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Chemo-enzymatic synthesis of (*S*)-α-cyano-3-phenoxybenzyl alcohol

Ayelet Fishman * and Michael Zviely †

IMI (TAMI) Institute for Research and Development Ltd, PO Box 10140, Haifa 26111, Israel

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Abstract

A chemo-enzymatic process for the preparation of (*S*)-α-cyano-3-phenoxybenzyl alcohol (*S*-CPBA), an important intermediate in the synthesis of many pyrethroids, was developed. The process consists of four stages, including lipase-mediated resolution. The first stage, the synthesis of racemic α-cyano-3-phenoxybenzyl acetate (CPBAc) from *m*-phenoxybenzaldehyde (*m*-PBA) and sodium cyanide in the presence of a phase transfer catalyst, resulted in a 75% yield with 95% purity. The second key step is the resolution of the racemic ester by a highly enantioselective lipase from *Pseudomonas* sp. The immobilized enzyme carried out the transesterification reaction to nearly full conversion (46% out of 50%) with an enantiomeric excess of >96%. The enzymatic reaction was accomplished in a batch system as well as in a fluidized bed column. The reaction was found to be inhibited by accumulation of the product and to a lesser extent, by the aldehyde. The separation of the enantiomerically pure alcohol from the undesired ester was performed by chromatographic techniques, as well as by extraction with hexane. The final racemization step of the (*R*)-ester was easily attained with the use of triethylamine in diisopropyl ether or toluene. The process was shown to be feasible on a gram scale and shows potential for scale up.

Pyrethrin, extracted from plants, has been known and used as an insecticide since early in the twentieth century. The rapid knock-down of insects, high level of activity and considerable degree of safety to mammals exhibited by the natural product, led to intense interest in synthesizing alternatives.[1] Many of the present commercially important pyrethrin analogues are esters of (*S*)-α-cyano-3-phenoxybenzyl alcohol (*S*-CPBA).[2,3] These products include Deltamethrin (Hoechst) and Esfenvalerate (Sumitomo Chemicals).

* Corresponding author. E-mail: ayelet@tami-imi.co.il

† Current address: FRUTAROM Ltd, PO Box 10067, Haifa 26110, Israel.

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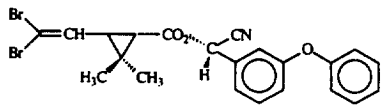
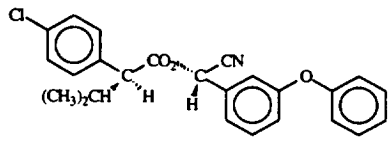
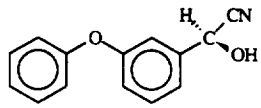


Deltamethrin



Esfenvalerate

The acid moiety in numerous pyrethroids is a derivative of chrysanthemic acid, whereas the alcohol moiety is a derivative of cyanohydrins. This work focuses on a route for the preparation of optically active cyanohydrins. The biologically active enantiomer is the (*S*) configuration.



(S)-α-cyano-3-phenoxybenzyl alcohol

Many stereoselective chemical methods for the preparation of chiral cyanohydrins are described in the literature, among them are the addition of trimethylsilyl cyanide and related cyanide-transfer agents to chiral aldehydes and the enantioselective addition of trimethylsilyl cyanide to aldehydes in the presence of chiral catalysts.[4,5] Cyclic dipeptides also catalyze the enantioselective addition of HCN to aldehydes.[6] Most of these methods are very complicated and require expensive chiral organic reagents. Enzymatic procedures leading to optically active cyanohydrins include the oxynitrilase-catalyzed enantioselective addition of HCN to aldehydes[7] and the enantioselective hydrolysis or transesterification of racemic cyanohydrin esters with lipases.[8,9] The main advantages of the enzymatic methods are the high enantioselectivity and conversions achieved and the ease of scale-up for the preparation of cyanohydrins in large quantities.

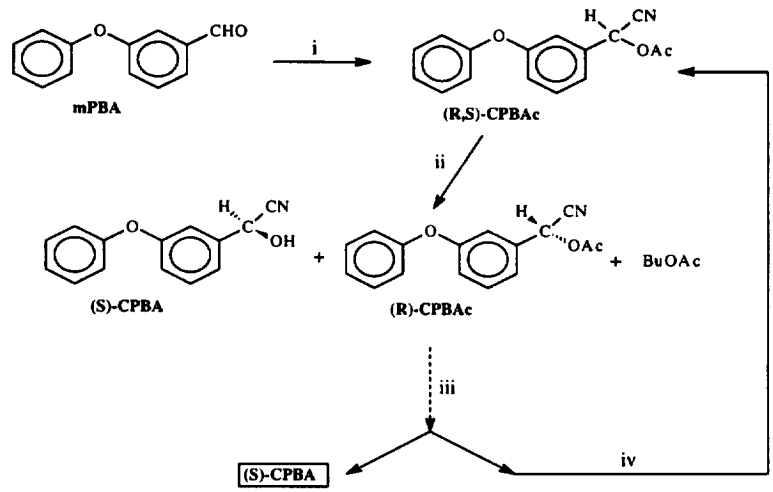
Mitsuda and co-workers[9] studied the hydrolysis of α-cyano-3-phenoxybenzyl acetate (CPBAc) in aqueous medium with *Arthrobacter* lipase. However, the immobilized enzyme had insufficient activity and low operational stability for repeated batch reactions. The main reason for the decrease in activity was the desorption of the enzyme from the support. As reported by Ohta et al.,[10] aqueous medium also facilitates the degradation of the product to the corresponding aldehyde.

The aim of this work was to design a practical process with industrial potential for the preparation of (*S*)-α-cyano-3-phenoxybenzyl alcohol using enzymatic resolution. Transesterification in an organic medium was the method of choice in order to minimize the decomposition and racemization of the product. In this paper, we report details of the enzymatic reaction including continuous use of the immobilized enzyme in a fluidized bed column. This work also describes a feasible, simple method for the purification of (*S*)-CPBA by extraction with hexane.

1. Results and discussion

The chemo-enzymatic process (Scheme 1) consists of four stages with *m*-phenoxy-benzaldehyde (*m*-PBA) as the starting material.

The first stage is the synthesis of racemic α-cyano-3-phenoxybenzyl acetate (CPBAc) from *m*-phenoxybenzaldehyde, sodium cyanide and acetic anhydride in the presence of a phase transfer catalyst. The second key step is resolution of the racemic ester by a highly enantioselective lipase from *Pseudomonas* sp. The enzymatic reaction was performed in a batch reactor as well as in a continuous fluidized bed column. The separation of the optically pure alcohol from the undesired ester was performed



Scheme 1. Reagents and conditions: (i) NaCN, Ac_2O, benzyltriethylammonium chloride, CH_2Cl_2/H_2O, 2–10°C; (ii) *Pseudomonas* sp. lipase, BuOH, diisopropyl ether, 35°C; (iii) separation by n-hexane extraction; (iv) racemization, Et_3N, diisopropyl ether, 65°C

by extraction with hexane, or by chromatographic techniques. The final racemization step of the (*R*)-ester was easily achieved with the use of triethylamine in diisopropyl ether or toluene

1.1. Synthesis of racemic CPBAc

m-PBA was reacted with sodium cyanide (1.4 molar ratio) and acetic anhydride. The reaction was performed in a water/dichloromethane medium in the presence of benzyltriethylammonium chloride, at a low temperature. The ester was formed in a yield of 80% with 95% purity.

1.2. Enzymatic resolution

Lipases from various *Pseudomonas* species were found to be highly selective towards (*S*)-CPBA. This is supported by the work of other groups (Effenberger,[7] Mitsuda[9]). CHIRAZYME® L-6 (crude lipase from *Pseudomonas* sp., Boehringer Mannheim) was generally used in this work due to its high reactivity and selectivity (>95% enantiomeric excess at full conversion). In our earlier work[11] butanol was found to be the preferred alcohol for the reaction and diisopropyl ether the most suitable solvent. In order to verify the structure and the stereochemistry of (*S*)-CPBA, the product was isolated and analyzed. Various factors affecting the transesterification reaction, such as temperature, concentration, water content and long-term enzyme stability, were evaluated in order to establish the feasibility of (*S*)-CPBA production.

1.3. Influence of temperature

The temperature can have a contradictory effect on the enzymatic process. On the one hand, the rate is increased with the rise in temperature. On the other hand, the stablility of the enzyme can be reduced in the long term and the product CPBA can decompose to HCN and *m*-PBA. The results presented in Table 1 confirm this assumption. At 45°C the enantioselectivity decreased and the formation of *m*-PBA was accelerated. The best compromise would be therefore to work at 38°C for a few hours (possibly

Table 1
Effects of temperature on transesterification of CPBAc with *Pseudomonas* sp. lipase[a]

	20°C		28°C		38°C		45°C	
composition (mol %)[b]	**5 hr**	**24 hr**	**5 hr**	**24 hr**	**5 hr**	**24 hr**	**5 hr**	**24 hr**
mPBA	6.0	7.2	5.7	4.3	*6.1*	14.4	7.6	11.1
CPBA	32.8	44.9	39.3	47.8	*45.8*	36.5	42.3	40.0
CPBAc	61.2	47.9	55.0	47.9	*48.1*	49.1	50.1	48.9
ee (%)[c]	97	97	96	95	*95*	95	92	87

a) Concentrations of CPBAc and immobilized enzyme in the reaction mixture were 5% and 2% (w/v) respectively. b) Measured according to NMR analyses with 1,1,2,2-tetrachloroethane as internal standard. c) of CPBA determined by chiral HPLC.

Table 2
Influence of water on the enzymatic reaction[a]

% water added	% CPBA[b]	% mPBA[b]	% CPBAc[b]
none	18.5	2.8	78.7
0.2	34.6	5.5	59.9
0.5	25	21.2	53.8
1	18	28.5	53.5
2	2.3	35.2	62.5

a) Concentration of substrate and immobilized enzyme in the reaction mixture were 5% and 2% (w/v) respectively. b) Measured by HPLC after 6 hours of reaction at 28°C.

less than 5) to retain both high activity and selectivity. Similar results were reported by Mitsuda et al.[9] regarding the hydrolysis of CPBAc with *Arthrobacter* lipase.

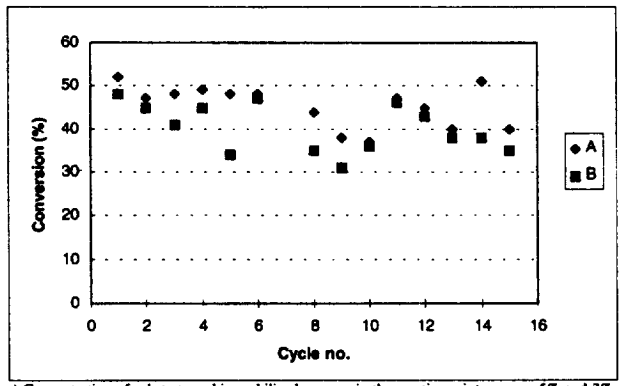
1.4. Influence of water

Many studies indicate that enzyme structure and function is strongly dependent on bound water. The addition of water to the organic reaction system was found to increase enzymatic activity in many cases.[12–14] Water was added to the anhydrous diisopropyl ether (routinely dried by molecular sieve) and the contents of the reaction mixture were determined after six hours of reaction (Table 2).

From the results it is evident that the addition of up to 1% water accelerates the reaction (decrease in substrate CPBAc). However, the addition of water also rapidly contributes to the decomposition of the product to the aldehyde. An optimum of ~0.2% water is desired although it is difficult to monitor this amount when considering industrial operation. A small deviation could result in major decomposition of the desired product and therefore it would be more practical to use anhydrous diisopropyl ether for the reaction.

1.5. Concentration of substrates

From a commercial point of view, it is desired to work at high substrate concentrations in order to reduce the volume of solvent and consequently the size of the reactor required. Conversely, a minimum amount of solvent is needed to enable proper mixing and mass transfer of the substrate to the enzyme. The enzymatic transesterification reaction was carried out at three substrate concentrations, while maintaining the ratio of substrate to enzyme constant. At a concentration of 20% (w/v) the conversion after 6 and 24 hours of reaction was 26 and 37% respectively, compared to 17 and 25% at 5% concentration.



a) Concentration of substrate and immobilized enzyme in the reaction mixture were 5% and 2% (w/v) respectively. Conversion was calculated from GC analyses. A - enzyme was kept in pure diisopropyl ether between cycles; B - enzyme was kept as a wet pellet between cycles

Fig. 1. Successive batch reactions with immobilized lipase[a]

Table 3
Experimental conditions and results of the continuous experiments

Parameter	Successive batch	Upward flow
flow-rate (ml/h)	--	18 (constant)
amount of immobilized enzyme (g)	0.2	0.3
temperature	35°C	35°C
initial conversion[a]	48 %	33.5 %
amount of total substrate passed[b] (g)	7.5 (15 cycles)	40
amount (S)-CPBA produced (g)	2.8	11.6
yield[c] (g product/g net enzyme[d])	140	386

a) subject to flow rate and amount of enzyme in the column. b) concentration of feed solution was 10% (w/v). c) the minimal yield determined at the end of the experiment, although the enzyme was still active. d) excluding the ion-exchanger.

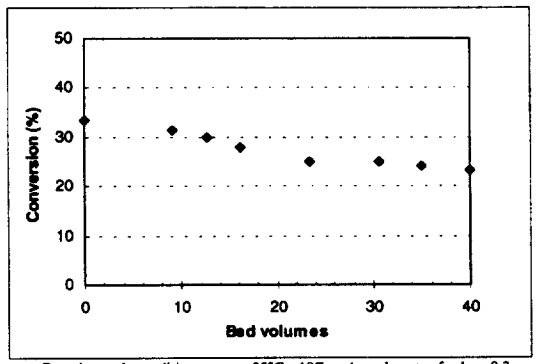
1.6. Successive batch reactions

CHIRAZYME® L-6 (immobilized on DEAE-Sephadex) was used in successive cycles of transesterification reactions in order to evaluate recycling. The conversion at the end of each three-hour cycle is presented in Fig. 1. In vessel A the enzyme was kept in diisopropyl ether over night between cycles, whereas in vessel B the enzyme was kept as a wet pellet. No significant differences were found between the two methods. The general trend following 15 cycles was a gradual decrease from 50 to 40% conversion.

1.7. Continuous system for preparation of S-CPBAc

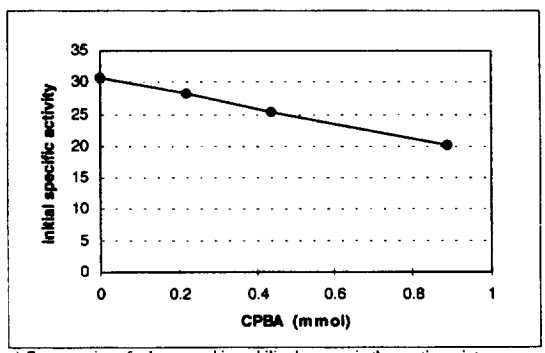
The immobilized enzyme was used in a continuous system similar to a fluidized bed reactor. The enzyme was confined in a jacketed column and maintained at 35°C. A peristaltic pump introduced the reactants (CPBAc, butanol, diisopropyl ether) into the column at the desired flow rate and the effluent liquid containing the product and the unreacted ester was collected in a fraction collector for analysis. The experimental conditions of the continuous experiments are summarized in Table 3. The progress of the reaction in terms of conversion is presented in Fig. 2.

The reasons for the decrease in conversion in the described experiments (and in the successive batch experiment as well) are assumed to be: (a) accumulation of an inhibitor present in the solution — either the aldehyde (present at a concentration of up to 5%) or the product itself; (b) natural denaturation of the enzyme with time; (c) physical loss of enzyme molecules due to the nature of the immobilized



a) Experimental conditions were 35°C, 10% w/v substrate feed, 0.3 g immobilized enzyme.

Fig. 2. Continuous production of of (*S*)-CPBA by immobilized lipase[a]



a) Concentration of substrate and immobilized enzyme in the reaction mixture were 5% and 2% (w/v) respectively. The initial specific activity (?mol/hr×mg CHIRAZYME® L-6) was calculated from GC analyses. The amount of CPBA is the initial quantity in the reaction.

Fig. 3. Product inhibition of the enzymatic reaction[a]

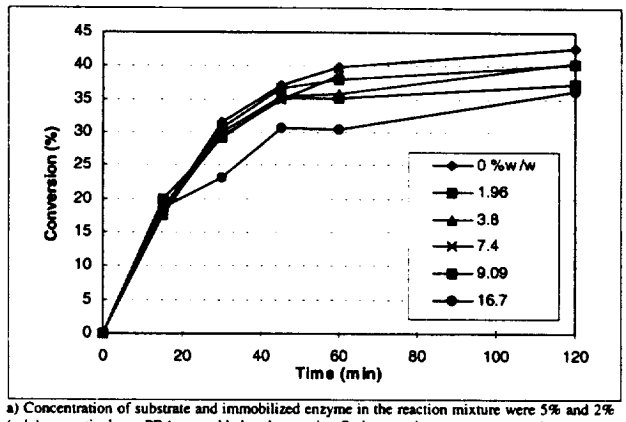
preparation. When the enzyme is immobilized by a non-covalent binding method, such as adsorption onto an ion-exchanger, there could be leakage of the enzyme with time.

1.8. Product inhibition

A preliminary experiment was performed to establish whether there is product inhibition in this system. Various amounts of CPBA were added to the standard reaction medium and the conversion was monitored. The initial specific activity of the reaction as a function of the initial amount of CPBA is presented in Fig. 3. 0.89 mmol CPBA is the maximum quantity that could be formed at full conversion. It is clearly evident that the presence of such an amount caused inhibition of the enzymes activity. The decrease in initial activity was proportional to the rise in initial amount of product present in the medium.

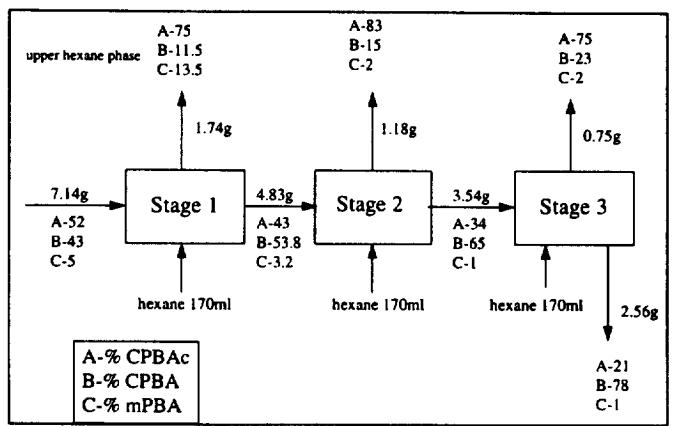
1.9. Inhibition by m-*PBA*

The possible inhibition by *m*-PBA was examined as well. Only when a large amount of aldehyde was present, (>10%w/w), was the reaction somewhat inhibited (Fig. 4). The usual amounts present in the beginning of the reaction are less then 5%. In the later stages of the reaction, if there is product degradation, the amount of aldehyde could reach 10% resulting in an inhibiting effect.



a) Concentration of substrate and immobilized enzyme in the reaction mixture were 5% and 2% (w/v) respectively. mPBA was added to the reaction flasks at various percentages (w/w).

Fig. 4. Inhibition of the enzymatic reaction by mPBA[a]



a) Experimental conditions are described in Experimental section.

Fig. 5. Separation procedure of CPBA by extraction with hexane[a]

1.10. Separation of the (S)-product from the undesired (R)-ester

The main problem related with optically active cyanohydrins is their instability.[5] Purification by means of distillation, crystalization or chromatography is accompanied by extensive degradation and racemization. Pure (*S*)-CPBA was obtained by column chromatography[9,15] however, degradation to *m*-PBA was noticed. Extraction with hexane was therefore used, based on the assumption that the distribution coefficients of the alcohol and the ester would be sufficiently different to enable separation. A mixture of CPBAc, *m*-PBA and CPBA was prepared, similar to the composition at the end of a typical enzymatic resolution. This mixture was extracted with cold hexane (0–4°C). Following removal of the upper hexane phase, the residue was again extracted with another portion of hexane. Three steps of extraction were carried out successively. Phases were analysed by NMR and the composition of each stream is given in Fig. 5.

Enrichment of the phases is clearly demonstrated by this method. The results were similar when the extraction was carried out at room temperature. It is important to note that the enantiomeric excess of the product in the final effluent was identical to the starting solution, indicating that there was no racemization during the processing.

The distribution of butyl acetate which is formed in the reaction, was also investigated. Most of it, as expected, was extracted into the upper organic phase (hexane). The preliminary experiments described above, show the feasibility of using extraction for purification of (*S*)-CPBA. However, many extraction steps may be needed (perhaps in a continuous mode of operation) for full separation.

1.11. Racemization of (R)-CPBAc

Racemization of the undesired (*R*)-CPBAc is crucial for process efficiency, because in the enzymatic resolution stage only 50% of the racemic substrate is converted to the optically active alcohol. The first racemization experiments were performed according to Mitsuda et al.[15] with triethylamine (10% molar solution) in toluene at 74°C. Partial racemization occured after 2 hours. When equimolar quantities of base and ester were used, full racemization was achieved at this time. Diisopropyl ether was used as a solvent in reflux (~65°C) and after 5–6 hours, the ester racemized completely. The racemate was subjected to enzymatic transesterification and the results were the same as with the untreated ester. The racemization was followed by optical rotation measurements until a value of 0 was reached. The best results were achieved with 1:1 molar ratio of base to ester, in a 13% (w/v) solution of diisopropyl ether or toluene.

2. Conclusions

Optically active (*S*)-α-cyano-3-phenoxybenzyl alcohol was successfully prepared in high enantiopurity (>95% ee) by a four-stage chemo-enzymatic process (Scheme 1). The process was shown to be feasible on a gram scale and shows potential for scale up. Two solvents, diisopropyl ether and hexane, could be used in the transesterification reaction, separation and racemization steps. *Pseudomonas* sp. lipase was immobilized onto DEAE-Sephadex and utilized in a continuous system based on a fluidized bed column. The enzyme retained its activity for ca. 40 bed volumes with only a slight gradual decrease towards the end of the experiment. Product inhibition was found to be a possible explanation for the loss of activity. Separation of the desired optically active alcohol from the undesired ester was accomplished by extraction with hexane; the hexane phase was evidently enriched with the ester, leaving the bottom phase enriched with the desired optically active product.

3. Experimental

3.1. General

^{1}H NMR analyses were conducted on a 200 MHz Bruker WP 200 SY spectrometer for 5% solutions in deuterochloroform. 1,1,2,2-Tetrachloroethane was used as an internal integration standard.

GC analyses were conducted on a Hewlett–Packard 5890 Series II Gas Chromatograph, using a 15 m capillary column (ID=0.25 mm) packed with Rtx-1. The temperature program was 100°C (1 min), 20°C min^{-1}, 260°C (5 min). Retention times were 5.1 and 6.7 min for *m*-PBA and CPBAc respectively. GC–MS analyses were performed on a Hewlett–Packard 5971A mass selective detector, in similar conditions to the GC analyses.

HPLC analysis were performed on a Hewlett–Packard 1050 Series instrument equipped with a Jasco UV-975 detector. The column was a Shandon Hypersil BDS C8 column (25 cm×4.6 mm) packed with

5 μm particles. Eluent composition: 60:40 acetonitrile:water; flow rate: 1 ml min^{-1}; detection: 230 nm. Samples for analysis were prepared in the following way: the solvent was evaporated from the mixture. 40 mg of mixture were dissolved in 25 ml acetonitrile and this solution was diluted 10 times before injection. Under these conditions the retention times were: CPBA 5.4 min; *m*-PBA 7.3 min; CPBAc 8.6 min.

Chiral HPLC analyses were performed on the same instrument with a Supelco LC-(*R*)-phenyl urea column (25 cm×4.6 mm), packed with 5 μm particles. Eluent composition: 97:3 hexane:isopropanol; flow rate: 1 ml min^{-1}; detection: 220 nm. Samples were diluted in diisopropyl ether. All solvents were of HPLC grade (Frutarom). Optical rotations were determined on a JASCO digital polarimeter DIP-370.

CHIRAZYME® L-6 (crude lipase from *Pseudomonas* sp.) was purchased from Boehringer Mannheim. Diisopropyl ether was purchased from Riedel de-Haen and was dried on 4 Å molecular sieve (Aldrich) prior to use. Triethyl amine was also purchased from Riedel de-Haen. Unless stated otherwise, all compounds were of analytical grade.

3.2. *Preparation of* (R,S)-*CPBAc*

Sodium cyanide (9.7 g, 0.198 mol), water (33 ml), benzyltriethylammonium chloride (1.65 g, 0.0073 mol) and methylene chloride (33 ml) were mixed together. Into the dropping funnel was introduced a solution of *m*-phenoxybenzaldehyde (BCL, 28.1 g, 0.142 mol), acetic anhydride (18.36 g, 0.18 mol) and methylene chloride (50 ml). The stirred reaction mixture was cooled to between 3 and 4°C using an ice bath and then the solution of *m*-PBA:Ac_2O:methylene chloride was added dropwise into the NaCN:$BzEt_3N^+Cl^-$:H_2O:methylene chloride cooled solution, at a rate which maintained the temperature between 2 and 10°C. The addition of *m*-PBA:Ac_2O:methylene chloride took ca. 7 hours. After the addition was completed, the reaction mixture was stirred at between 3 and 4°C for an additional 15 min. The ice bath was removed and the reaction mixture was stirred at ambient temperature overnight.

The phases were separated, the methylene chloride from the lower organic layer was removed in vacuo to obtain 35.1 g crude brown oily product. The product was analyzed by GC to give the following results: (*R,S*)-CPBAc 95.3%; *m*-phenoxybenzaldehyde 3.8%; *m*-phenoxyphenylpropane-1,2-dione 0.8%; *m*-phenoxyphenylbenzoin 0.01%.

The aqueous phase was neutralized using NaOCl (aq.). The organic phase was extracted using a solvent mixture of ethylacetate:petroleum ether (40–60°C) (1:9). The solvent weight was seven times that of the crude product. The upper phase contained the desired product. After evaporating in vacuo, its color was pale-yellow to dark orange: ^1H NMR ($CDCl_3$) δ 2.17 (3H, s), 6.36 (1H, s), 7.01–7.42 (9H, m). MS: m/z 267 (M, 36), 241 (29), 196 (100), 181 (43), 170, (37), 43 (38).

3.3. *Immobilization of lipase onto DEAE-Sephadex*

DEAE-Sephadex A-25 (5 g, Sigma) was mixed with 100 ml of 0.1 N NaOH solution and 20 ml distilled water for 3 hours at room temperature. The slurry was filtered (Whatman filter no. 41) and washed extensively (~170 ml water) until neutral pH was reached. The polymer was kept under refridgeration overnight. To the wet Sephadex, CHIRAZYME® L-6 (0.5 g) and 35 ml of distilled water were added. Reaction continued under magnetic stirring for 3 hours at room temperature (pH=8). The solids were filtered and washed with distilled water. The immobilized enzyme was lyophilized overnight (Heto CD-8 instrument). And the dried enzyme was stored in a dessicator (over silica gel) under cooling (4°C).

3.4. Enzymatic resolution of CPBAc

(*R*,*S*)-CPBAc (0.5 g, 1.88 mmol), butanol (87 μl, 0.94 mmol) and diisopropyl ether (10 ml) were mixed together. Lipase from *Pseudomonas* sp. (CHIRAZYME® L-6, 0.2 g physically immobilized onto Sephadex-DEAE) was added to the solution and the reaction mixture was placed in a shaking bath (Haake SWB 20) at 150 rpm, 35°C and examined periodically by GC and HPLC. The final reaction mixture contained: 46% (*S*)-CPBA, 6% *m*-PBA and 48% mostly (*R*)-CPBAc. Separation of the mixture was achieved by column chromatography using silica gel 40 (Merck) and n-hexane:ethyl acetate (96:4) as an eluent, at 10°C. (*S*)-CPBA: ^{1}H NMR ($CDCl_3$) δ 5.49 (1H, s), 7.01–7.40 (9H, m); $[\alpha]_D^{25}=-24.1$ (c 1, in $CHCl_3$), lit.[15] $[\alpha]_D^{25}=-24.8$ (c 1, in $CHCl_3$); MS (EI): m/z 198 (M^+-HCN); (*R*)-CPBAc: $[\alpha]_D^{25}=-7.02$ (c 1, in $CHCl_3$), lit.[15] $[\alpha]_D^{25}=-8.1$ (c 1, in $CHCl_3$); MS (EI): m/z 267 (M, 36), 241 (29), 196 (100), 181 (43), 170 (37), 43 (38). *m*-PBA: ^{1}H NMR ($CDCl_3$) δ 9.94 (1H, s), 7.01–7.40 (9H, m).

3.5. Influence of temperature on the enzymatic reaction

The experiment was carried out as described above. Each experiment was carried out at a different temperature: 20, 28, 38, and 45°C. The solvent was evaporated from the samples and they were analysed by ^{1}H NMR with 1,1,2,2-tetrachloroethane as an internal standard.

3.6. Influence of water on the transesterification reaction

The control flask contained the same compounds as described above. 20, 50, 100 and 200 μl distilled H_2O were added to the other Erlenmeyer flasks.

The flasks were placed in a shaking bath at 28°C. Samples of 40 μl were taken out periodically and analyzed by GC. At the end of the experiment the solvent was evaporated at 40°C and the remaining organic mixture was analyzed by HPLC.

3.7. Resolution at various substrate concentrations

The reaction mixture contained CPBAc (0.5 g), butanol (87 μl) and immobilized lipase (0.3 g). The amount of diisopropyl ether was 10, 5 and 2.5 ml. The flasks were placed in a shaking bath at 150 rpm and 35°C.

3.8. Successive batch reactions with immobilized lipase

The reaction was carried out in a 50 ml centrifuge glass tube containing (*R*,*S*)-CPBAc (0.5 g), butanol (87 μl), diisopropyl ether (10 ml) and CHIRAZYME® L-6 (0.2 g, immobilized onto DEAE-Sephadex). The tube was placed in a shaking bath at 150 rpm and 35°C. The conversion was calculated according to GC. At the end of each 3 h cycle, the enzyme was centrifuged (Eppendorf centrifuge 5403, 5000 rpm and 4°C) and the organic phase removed. The enzyme was then washed 2 to 3 times with 10 ml of diisopropyl ether until no product was detected. The enzyme was kept overnight in the refridgerator either as a wet pellet or in pure solvent. On the following day, a mixture of CPBAc, butanol and solvent was added and a new cycle began.

3.9. Continuous enzymatic reaction in a fluidized bed column

The inner dimensions of the glass column were approximately 10 mm diameter, 100 mm length, with a bed volume of 10 ml. It had a sinter at both ends to retain the enzyme. A peristaltic pump (Watson–Marlow type 101/R MkII) with Viton® tubings introduced the feed solution into the bottom of the jacketed column. The 10% (w/v) feed solution contained: (*R,S*)-CPBAc (20 g), butanol (3.5 ml), diisopropyl ether (200 ml). Flow rate was set at 18 ml h^{-1} and the temperature was 35°C. Immobilized CHIRAZYME® L-6 (0.3 g) was confined in the column.

3.10. Extraction with hexane

The extraction experiments were conducted in a 500 ml round bottom flask. The mixture contained 43, 5 and 52% of CPBA, *m*-PBA and CPBAc respectively (total 7.14 g) and n-hexane (170 ml). The compounds were vigorously mixed for 30 minutes with a magnetic stirrer and then left for phase separation. Following phase separation the solvent was evaporated and both fractions were analyzed by quantitative NMR with tetrachloroethane as an internal standard. To the lower phase an additional amount of 170 ml hexane was added and mixed as previously described. Following phase separation and solvent evaporation, a third extraction step was performed.

3.11. Racemization of (R)-CPBAc

(*R*)-CPBAc (1.5 g, 5.6 mmol) and triethyl amine (0.65 g, 6.4 mmol) were mixed in diisopropyl ether or toluene (12 ml) under reflux (65°C) for 6 hours. At the end of the reaction the solvents were evaporated and the optical rotation measured. The $[\alpha]_D^{25}$ (c 1, $CHCl_3$) changed from −5.0 to 0.0, indicating that full racemization had occured. The racemized ester was subjected to enzymatic transesterification with untreated CPBAc as a control. The kinetics and optical purity of the product were the same.

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