ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 213–218

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Enzymatic formation and esterification of (*S*)-mandelonitrile

Ulf Hanefeld *, Adrie J.J. Straathof, Joseph J. Heijnen

Kluyver Laboratory for Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, Netherlands

Abstract

The (*S*)-selective hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) catalyzes the *trans*-cyanohydrin reaction (transcyanation). The equilibrium of this two-step reaction sequence is not favorable unless a large excess of acetone cyanohydrin (**1**) is used. Therefore, the coupling of this reaction with a follow-up reaction was investigated. It was established that the *trans*-cyanohydrin reaction could be performed in organic media, making it possible to couple it with a lipase-catalyzed acylation. *Candida antarctica* lipase B (CAL-B) shows a high selectivity ($E = 100$) for (*S*)-mandelonitrile (**4**) and is, therefore, the ideal candidate for this type of multi-step one-pot reaction.

Keywords: Hydroxynitrile lyase; Oxynitrilase; *Hevea brasiliensis*; *Candida antarctica* lipase B; *Trans*-cyanohydrin reaction; Transcyanation; (*S*)-Cyanohydrin

1. Introduction

The hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) is a versatile catalyst for the highly enantio-selective synthesis of (*S*)-cyanohydrins [1,2]. In the reversal of its natural task, it can be used to add HCN to prochiral aldehydes or methylketones, forming important (*S*)-cyanohydrin building blocks [3]. *Hb*HNL has been overexpressed in *Pichia pastoris*, making it readily available for large-scale synthesis [4]. Indeed, several synthetic applications [5] and even the industrial application of *Hb*HNL have recently been described [6]. A number of investigations into the stability of *Hb*HNL showed that it is stable in the pH range 5.0–7.0, but its stability is highly dependent on the type and concentration of buffer, on the presence of solvents, and on the reactants [7–10]. A new study devoted to the immobilization of *Hb*HNL demonstrated its successful use in a variety of organic media [11]. Moreover, the structure of this dimeric, unglycosylated enzyme has been elucidated by X-ray studies [12]. These, together with electrospray ionization mass spectrometry investigations [10], indicate a mechanism similar to the chemical reaction and to the mechanism of the hydroxynitrile lyase from *Manihot esculenta* [13].

In order to avoid the use of toxic and potentially explosive HCN acetone cyanohydrin (**1**), the natural substrate of *Hb*HNL, can be used as the cyanide source [3,14–16]. This *trans*-cyanohydrin reaction (transcyanation) proceeds in two steps [10]. First, **1** is split into acetone (**2**) and HCN, then the carbonyl compound reacts enantio-selectively with the HCN,

* Corresponding author. Current address: Laboratory for Applied Organic Chemistry and Catalysis, DelftChemTech, Delft University of Technology, Julianalaan 136, 2628 BL Delft, Netherlands. Tel.: +31-15-278-9304; fax: +31-15-278-4289.

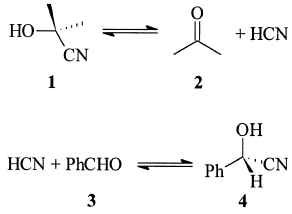
E-mail address: u.hanefeld@tnw.tudelft.nl (U. Hanefeld).

1381-1177/01/$ - see front matter
PII: S1381-1177(00)00061-8

again catalyzed by the enzyme. There is no enzyme HCN complex and it is the carbonyl compound that enters into the active site first, followed by the HCN [17]. In these studies [10], benzaldehyde (**3**) was used as the carbonyl compound (Scheme 1). In order to suppress the racemic chemical reaction that is base-catalyzed, a low pH value (3.75) was used. In this manner, it was possible to observe only the enzyme-catalyzed reaction and to avoid the formation of (*R*)-mandelonitrile.

For a successful application of the *trans*-cyanohydrin reaction, it is essential that the equilibrium lies on the product side. The earlier measurement [10] of the separate K_{eq}'s of the separate reactions, which is the conversion of **1** into HCN and acetone and its reversal, as well as the reaction of **3** with HCN to give (*S*)-mandelonitrile (**4**), gave the following results: $K_{eq1} = [\mathbf{2}][\mathrm{HCN}]/[\mathbf{1}] = 45.0$ mM and $K_{eq2} = [\mathbf{3}][\mathrm{HCN}]/[\mathbf{4}] = 5.6$ mM. These results already indicate that the overall reaction sequence will not give a complete conversion of one equivalent of **1** into one equivalent of **4**. In order to confirm this, the influence of the ratio of **1** to **3** needs to be investigated.

As a second line of investigation, it was thought of coupling the *trans*-cyanohydrin reaction with another, if possible irreversible, reaction. In this manner, the overall reaction equilibrium should be shifted towards the product side. One possibility would be to form a derivative of the alcohol group of the product. **4** is a secondary alcohol, which could easily be converted into an ester, while **1** is an unreactive tertiary alcohol. A lipase should be the suitable catalyst for this reaction. Indeed, there is precedence for the acylation of cyanohydrins catalyzed by lipases [18–24]. When an enol ester such as the *iso*-propenyl acetate is used, the acylation is almost irreversible. Furthermore, the released **2** is already a constituent of the reaction mixture, so that neither the number of compounds to which the enzymes is exposed is increased unnecessarily nor is the product recovery complicated by additional compounds. As mentioned above, immobilized *Hb*HNL can be used in organic solvents for the reaction of HCN with carbonyl compounds. Therefore, studies towards this three-step conversion (Scheme 3) were undertaken.



Scheme 1. The *trans*-cyanohydrin reaction proceeds in two steps.

In this context, it is important that *Hb*HNL is an α/β hydrolase fold enzyme [12]. It is closely related to hydrolases, carboxypeptidases, lipases and thioesterases. Indeed, it does contain a potential catalytic triad consisting of Ser80, Asp207 and His235. These amino acids are correctly positioned in space [12] and might, therefore, cause hydrolytic activity. Before any acylation of the product cyanohydrin by a lipase could be investigated, it had therefore to be established whether *Hb*HNL would interfere or itself catalyze this reaction.

2. Materials and methods

2.1. Chemicals and enzymes

Purified *rac*-mandelonitrile was a gift from DSM-Chemie Linz and contained less than 0.5% benzaldehyde. Benzaldehyde, acetone cyanohydrin and *iso*-propenyl acetate were distilled prior to use and stored under nitrogen. All solvents were of p.a. quality. They were dried over molecular sieves, degassed and stored under nitrogen. Buffers were also degassed and stored under nitrogen. *Rac*-mandelonitrile acetate was synthesized from the commercially available *rac*-mandelonitrile according to Ref. [21]. All other chemicals were of p.a. quality. Celite R 633 (World Minerals, Santa Barbara, USA) was used for the enzyme immobilization. The immobilization of *Hb*HNL was performed according to Ref. [11].

The *Hb*HNL (5100 U/ml; 53 mg/ml) and the *Candida antarctica* lipase B (CAL-B; chirazyme L-2, c.-f.,C2,lyo, equivalent to Novozym 435) were made available by Roche Diagnostics (Penzberg, Germany). *Pseudomonas fluorescens* lipase (PFL) was purchased from Biocatalysts. *Porcine pancreas* lipase (PPL; Sigma type II) and *C. rugosa* lipase

(CRL; Sigma type VII) were purchased from Sigma (USA). Amano lipase PS (Amano PS = *P. cepacia* lipase) and Amano lipase 20 (Amano 20) were purchased from Amano Pharmaceutical (Japan).

2.2. Reaction assay and HPLC analysis

Analysis of the reaction mixtures, determination of the degree of conversion and determination of the enantiomeric excess (*ee*) were performed by HPLC (Waters Alliance with a diode array detector), all within one run. A CHIRACEL OB-H 0.46 cm ∅ × 25 cm column was used. Samples were filtered, and if necessary, diluted with the eluent. 90% and 10% *iso*-propanol were used as eluents and with a flow rate of 1 ml/min, the retention times were: *iso*-propenyl acetate 4.7 min, acetone 5.6 min, benzaldehyde 5.9 min, (*R*)-mandelonitrile 8.5 min, (*S*)-mandelonitrile 9.0 min, (*S*)-mandelonitrile acetate (**7**) 9.9 min, and (*R*)-mandelonitrile acetate 14.4 min.

2.3. Methods

2.3.1. Test for hydrolase activity of HbHNL

100 mg (0.57 mmol) rac-mandelonitrile acetate (**5**) were added to 25 ml degassed sodium citrate buffer (50 mM, pH = 5.5) containing 2650 U *Hb*HNL. The mixture and a blank reaction were stirred at 25°C. No conversion was observed in either case.

2.3.2. Test of the trans-cyanohydrin reaction in organic solvents

531 mg (5 mmol) **3** and 435 mg (5 mmol) **1** were dissolved in A: 25 ml hexane/*tert*-butyl methyl ether (3:1), B: 25 ml *tert*-butyl methyl ether or in C: 25 ml toluene. Then, 100 μl (510 U) *Hb*HNL in phosphate/citrate buffer (25 mmol/l, pH = 5.5) were added. The mixtures were stirred at RT. A: After 18.75 h, 64% **4** (*ee* = 99%) were formed; B: after 18.5 h, 29% **4** (*ee* = 97.3%) were formed; C: after 25.5 h, 42% **4** (*ee* = 96%) were formed.

2.3.3. Test for the dependence of immobilized HbHNL [11] on the water activity

531 mg (5 mmol) **3** and 435 mg (5 mmol) **1** were dissolved in A: 25 ml toluene dried over molecular sieves containing 100 mg (40 U) *Hb*HNL on Celite R 633; B: 25 ml toluene stored over $Na_2SO_4/Na_2SO_4 \times 10H_2O$ containing 80 mg (32 U) *Hb*HNL on Celite R 633. C: To 250 mg (100 U) *Hb*HNL on Celite R 633, 150 mg Na_2SO_4 and 100 mg $Na_2SO_4 \times 10\ H_2O$ 10 ml toluene and then 266 mg (2.5 mmol) **3** and 212 mg (2.5 mmol) **1** were added. The mixtures were stirred at RT. A and B: no reaction occurred. C: after 18 h, 41% **4** (*ee* = 94%) were formed.

2.3.4. Screening of the lipases

To A: 140 mg CAL-B, B: 60 mg Amano PS, C: 77 mg Amano 20, D: 440 mg PPL, E: 50 mg PFL or F: 118 mg CRL, 10 ml toluene, then, 100 mg (0.57 mmol) **5** and 50 μl methanol (1.20 mmol) were added. The mixtures were stirred at RT. After 17 h, only the reaction A (CAL-B) was complete: **4** (*ee* = 99%) was formed and **6** (*ee* = 99%) remained unhydrolyzed. For the other lipases, the following conversions were measured after 115 h: B (Amano PS): 14% **4** (*ee* = 99%), 36% **7** and 50% **6**; C (Amano 20): 36% **4** (*ee* = 98%), 14% **7** and 50% **6**; D (PPL): 5% **4** (*ee* = 70%), 46% **7** and 49% **6**; E (PFL): 10% **4** (*ee* = 99%), 40% **7** and 50% **6**; and F (CRL): 27% (*R*)-mandelonitrile (*ee* = 99%), 50% **7** and 23% **6**.

2.3.5. CAL-B catalyzed acylation of rac-mandelonitrile

To 100 mg CAL-B (dried in vacuo) in 10 ml toluene, 133 mg (1 mmol) *rac*-mandelonitrile and 250 mg (2.5 mmol) *iso*-propenyl acetate were added. The reaction was stirred at RT. After 14 days, the conversion was complete: 50% **7** (*ee* = 93%) were formed and 50% (*R*)-mandelonitrile (*ee* = 95%) remained.

2.3.6. Trans-cyanohydrin reaction in the presence of acetic acid

266 mg (2.5 mmol) **3** and 212 mg (2.5 mmol) **1** were dissolved in 10 ml toluene. 30 mg (0.5 mmol) acetic acid and 200 μl (510 U) *Hb*HNL in phosphate/citrate buffer (25 mmol/l, pH = 5.5) were added. The *Hb*HNL precipitated and no reaction occurred.

*2.3.7. Delayed addition of iso-propenyl acetate to **1**, **3**, CAL-B and HbHNL*

To 15 mg Na_2SO_4, 20 mg $Na_2SO_4 \times 10H_2O$, 150 mg immobilized *Hb*HNL (60 U) on Celite R 633 and 75 mg CAL-B 12.5 ml toluene were added. Then, 266 mg (2.5 mmol) **3** and 212 mg (2.5 mmol) **1** were added and the mixture was stirred at RT. After 3.5 h, 250 mg (2.5 mmol) *iso*-propenyl acetate were added. After 22 h, the reaction mixture contained 84.5% **3**, 10.8% **4** and 4.7% **7**. This is equivalent to 30% conversion of **4** to **7**. After 28 h, the amount of **3** was unchanged, but 36% **4** were converted to **7** to give a final concentration of 84.5% **3**, 9.9% **4** and 5.6% **7**.

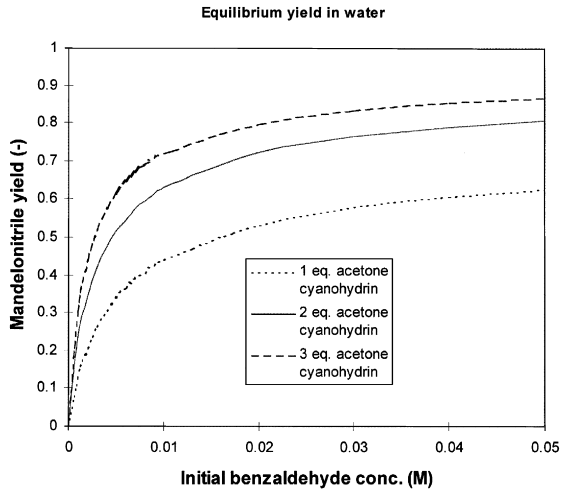


Fig. 1. Calculated equilibrium yield of the *trans*-cyanohydrin reaction, using 1, 2 or 3 equivalents of **1**.

3. Results and discussion

The equilibrium position of the transcyanation was calculated from the two reaction equilibrium equations (see Section 1) and the molar balances for the conserved parts of the reacting compounds:

2-propyl balance: $n_{1,0} = n_1 + n_2$
cyano balance: $n_{1,0} = n_1 + n_4 + n_{HCN}$
benzyl balance: $n_{3,0} = n_3 + n_4$

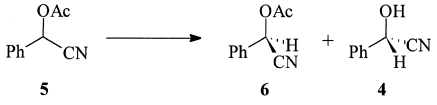
In these equations, n_x is the number of moles of compound **X** and the subscript 0 indicates the initial condition. When the initial amounts are known and the aqueous reaction volume is given, the five unknown concentrations can be found by simultaneously solving the five equations. The equilibrium yield was calculated by dividing the equilibrium amount of **4** by the initial amount of **3**. The results indicate (Fig. 1) that it is impossible to reach a conversion of more than 60% when one equivalent of **1** is used per equivalent of **3**. Even when three equivalents of **1** are used, the maximum yield of the target compound is below 90%, whilst all the advantages of using **1** instead of HCN are lost. The reaction mixture will contain significant amounts of HCN, **1**, **2** and **3**, complicating the product recovery and generating significant amounts of toxic waste. It has to be noted that, since the K_{eq} of the racemic reaction is half the K_{eq} of the enantio-selective reaction, the equilibrium yields would be higher if the undesired *R*-enantiomer had been formed, too.

These results led to the investigation of potential follow-up reactions that would irreversibly shift the equilibrium of the *trans*-cyanohydrin reaction towards a stabilized product. For the reasons mentioned in Section 1, a lipase-catalyzed acylation with *iso*-propenyl acetate was chosen. Thus, it was investigated whether the *trans*-cyanohydrin reaction also works in organic solvents. Indeed, *Hb*HNL does catalyze this reaction. When comparing the activity of aqueous *Hb*HNL solutions (even for immobilized *Hb*HNL, at least 1% water was recommended by Ref. [11]), hexane/*tert*-butyl methyl ether (3:1), toluene and *tert*-butyl methyl ether as solvents, the reaction proceeded the fastest in hexane/*tert*-butyl methyl ether while the pure ether was less suitable for the reaction than toluene. In line with Ref. [11], the immobilized enzyme was found to be inactive in dry solvents, while it was still active in the presence of $Na_2SO_4/Na_2SO_4 \times 10H_2O$. Since *Hb*HNL is an α/β hydrolase fold enzyme, it was tested whether it could catalyze the hydrolysis of **5**. No reaction was observed under ideal conditions for the enzyme. A possible esterase activity could, therefore, also be excluded.

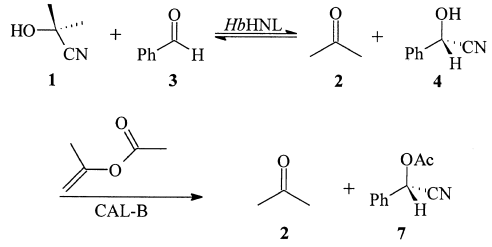
Several lipases are known to catalyze the acylation of cyanohydrins. Since the reaction conditions were not always comparable, PPL, PFL, CRL,

Amano PS and Amano 20 were tested under identical conditions (Scheme 2). CAL-B, which has, to the best of the authors' knowledge, never been used for the acylation of cyanohydrins, was also included into this screening. In this approach, it is important that the lipase accepts **4**; the selectivity, however, can be low since *Hb*HNL provides the enantio-selectivity. Except for CRL, all the other lipases showed the selectivity predicted by Kazlauskas et al.'s rule [25]. After 17 h, the enantio-selective hydrolysis of **5** was only complete (50% conversion of the racemate) when CAL-B was used. Indeed, the *ee* of the product **4** and of the remaining (*R*)-mandelonitrile acetate were 99%. The selectivity of CAL-B is significantly higher ($E = 1060$, according to a calculation from the *ee* values [26]) than that of the other lipases used for this type of reaction to date. Even for the acylation of *rac*-mandelonitrile with *iso*-propenyl acetate, the selectivity of CAL-B was still high ($E = 100$), yielding 50% **7** (*ee* = 93%). The other lipases did not give satisfactory results. CAL-B was, therefore, the lipase of choice for the combined reactions (Scheme 3).

With these results at hand, it was attempted to combine both reactions. *Hb*HNL and CAL-B are both active under compatible conditions and with toluene as solvent, it was hoped to achieve the overall reaction. Whether a solvent is particularly suitable for *Hb*HNL depends to a large degree on the amount of HCN present during the reaction [11]. With the coupled reactions, only extremely low concentrations of HCN were expected. However, all initial experiments failed. Since neither the presence of CAL-B nor of *iso*-propenyl acetate suppressed the *trans*-cyanohydrin reaction, a different reason had to be at the heart of this unexpected failure. Indeed, it was recently reported that even under 'dry' conditions, the hydrolysis of vinyl acetate is faster than the CAL-B catalyzed formation of an ester [27]. Since the salt pair used in the experiments described here does constitute a relevant amount of water, this type



Scheme 2. Test reaction for the screening of lipases.



Scheme 3. Proposed one pot three-step enzyme-catalyzed synthesis of (*S*)-mandelonitrile acetate **7**.

of hydrolysis could not be ruled out. The thus liberated acetic acid is known to deactivate *Hb*HNL. To test this hypothesis, the *trans*-cyanohydrin reaction was performed in the presence of an amount of acetic acid equivalent to the hydrolysis of 20% of the *iso*-propenyl acetate. No reaction occurred and the *Hb*HNL precipitated. In another attempt to combine both reactions, the *iso*-propenyl acetate was only added after some **4** had already formed. No more **4** was formed but it was transformed into the target compound **7**.

4. Conclusions

The equilibrium situation for the *Hb*HNL catalyzed *trans*-cyanohydrin reaction is not favorable. Unless a large excess of **1** is used, the conversions are not very high. A large excess of **1**, however, would also mean loss of the advantages of using it as a cyanide donor. To shift the equilibrium, the reaction therefore needs to be coupled with an irreversible third step such as an acylation with *iso*-propenyl acetate. Conditions were therefore established to perform the *trans*-cyanohydrin reaction in organic solvents. Since *Hb*HNL showed no hydrolase/esterase activity, several lipases were screened. This is the first report of the use of CAL-B for this reaction ($E = 100$). However, the water activity necessary for the activity of *Hb*HNL caused the formation of acetic acid that deactivated the *Hb*HNL. Investigations into alternative reaction conditions, which neutralize the acid, and alternative acylation agents, which do not release deactivating acids upon hydrolysis, are in progress. Furthermore, the high enantioselectivity of CAL-B shows the potential of combin-

ing the enzymatic esterification with the chemical in-situ formation of *rac*-mandelonitrile, a dynamic kinetic resolution [23,24].

Acknowledgements

We thank Roche Diagnostics Penzberg (W. Tischer) for providing the *Hb*HNL and the CAL-B, DSM-Chemie Linz (P. Pöchlauer) for providing pure mandelonitrile and the EU for generous financial support (BIO4 CT960112).

References

[1] M. Schmidt, H. Griengl, in: W.D. Fessner (Ed.), Top. Curr. Chem. 200, 1999, pp. 193–226.
[2] H. Griengl, A. Hickel, D.V. Johnson, C. Kratky, M. Schmidt, H. Schwab, Chem. Commun. (1997) 1933–1940.
[3] N. Klempier, H. Griengl, M. Hayn, Tetrahedron Lett. 34 (1993) 4769–4772.
[4] M. Hasslacher, M. Schall, M. Hayn, R. Bona, K. Rumbold, J. Lückl, H. Griengl, S.D. Kohlwein, H. Schwab, Protein Expression Purif. 11 (1997) 61–71.
[5] H. Griengl, N. Klempier, P. Pöchlauer, M. Schmidt, N. Shi, A.A. Zabelinskaja-Mackova, Tetrahedron 54 (1998) 14477–14486.
[6] P. Pöchlauer, Chim. Oggi 16 (1998) 15–19.
[7] A. Hickel, M. Graupner, D. Lehner, A. Hermetter, O. Glatter, H. Griengl, Enzyme Microb. Technol. 21 (1997) 361–366.
[8] M. Bauer, R. Geyer, M. Boy, H. Griengl, W. Steiner, J. Mol. Catal. B: Enzym. 5 (1998) 343–347.
[9] M. Bauer, H. Griengl, W. Steiner, Enzyme Microb. Technol. 24 (1999) 514–522.
[10] U. Hanefeld, A.J.J. Straathof, J.J. Heijnen, Biochim. Biophys. Acta 1432 (1999) 185–193.
[11] D. Costes, E. Wehtje, P. Adlercreutz, Enzyme Microb. Technol. 25 (1999) 384–391.
[12] U.G. Wagner, M. Hasslacher, H. Griengl, H. Schwab, C. Kratky, Structure 4 (1996) 811–822.
[13] H. Wajant, K. Pfizenmaier, J. Biol. Chem. 271 (1996) 25830–25834.
[14] V.I. Ognyanov, V.K. Datcheva, K.S. Kyler, J. Am. Chem. Soc. 113 (1991) 6992–6996.
[15] T.T. Huuhtanen, L.T. Kanerva, Tetrahedron: Asymmetry 3 (1992) 1223–1226.
[16] B. Danieli, S. Frattini, G. Roda, G. Carrea, S. Riva, J. Mol. Catal. B: Enzym. 5 (1998) 223–228.
[17] M. Bauer, H. Griengl, W. Steiner, Biotechnol. Bioeng. 62 (1999) 20–29.
[18] T. Sakai, Y. Miki, M. Nakatani, T. Ema, K. Uneyama, M. Utaka, Tetrahedron Lett. 39 (1998) 5233–5236.
[19] K. Lundell, T. Raijola, L.T. Kanerva, Enzyme Microb. Technol. 22 (1998) 86–93.
[20] J. Roos, U. Stelzer, F. Effenberger, Tetrahedron: Asymmetry 9 (1998) 1043–1049.
[21] F. Effenberger, B. Gutterer, T. Ziegler, E. Eckhardt, R. Aichholz, Liebigs Ann. Chem. (1991) 47–54.
[22] A. Fishman, M. Zviely, Tetrahedron: Asymmetry 9 (1998) 107–118.
[23] L.T. Kanerva, K. Rahiala, O. Sundholm, Biocatalysis 10 (1994) 169–180.
[24] M. Inagaki, J. Hiratake, T. Nishioka, J. Oda, J. Org. Chem. 57 (1992) 5643–5649.
[25] R.J. Kazlauskas, A.N.E. Weissfloch, A.T. Rappaport, L.A. Cuccia, J. Org. Chem. 56 (1991) 2656–2665.
[26] J.L.L. Rakels, A.J.J. Straathof, J.J. Heijnen, Enzyme Microb. Technol. 15 (1993) 1051–1056.
[27] H.K. Weber, H. Weber, R.J. Kazlauskas, Tetrahedron: Asymmetry 10 (1999) 2635–2638.