases with a somewhat higher catalyst loading of 40 U per g, lens-shaped capsules led to 74% yield and slightly decreased 91% ee (Table 1, entry 3).

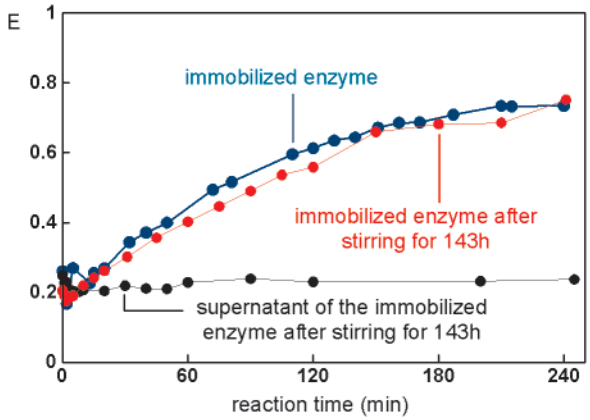
Next we were interested in the recycling abilities of the entrapped enzymes. These experiments were carried out on the basis of the reaction parameters described in Table 1, entry 3. In total, the lens-shaped hydrogels were recycled 20 times. The results, shown in Scheme 2, indicate the potential to recycle the catalyst efficiently. Even after reusing the lens-shaped catalysts 20 times, no decrease of enantioselectivity was observed. In contrast, the ee slightly increased from 91% ee to 95% ee. This might be due to an increased stabilization of the enzyme within the hydrogel matrix. Also, the yield remains in the same range of ca. 80%. Furthermore, the oxynitrilase-containing hydrogels do not change their elasticity, size, and flexibility. The long-term-stability of the enzyme, however, might mainly result from cross-linking the enzyme.

Scheme 2. Recycling of Entrapped (*R*)-Oxynitrilases



In addition, we further investigated a potential catalyst leaching. These experiments were carried out with respect to the dehydrocyanation reaction of mandelonitrile.[12] The results underline the recycling abilities of the entrapped oxynitrilase catalyst (Scheme 3): No catalyst leaching was observed during a time of 143 h. This conclusion is supported by the (nearly) identical course of the enzyme activities determined from the reactions with "fresh" hydrogels and hydrogels after stirring for 143 h, respectively.

Scheme 3. Investigation about Leaching of the Entrapped Enzyme via Photometrical Detection of the Concentration of Benzaldehyde (Extinction E) during the Reaction Course (for Details of This Experiment, see Supporting Information)[12]



In addition, no activity of the supernatant (of the immobilized enzyme after stirring for 143 h in aqueous solution) was found, indicating that there is no diffusion of the enzyme into the solution. Thus, neither a catalyst leaching nor a decrease of the catalyst activity (due to deactivation) has been observed.

(12) In general, the catalytic activity of oxynitrilases is determined via this cleavage reaction of mandelonitrile (dehydrocyanation reaction). The reaction course has been determined according to the increase of the concentration of benzaldehyde (extinction E) which has been detected with a photometer (at 250 nm). For further details, see Supporting Information.

With an efficient entrapped catalyst in hand, we focused on the optimization of the reaction conditions. In particular, a high enantioselectivity even when using a nonpurified, entrapped enzyme was desirable.

A remarkable dependence of enantioselectivity and yields on the nature of the solvent has been observed. The catalytic amount also plays a significant role. It is noteworthy that an enantioselectivity of 99% ee was obtained when carrying out the reaction in diisopropyl ether and with a catalytic amount of 100U/mmol (Table 1, entry 6). Interestingly, ≥99% ee was also found in the subsequent recycling stages (in total seven reactions). The average yields are in the range of >80%.

In conclusion, we reported a new type of entrapped oxynitrilase catalyst which not only shows a long-term-stability, high activity, and good recycling rates but also gives high enantioselectivities of up to 99% ee. This new type of entrapped biocatalyst is further characterized by the following properties: (i) recyclable without loss of enzymatic activity; (ii) no catalyst leaching; (iii) macroscopic well-defined size with a diameter in the mm range, thus making simple filtration steps possible; (iv) suitable for technical reactors due to high elasticity; (v) very low price of the encapsulation materials; (vi) nontoxicity of the encapsulation materials; (vii) simple encapsulation technique; and (viii) a high synthetic efficiency of the lens-shaped catalyst capsules. The use of these entrapped oxynitrilases enables an efficient synthesis of (*R*)-mandelonitrile, which is a starting material for the manufacture of the industrially important α-hydroxy carboxylic acid (*R*)-mandelic acid.

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Supporting Information Available: An experimental protocol for the preparation of the (*R*)-oxynitrilase-containing lens-shaped PVAL-hydrogels (according to Scheme 1) as well as experimental protocols and data referring to Schemes 2 and 3, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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