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Coprecipitation of *Pseudomonas fluorescens* Lipase with Hydrophobic Compounds As an Approach to Its Immobilization for Catalysis in Nonaqueous Media

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Abstract—The precipitation of *N*-cetylamine, *N*-cetylacetamide, hexadecane-1,2-diol, cetyl alcohol, and poly(butyl metacrylate) in acetone–water media in the presence of the lipase from *Pseudomonas fluorescens* was found to be accompanied by the coprecipitation of the enzyme. Within the lyophilized coprecipitates, the lipase exhibits a high catalytic activity and enantioselectivity in the reaction of (1*RS*)-phenylethanol acetylation with vinyl acetate in *t*-butyl methyl ether. In order of increasing lipase activity, the coprecipitates can be arranged in the series: cetyl alcohol, poly(butyl metacrylate), hexadecane-1,2-diol, *N*-cetylamine, and *N*-cetylacetamide, with the activity 2.5- to 19-fold exceeding the activity of the native enzyme. Immobilization of the lipase on solid supports, such as Celite 545 (physical sorption) and Eupergit C250L (covalent binding), in the presence of hexadecane-1,2-diol was found to increase the esterifying activity of the enzyme.

Key words: *cetyl alcohol, ester synthesis, hexadecane-1,2-diol, immobilization, N-cetylacetamide, poly(butyl metacrylate)*

INTRODUCTION

The enzyme-catalyzed enantioselective synthesis of organic compounds has recently become an intensively developed field of bioorganic and organic chemistry.[2] This is dictated by the necessity to have a wide assortment of chiral organic compounds of high optical purity, including some medicines and their components and various biochemical agents. Lipases (EC 3.1.1.3) occupy a special place in the series of hydrolytic enzymes used as catalysts for many transformations in organic chemistry, as they can be used both for the hydrolysis of lipids and esters and for the enantioselective esterification and transesterification in organic solvents or water–organic media [1–7]. Factors like strict stereospecificity and the high rate of the enzymatic reactions and the possibility to perform these processes under mild conditions favor the industrial use of lipases. However, in industry, only the use of immobilized enzymes is highly profitable, because this makes the separation from reaction systems and repeated use of the enzymes much easier.

Various approaches to lipase immobilization, such as physical sorption on hydrophobic adsorbents [8–10], inclusion into hydrophobic gels [11] and hydrophobic coprecipitates [12], and treatment of the enzymes with synthetic lipid-like agents [13], are thus studied intensively. Numerous immobilization methods ensure that we obtain catalysts with wide-ranging characteristics. For the preparations of lipases immobilized on a number of solid supports, the highest initial rate of alcohol esterification in a model reaction (6- to 7-fold higher than that for the nonimmobilized enzyme) was observed in the case of the enzyme adsorption on Celite 545 particles, whereas the operational stability (the enzyme activity after ten working cycles) was higher in the case of the enzyme covalent binding to the Eupergit C250L support [10].

We demonstrated previously that the incorporation of lipases into hydrophobic coprecipitates with HDD [12] increases significantly (more than tenfold) the catalytic enzyme activity in the enantioselective esterification. A marked increase in the enzymatic activity is also observed upon enzyme treatment with lipid-like reagents [13]. However, these immobilization methods are still poorly investigated. Hence, the study of the compounds structurally similar to HDD and also some polymers with similar solubilities and the balance of hydrophilic and hydrophobic properties is of interest.

In this study, we focused on lipase inclusion into coprecipitates with a number of hydrophobic compounds, developed approaches to the preparation of recoverable catalysts on the basis of the Celite 545 and

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[2] Abbreviations: AIBN, azoisobutyronitrile; CeAA, *N*-cetylacetamide; $CeNH_2$, *N*-cetylamine; CeOH, cetyl alcohol; HDD, hexadecane-1,2-diol; PBMA, poly(butyl metacrylate); and TBME, *t*-butyl methyl ether.

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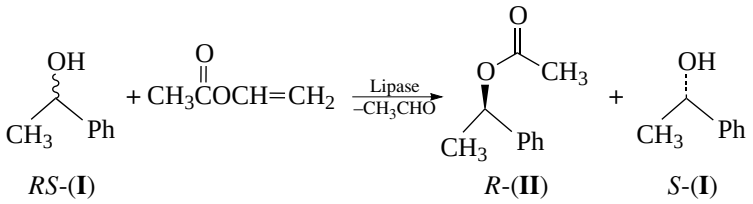

RS-(**I**) *R*-(**II**) *S*-(**I**)

The scheme of the enzymic esterification of (1*RS*)-phenylethanol with vinyl acetate.

Eupergit C250L solid particles, and suggested a modified procedure for measuring the rate of the model reaction: enantioselective acetylation of (1*RS*)-phenylethanol (*RS*-(**I**)) with vinyl acetate in the TBME medium (see scheme).

RESULTS AND DISCUSSION

The Composition of the Reaction Mixture in the Model Reaction

The pure product of enzymatic acetylation, (1*R*)-phenylethyl acetate (*R*-(**II**)) (scheme) was isolated by HPLC instead of gas chromatography, which was used previously [10]. Under standard conditions of rpHPLC (elution with 1 : 1 water–acetonitrile isocratic system), more hydrophobic compounds are retained stronger by the sorbent with octadecyl groups. As hydrophobicity increases in the order (**I**), vinyl acetate, and (**II**), the retention times for these compounds increase in a similar order and are 5.9, 6.2, and 13.5 min, respectively (Fig. 1). The content of the product (**II**) in the reaction mixture was determined from the square of its chromatographic peak in the optical density–time coordinates taking into account its known molar extinction coefficient ε_{260} (80 M^{-1} cm^{-1}). The correlation between the content of (**II**) measured by this method and determined by GC showed the difference to be less than 3%.

Preparation and Properties of the Lipase Coprecipitates with Hydrophobic Compounds

Coprecipitates of the lipase with hydrophobic compounds were obtained as described for the lipase coprecipitate with HDD [12]. The lipase coprecipitants with the same length of hydrophobic moiety (C_{16}), but varying in the terminal functional group, as well as PBMA, were used. The enzyme content in the coprecipitates was calculated from its residual activity in the supernatant:

$$\text{lipase incorporation into precipitate (\%)} = \left(1 - \frac{\text{lipase activity in supernatant}}{\text{starting lipase activity}}\right) \times 100.$$

The maximal enzyme inclusion (more than 99%) into the coprecipitates was observed upon coprecipitation with $CeNH_2$ (Table 1). This effect can be explained by the contribution of the electrostatic interaction between the positively charged $CeNH_2$ particles and the negatively charged lipase molecules. The efficiency of the lipase incorporation into the precipitates decreases in the series of coprecipitants: $CeNH_2$ –

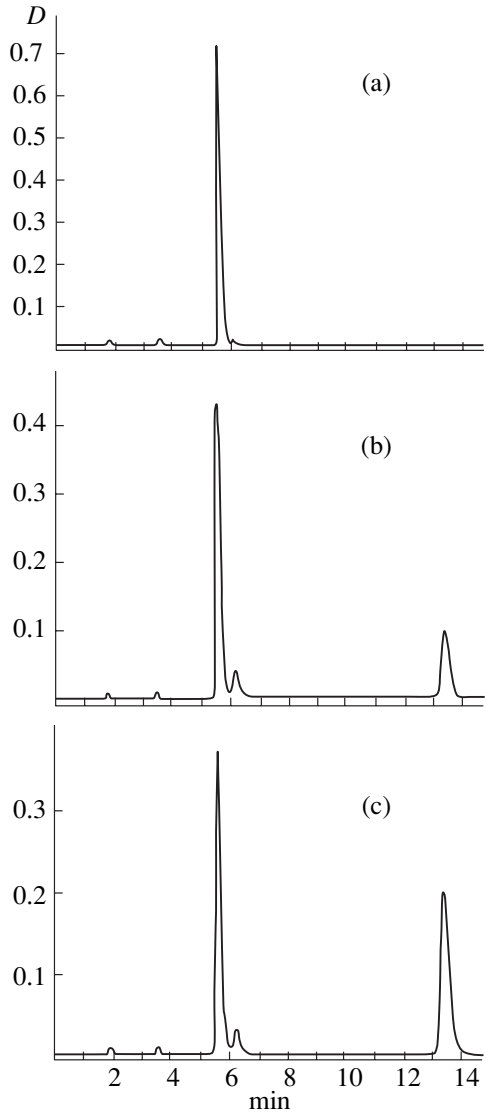

Fig. 1. The rpHPLC analysis of the reaction mixture upon the enantioselective acetylation of (1*RS*)-phenylethanol with vinyl acetate catalyzed by the native lipase. The reaction time: (a) 0, (b) 24, and (c) 97 h; conversion of (1*RS*)-phenylethanol: (b) 25 and (c) 50%.

Table 1. Enzymic activity of the native lipase and its coprecipitates

Lipase coprecipitant	Enzyme inclusion in coprecipitate, %	Activity*	
		in triacetin hydrolysis	in (1*RS*)-phenylethanol acetylation
–	–	435	1530
CeAA	65	200	19000
$CeNH_2$	99	250	13600
HDD	85	490	9300
CeOH	80	150	2500
PBMA	90	46	2900

* In (μmol of product) h^{-1} (mg of enzyme)$^{-1}$.

PBMA–HDD–CeOH–CeAA. However, in the case of CeOH, the supernatant was found to contain very small particles of the coprecipitate that do not sediment under the conditions used for its isolation. Therefore, the enzyme content in this coprecipitate (80%) is only tentative. Another regularity is observed upon the determination of the catalytic activity of coprecipitates for triacetin hydrolysis (Table 1). The hydrolytic activity of the lipase does not remain intact: the activity falls in the series of coprecipitants HDD–$CeNH_2$–CeAA–CeOH–PBMA. Some activation of the lipase (1.13-fold) is observed only in the case of its coprecipitate with HDD. The stabilization of lipases by *cis*-diols (e.g., sorbitol) was observed [14], and this may be the reason for the high activity of the enzyme in its coprecipitates with HDD.

The most interesting results were obtained upon the determination of the activity of the coprecipitates in a model esterification reaction in an organic medium (Fig. 2). The growth of enzymatic activity as compared to that of the native enzyme was observed in all cases

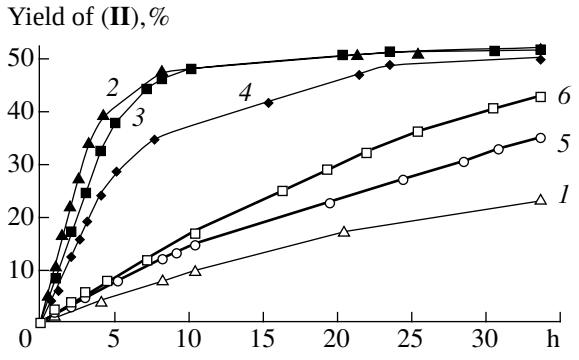

Fig. 2. The kinetic curves of 1-phenylethyl acetate accumulation in the model esterification reaction catalyzed by (*1*) the native lipase and the lipase included in the coprecipitates with (*2*) CeAA, (*3*) $CeNH_2$, (*4*) HDD, (*5*) CeOH, and (*6*) PBMA. The lipase content is 0.03 g.

of the phenylethanol (*RS*-(**I**)) acetylation with vinyl acetate catalyzed by various lipase coprecipitates (Table 1). The esterifying activity of the enzyme coprecipitates decreases in the series of the coprecipitants CeAA–$CeNH_2$–HDD–PBMA–CeOH. Note that the catalysts active in organic solvent are less active in aqueous medium (Table 1). This may be due to the different wettability of the coprecipitates and also to the differences in their structural organization that affects the transport of substrates to the lipase active sites. At the same time, the marked difference in the activity of the coprecipitates in esterification suggests that more or less specific binding of the lipase to coprecipitants exists. Taking into consideration the equal or very close lengths of the hydrophobic substituents in the coprecipitants (except for PBMA), one can assume that the binding is dictated by polar interactions (electrostatic interactions and hydrogen bonding) with the coprecipitants, which result in the most active coprecipitates (CeAA and $CeNH_2$). It should be noted that all the obtained coprecipitates could be isolated from reaction mixtures and, hence, used repeatedly. This fact is important from practical point of view, and, in this regard, PBMA offers the greatest promise as a coprecipitant, since its coprecipitates with the lipase can most easily be isolated from both the aqueous medium and TBME. Therefore, the use of polymeric coprecipitants could create new methods for obtaining recoverable catalysts. In addition to high catalytic activity, the cheapness of the starting materials is one of the advantages of the resulting coprecipitates and profitably differentiates them from the previously suggested lipid-like agents, which are difficult to obtain [13].

The Enantioselectivity of Esterification

The study of the optical activity of the samples of 1-phenylethyl acetate obtained by esterification catalyzed by the lipase and its coprecipitates testified to the retaining of the enantioselectivity of esterification (Table 2). For all of the coprecipitates, the values of specific optical rotation are of the same sign and fall within the same range (70°–80°). The differences in the optical rotation values are due to the product isolation at various degrees of the *RS*-(**I**) to *R*-(**II**) conversion. Since the acetylation of the *R*-form of the alcohol is accompanied by the slow acetylation of its *S*-form, the optical rotation of the product decreases with an increase in the *RS*-(**I**) conversion. Comparative experiments with the native lipase and the coprecipitate of the lipase with CeOH (Table 2) clearly demonstrated this statement. More than 99% enantiomeric purity of *R*-(**II**) was observed in the case of the catalysis by the native lipase at 48% conversion of *RS*-(**I**) [7]. Based on the data represented, one can conclude that the enantiomeric purity of the product in the model reaction remains high upon the use of the lipase coprecipitates as catalysts.

Table 2. Optical rotation of 1-phenylethyl acetate (**II**) obtained via acetylation of (1*RS*)-phenylethanol with vinyl acetate catalyzed by the lipase coprecipitates with various coprecipitants

Lipase coprecipitant	$[\alpha]_{589}$,* deg	Conversion of (1*RS*)-phenylethanol, %
–	80.4	42.5
CeAA	76.0	46.5
$CeNH_2$	77.2	46.0
HDD	73.9	45.5
CeOH	70.5	48.0
PBMA	79.2	44.8

* 20°C, 1 : 1 acetonitrile–water.

Table 3. The initial lipase activity and the activity of various preparations of immobilized lipase in the acetylation of (1*RS*)-phenylethanol with vinyl acetate in *tert*-butyl methyl ether

Preparation	Activity*	Relative activity of preparations, %
Native lipase	1.528	100
Lipase on Celite	3.266	213
Lipase and CeAA on Celite	4.628	303
Lipase and HDD on Celite	4.208	276
Lipase on Eupergit C250L	1.131	74
Lipase and HDD on Eupergit C250L	1.200	78

* In (mmol of 1-phenylethyl acetate) h^{-1} (mg of lipase)$^{-1}$.

Immobilization of the Lipase on Solid Supports

Despite the possibility of using lipase coprecipitates with hydrophobic coprecipitants as highly active and stereospecific catalysts of acetylation in nonaqueous media, the immobilization of the enzyme on solid carriers offers more promise because of the mechanical stability of such catalysts. In this connection, the immobilization of the lipase on solid supports in the presence of coprecipitants described above is of interest as an attempt to obtain highly active and, at the same time, mechanically rigid catalysts. We have chosen two principally different methods, physical sorption of the lipase on Celite 545, a zeolite carrier, and a covalent binding of the protein to the Eupergit C250L [a commercially available poly(methyl metacrylate) support that bears epoxy groups] in the presence of those hydrophobic coprecipitants that displayed high catalytic activity (Table 3, Fig. 3).

Covalent binding of the lipase to Eupergit C250L led to 74% of the enzymatic activity being retained, whereas the binding of the lipase treated with HDD to the same support resulted in 78% of the enzymatic activity being retained. Therefore, the covalent binding in the presence of hydrophobic HDD increases, though not substantially, the esterifying activity of the immobilized enzyme. A much more marked effect was observed in the case of the lipase sorption on Celite 545: the esterifying activity was 2.1 times more than that of the native enzyme. Moreover, in the presence of HDD and CeAA, the esterification rate was further increased by 1.3 and 1.4 times, respectively. It is obvious that the presence of hydrophobic coprecipitants plays an important role in obtaining solid catalysts by physical sorption of the protein. The physical precipitation of active coprecipitates on the support surface probably occurs in this case. At the same time, the coprecipitates cannot be chemically bound to Eupergit C250L, and, therefore, their activity is almost the same as that in the absence of coprecipitants. Thus, hydrophobic coprecipitation of the lipase can offer both by itself and in the combination with the adsorption on solid supports.

EXPERIMENTAL

A commercially available preparation of the lipase from *Pseudomonas fluorescens* (the enzyme content was 0.25 wt %) with the specific activity for triacetin hydrolysis of 1200 (μmol of acetic acid) h^{-1} g^{-1} was purchased from RÖHM Pharma Polymers (Germany). Celite 545 was from Serva (Germany). Eupergit C250L, a poly(methyl metacrylate) bearing epoxy groups, was from Pharma Polymers (Germany). Ethyl acetate (bp 73°C), dioxane (bp 101°C), and butyl metacrylate (bp 53°C/5 mm Hg) (Reakhim, Russia) were purified by distillation. 1-Phenylethanol, vinyl acetate, *tert*-butyl methyl ether, and triacetylglycerol (triacetin) were from Merck (Germany); *N*-cetylamine,

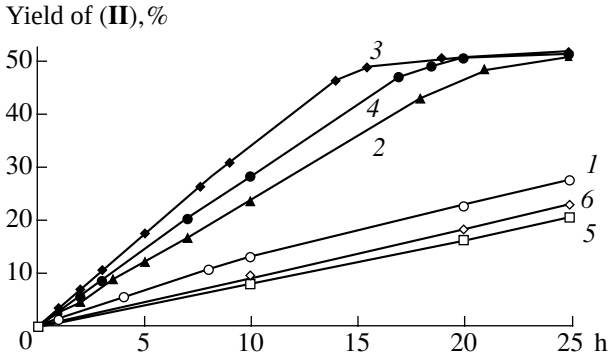

Fig. 3. The kinetic curves of 1-phenylethyl acetate accumulation in the esterification reaction catalyzed by the lipase immobilized on various supports. The lipase content is 0.036 mg; the lipase preparations are (*1*) native lipase; (*2*)–(*4*) the lipase adsorbed on Celite 545 (*2*) without coprecipitants and in the presence of (*3*) CeAA and (*4*) HDD; the lipase immobilized on Eupergit C250L (*5*) without coprecipitants and (*6*) in the presence of HDD.

hexadecane-1,2-diol, and cetyl alcohol from Fluka (Germany); and basic alumina L5/40 from Chemapol (Czech Republic). AIBN, acetonitrile, acetic anhydride, triethylamine, hexane, acetone, potassium dihydrogen phosphate (analytical grade), sodium chloride, calcium chloride (high-purity grade), and sodium hydroxide (Reakhim, Russia) were used without additional purification.

(1)-Phenylethyl acetate (II). Acetic anhydride (0.15 mol), triethylamine (0.15 mol), and ethyl acetate (25 ml) were added to 0.05 mol of *RS*-(**I**). The reaction mixture was refluxed for 4 h and washed three times with 1 M KH_2PO_4 (pH 4.5), and the organic phase was distilled to give *RS*-(**II**), bp 89°C/5 mm Hg [15].

***RS*-(I), vinyl acetate, and (II) were separated in a model experiment** on a LiChroCART RP 18 (5 μm) column (240 × 4 mm, Merck, Germany) on a Beckman System Gold instrument (United States). Optical absorption was measured at 260 nm. Optimal conditions for HPLC were as follows: flow rate of 0.8 ml/min, 1 : 1 acetonitrile–water as an eluent, tenfold dilution of the mixture under study with the eluent, and an applied sample volume of 20 μl at (*RS*-(**I**) and vinyl acetate contents of 0.5 and 1.5 μmol, respectively. Under these conditions, the retention times for *RS*-(**I**), vinyl acetate, and *RS*-(**II**) were 5.9, 6.2, and 13.5 min, respectively. Mixtures of (**I**) and (**II**) containing 10, 20, 31.4, and 40% of (**II**) according to GC were used as control samples. Contents of (**II**) determined by rpHPLC were 9.7, 19.4, 30.3, and 41.7%, respectively.

***N*-Cetylacetamide.** Acetic anhydride (50 mmol) was added dropwise to a stirred solution of $CeNH_2$ (25 mmol) in ethyl acetate (200 ml), and the mixture was stirred for an additional 2 h. The reaction mixture was washed three times with water and evaporated in a vacuum, and the resulting CeAA was recrystallized from hexane and dried in a desiccator, mp 85°C [16].

Poly(butyl metacrylate). To remove stabilizing hydroquinone, monomeric butyl metacrylate was dissolved in hexane, passed through the column with alumina, and distilled. It was polymerized in bulk at 80°C after preliminary nitrogen bubbling with AIBN (3% of polymer mass) as an initiator. The resulting polymer was dissolved in dioxane, separated from the monomer by reprecipitation with ethanol, and dried in a desiccator with a yield of 60%. The molecular mass of PBMA determined viscosimetrically by the standard procedure [17] was 20 000.

Lipase inclusion into the hydrophobic coprecipitates with CeOH, $CeNH_2$, CeAA, and PBMA was carried out as described for its coprecipitates with HDD according to the procedure reported earlier [12]. The lipase (0.2 g) was dissolved in 10 ml of 0.01 M phosphate buffer, pH 7.5 (buffer A) at 0°C and separated from insoluble impurities by filtration. A solution of HDD (20 mg) in warm acetone (0.7 ml) was added dropwise to the lipase solution under stirring. Acetone was removed in a vacuum, and the aqueous solution was kept for 24 h at 6°C. The solid phase was separated by centrifugation (8000 rpm, 20 min), lyophilized, and stored at 6–10°C. The protein content in coprecipitates was determined from the residual hydrolytic activity of the enzyme measured in the supernatant taking into account the lipase content in its commercial preparation and the starting activity of the preparation.

Immobilization of the lipase on Celite 545 particles and Eupergit C250L support. The lipase powder (80 mg) was dissolved at 0°C in buffer A (10 ml), and separated from insoluble impurities by filtration. Celite 545 (1 g) was added, the mixture was incubated for 72 h at 6°C, and then the water was removed. The resulting enzyme preparation was stored at 6–10°C.

Preparation of the immobilized enzyme was similarly obtained from 100 mg of the lipase powder solution in 10 ml of buffer A and 1 g of Eupergit C250L by incubation for 24 h at 6°C. The supernatant was decanted, and its hydrolytic activity was measured to calculate the amount of lipase covalently bound to the support. The preparation of immobilized enzyme was dried on a porous glass filter by blowing air and stored at 6–10°C.

The preparations of the lipase suspension with coprecipitants obtained as described above were immobilized similarly.

The hydrolytic activity of the dissolved and immobilized lipase was determined in the reaction of triacetin hydrolysis. A solution (50 μl) containing the enzyme (2.5 μg) or the dry coprecipitate (0.5 mg) was added to a solution (5 ml) of the substrate containing 0.1 M triacetin, 0.05 M sodium chloride, and 0.05 M calcium chloride in the cell of a Radiometer Copenhagen TTT 60 titrator. Acetic acid released in the enzymic reaction was titrated with 0.01 M NaOH solution to pH 7.0 for 10–15 min. The hydrolytic activity of the enzyme was calculated from the titration results in (μmol of acetic acid) h^{-1} (mg of the enzyme)$^{-1}$.

The activity of the lipase and its immobilized preparations in the esterification reaction was determined from the initial rate of the of *RS*-(**I**) acetylation with vinyl acetate [7]. *RS*-(**I**) (5 mmol) and vinyl acetate (15 mmol) were added to TBME (10 ml). Then the lipase powder (14 mg), the lipase coprecipitate (2 mg), or the solid carrier with the immobilized lipase (300 mg) was added, and the mixture was shaken at 37°C. The Aliquots (0.5 ml) were sampled at defined intervals (Figs. 2, 3), the solid catalyst particles were separated by centrifugation (10 000 rpm, 5 min), and the supernatant was analyzed as described above. The initial rate of the enzymic reaction was calculated from the accumulation rate of the reaction product (**II**) in μmol h^{-1} (mg of the enzyme)$^{-1}$.

The determination of the acetylation enantioselectivity under the catalysis by lipase preparations. To isolate (**II**), obtained via catalysis by various lipase coprecipitates, the reaction mixture (10 ml) was concentrated in a vacuum to the volume of 2.5 ml, solid

particles of catalyst were separated by centrifugation, the supernatant was diluted fivefold with the eluent, and the aliquot was chromatographed as described above. The fraction whose retention time corresponded to that of model (**II**) was isolated by HPLC. The optical rotation of the product was measured on a Digital Polarimeter DIP-360 (Jasco, Japan) at 589 nm (Na D-line) in 1 : 1 acetonitrile–water at 20°C.

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