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Lipase-based HPLC stationary phase: enantioselective synthesis of 2-substituted 1,3-propanediol monoacetates

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Abstract

Pseudomonas cepacia lipase (PCL) has been immobilized by coating the enzyme on an epoxysilica HPLC column. The biocatalyst has been successfully used for the preparation of both the enantiomers of 3-acetoxy-2-benzyl-propan-1-ol and of 3-acetoxy-2-methylpropan-1-ol. The immobilized enzyme is active after months of use either in aqueous or in organic media.

1. Introduction

The asymmetrization of prochiral or *meso* diols or diesters together with the resolution of racemic alcohols or esters by lipases represents one of the most useful methods to prepare enantiomerically pure compounds. The lipase catalyzed enantioselective esterification in the presence of an acyl donor and enantioselective hydrolysis have been shown to be efficient methods for the preparation of pharmacologically active compounds or important chiral building blocks with defined stereochemistry.[1–4] These enzymes are available from different sources and are very stable either in aqueous or organic solvents. Several attempts have been made to immobilize lipases.[5,6] It is well known that immobilization can raise an enzyme's potential, such as performance at high substrate concentration, increased operating stability, feasibility of continuous operation, repeated usage and retention of the enzyme in a bioreactor. Among the different examples reported, the immobilization of lipases on a membrane allows the enzyme to remain active in the preparation of enantiomerically pure compounds which is important for pharmaceutical large scale applications.[7,8] Recently a new procedure has been reported in which lipases are incorporated in hydrophobic organic–inorganic hybrid materials.[9] These heterogeneous biocatalysts show much higher activity for esterifications in organic solvents and remarkably long-term stability. Non-covalent binding of lipase has also been reported using an immobilized artificial membrane stationary phase (IAM-SP): the enzyme was active and compatible with the on-line syntheses and purification of stereochemically

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pure products.[10] Thus, the key to scale-up a lipase catalyzed enantioselective synthesis can be considered to reside in the immobilization technology.[11] This expectation provides the reasons for the development of so many methods to immobilise these enzymes. No reports, however, describe the immobilization of lipase on high performance liquid chromatography (HPLC) supports. We were interested in preparing a lipase-based HPLC column to be used as a bioreactor, i.e. for the enantioselective synthesis of optically active compounds, starting either from chiral or prochiral substrates.

In order to evaluate reliably the performances of the immobilized enzyme, reactions were selected which are well known to occur in bulk solution for the preparation of synthetically useful compounds.

2. Results and discussion

The properties of the support material can influence the activity of the immobilized enzyme and epoxysilica was chosen because it has been successfully used in the immobilization of proteins[12] and enzymes.[13] The anchoring of lipase can be realised either in bulk solution or in situ and these supports have been proved to guarantee high chromatographic performances, once they have been completely derivatized.[14] The column used for this investigation has been prepared by in situ coating of *Pseudomonas cepacia* lipase (PCL) on the packed epoxysilica matrix. The epoxysilica support was prepared by reacting activated silica with (3-glycidoxypropyl)trimethoxysilane according to a reported procedure.[15] An HPLC column (5×0.46 cm i.d.) was then conventionally slurry packed. The enzyme (100 mg suspended in phosphate buffer 50 mM, pH 7.5) was allowed to coat the epoxysilica matrix. The obtained lipase-based column was finally stabilized by treating the support with glutaraldehyde as a cross-linking agent.

Indeed, there has been much interest in the synthesis of enantiomerically pure derivatives of 2-substituted 1,3-propanediols, as they can be used in the preparation of a wide range of natural products and biologically active compounds, such as muscone, microbial growth factors and renin inhibitors.[16–20] Thus, the PCL-based HPLC column was used to prepare both enantiomers of 3-acetoxy-2-benzyl-propan-1-ol. Compound (*R*)-**3a** was obtained by esterification of 2-benzyl-1,3-propan-diol, **1a**, using vinylacetate as the acyl donor, while (*S*)-**3a** was obtained by hydrolysis of 1,3-diacetoxy-2-benzyl-propane, **2a**, easily prepared by acetylation of the corresponding diols **1** (Scheme 1 and Table 1). Both reactions occurred in bulk solution, with high conversion, and essentially enantiomerically pure fractions were obtained, in agreement with the literature data.[18,19]

The enzyme-based HPLC column furnished comparable results for the hydrolysis and esterification reactions (entries 1–4), but over longer reaction times. Thus, the immobilized enzyme maintains its activity either using aqueous medium or vinylacetate as the mobile phase. An HPLC method was developed which allowed the simultaneous determination of the conversion of the substrate and of the enantiomeric excesses (ees) of the product by using a chiral stationary phase. As an example, the analysis of **3a**, obtained by hydrolysis of **2a**, is reported in Fig. 1. The target product, **3a**, was obtained in an enantiomerically pure form, and the starting compound **2a** and the obtained diol **1a** are determined as well. The absolute configuration of the prevailing enantiomer was assigned by measuring the optical rotation at 589 nm and comparing its sign with that reported for (*R*)-**3a**.[18]

To enhance characterization of the performances of the immobilized PFL, two other substrates were investigated in the esterification and hydrolysis reactions both in bulk solution and on the column (Scheme 1). Esterification of **1b** in bulk solution gave (*R*)-**3b** with 100% conversion and 24% ee after 4 h; on the contrary, the conversion was much slower on the column, at only 7% after 48 h. Compound (*R*)-**3b** was obtained by hydrolysis of **2b** in bulk solution with very low yield and enantioselectivity; again the reaction on the column was very slow (3% conversion after 48 h) so we decided not to investigate further the 2-phenyl derivatives. On the contrary, very high ees (98%) were obtained in the esterification of **1c** in

Table 1
Lipase catalyzed enantioselective esterification and hydrolysis

Entry	Substrate	Time (h)	Conversion (%)	Yield (%)	Ee (%)	Abs. Conf.
1	**1a**[a]	0.5	100	100	>99[b]	*R*
2	**1a**	10	100	100	>99	*R*[c]
3	**2a**[a]	5	66	40	>99	*S*
4	**2a**	48	68	42	>99	*S*
5	**1c**[a]	0.3	93	57	58[d]	*S*
6	**1c**	138	85	57	36	*S*
7	**2c**[a]	0.9	40	40	96	*R*[e]
8	**2c**	45	35	35	86	*R*

[a]The reaction was conducted in bulk solution: see experimental section.

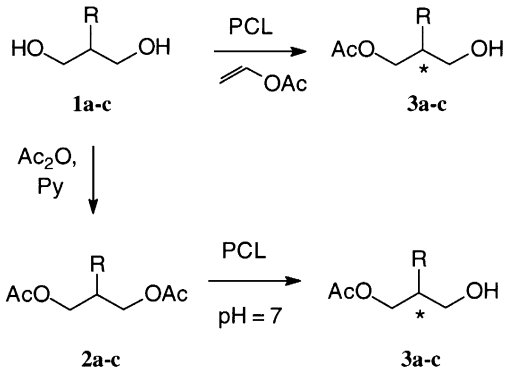
[b] Determined by HPLC analysis of the monoacetate on Chiralcel OD, hexane/IPA 95/5, flow rate = 1 ml/min.

[c]Determined by direct comparison of optical rotation: $[\alpha]_D^{25}$ = +31.9 (c 1.2, $CHCl_3$), to be compared with the value[18] $[\alpha]_D^{20}$ = +27.7 (c 1.3, $CHCl_3$) for (*R*)-**3a** (e.e. 97%).

[d] Determined by HPLC analysis of the corresponding benzoate on Chiralcel OD, hexane/IPA 99/1, flow rate 0.5 mL/min.

[e]Determined by direct comparison of optical rotation: $[\alpha]_D^{25}$ = -9.6 (c 2, EtOH), to be compared with the value[21] $[\alpha]_D^{20}$= +10 (c 2, EtOH) for (*S*) **-3c** (e.e. >98%).

bulk solution, after the substrate had been completely converted and the formation of diacetate **2c** from **3c** had started. This suggested a very high enantioselectivity in the esterification of the second hydroxyl group of **3c**, in agreement with literature data.[22] This behaviour was also observed when the reaction was performed on the PCL-based column. The reaction was monitored up to 85% conversion, because of the much longer time needed on the column, and a significant increase in the ee (from 16 to 36%)



a: R = CH_2-Ph
b: R = Ph
c: R = CH_3

Scheme 1.

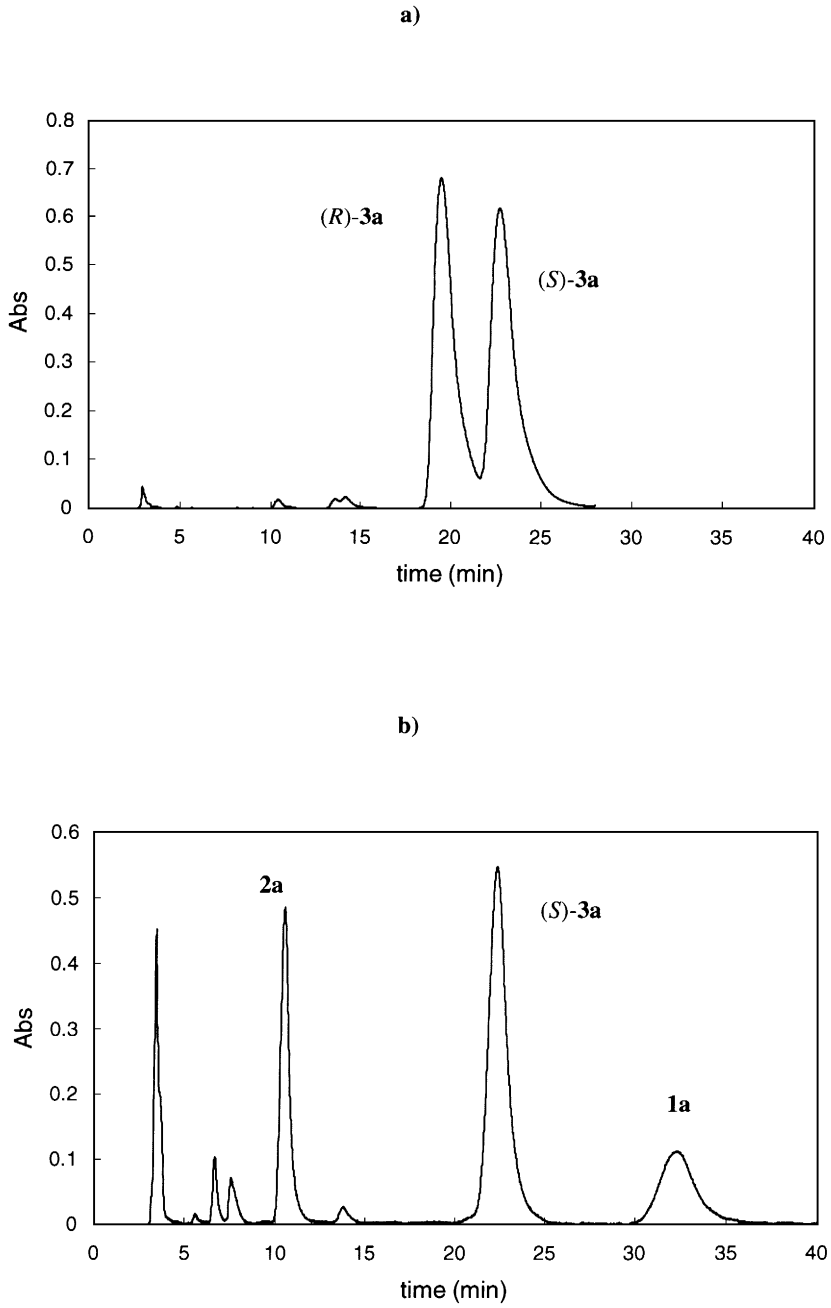


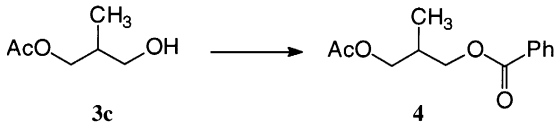
Fig. 1. HPLC analysis of (a) racemic monoacetate **3a**; (b) hydrolysis reaction mixture containing **3a** (Chiracel OD, hexane:2-propanol 95:5, flow 1 mL/min, λ 212 nm)

was observed at higher conversion. The chemical yield was comparable at the same conversion values for both the reactions in bulk solution and on the column (entries 5 and 6).

High enantioselectivity was obtained at about 40% conversion in the hydrolysis of **2c**, both in bulk solution (after 0.9 h) and on the column (after 45 h) (entries 7 and 8). A decrease of the enantioselectivity was observed at higher conversion of the substrate: for example, at about 60% conversion, the ee of (*R*)-**3c** was 92% in bulk solution (after 2 h) and 72% on the column (after 96 h). A significant difference in

the times needed to perform the reaction in bulk solution or on the column has also been observed with 2-methyl derivatives.

The conversion of the substrate (**1c** or **2c**) was followed by GC analysis, while the enantiomeric excess of the product was determined by HPLC analysis of the benzoate derivative **4**, obtained by reaction of **3c** with benzoyl chloride (Scheme 2). This allowed easier monitoring and a reliable determination of the enantiomeric composition.



3c **4**

Scheme 2.

As an example, the HPLC analysis of a sample of the benzoate derivative **4** is reported in Fig. 2.

The absolute configuration of the prevailing enantiomer has been assigned by measuring the optical rotation and comparing its sign with that reported for (*R*)-**3c**; the elution order of the derivative **4** on Chiralcel OD has been determined by HPLC analysis of a sample of known absolute configuration.[‡]

3. Conclusion

The PCL-based HPLC column has been proved to be efficient for the enantioselective synthesis of 2-substituted 1,3-propanediol monoacetates. The enantioselectivity of the enzymatic processes on column and in bulk solution are comparable, the conversion being generally slower for the immobilized enzyme.

The PCL-based column can be used in organic and aqueous media and it is stable for months after continuous use. This makes the column useful for preparative purposes.

4. Experimental

4.1. General

^{1}H NMR (200 MHz) spectra were recorded in $CDCl_3$ with a Varian Gemini 200 spectrometer using tetramethylsilane as internal standard. Optical rotations were measured on a Jasco DIP 360 automatic polarimeter. Analytical TLC was performed on 0.2 mm silica gel plate Merck 60 F-254. GC (FI detector) analysis was performed on a SGE BP1 column (25 m×0.53 mm i.d., 1.0 μm film). The enantiomeric excesses were determined by HPLC analysis of the reaction mixture on an HPLC system consisting of a Jasco PU-980 pump and a Jasco MD 910 multiwavelength detector (Jasco, Tokyo, Japan). The chromatographic retention of the solutes were monitored by measuring the absorption at 212 nm (**3a**, **3b**). The enzymatic reactions were carried out by eluting the reaction mixture on an HPLC system consisting of a Jasco 880-PU pump and a Jasco 875-UV–vis detector (Jasco, Tokyo, Japan).

4.2. Starting materials, reagents and solvents

Lipase PS from Amano was kindly provided by Prof. P. Crotti, University of Pisa, Italy.

[‡] A sample of compound (*S*)-**3c** with 98% ee was derivatized with benzoyl chloride and analyzed by optical rotation, obtaining a value of $[\alpha]_D^{25}=-0.8$ (c 1, $CHCl_3$) to be compared with the value[22] of $[\alpha]_D^{25}=-0.8$ (c 0.6, $CHCl_3$) for the enantiomerically pure (*R*) compound.

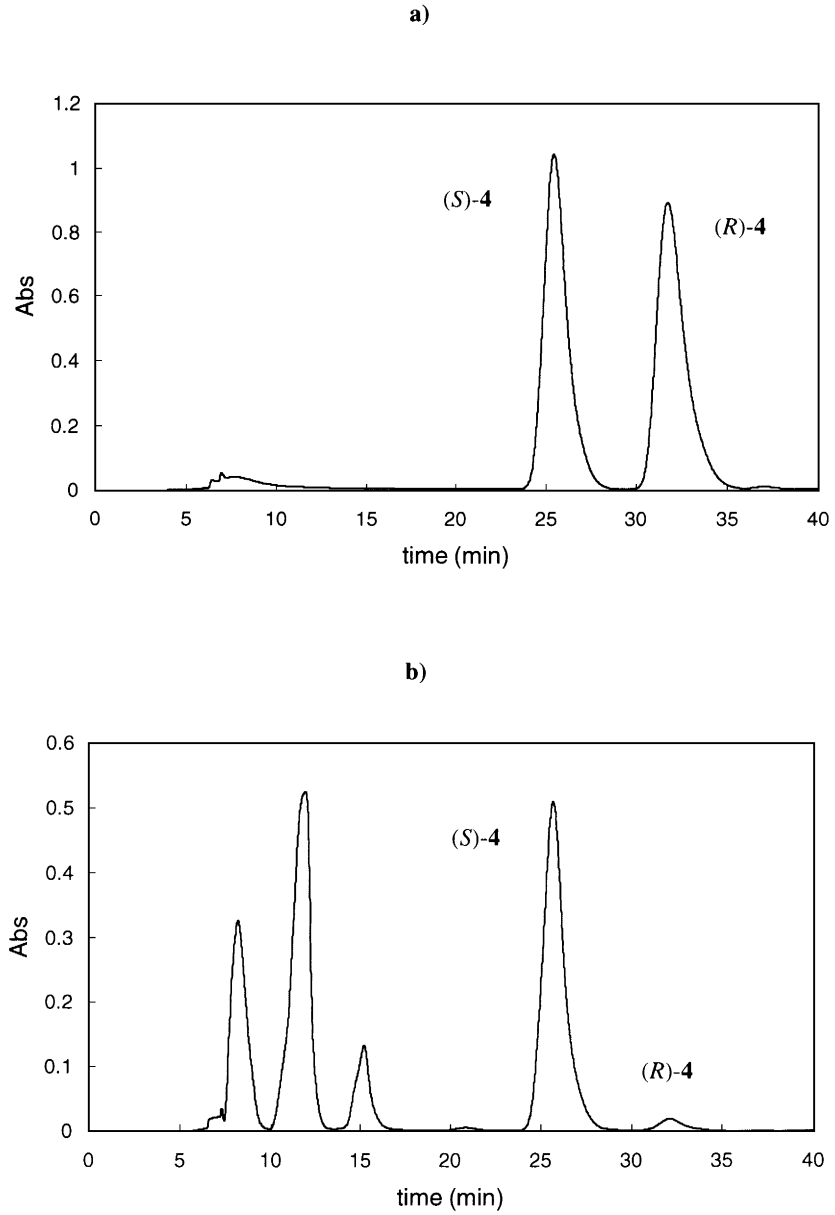


Fig. 2. HPLC analysis of (a) racemic benzoate **4**; (b) benzoate **4** obtained by derivatization of a hydrolysis reaction mixture containing **3c** (Chiracel OD, hexane:2-propanol 99:1, flow 0.5 mL/min, λ 230 nm)

Acetic anhydride, benzoyl chloride, diethyl ether, methylene chloride, pyridine, tetrahydrofuran, triethylamine were refluxed and distilled under an inert atmosphere. 2-Methyl-1,3-propanediol **1c**, 2-benzyldiethyl malonate, 2-phenyldiethyl malonate and vinyl acetate were commercially available. Diols **1a**–**b** were prepared by $LiAlH_4$ reduction of 2-benzyldiethyl malonate and 2-phenyldiethyl malonate following the procedure described in Ref. 20. The obtained products were characterized by ^{1}H NMR. Diacetates **2a**–**c** were obtained by reaction of diols **1a**–**c** with 2.5 equiv. of acetic anhydride and 2.5 equiv. of pyridine. After stirring overnight, the mixture was diluted with toluene and evaporated under reduced pressure to give the products in quantitative yields, which were characterized by ^{1}H NMR.

Racemic monoacetate **3a**–**c** were prepared as described in Ref. 20. The products were characterized by ^{1}H NMR and HPLC on a chiral column.

4.3. Monoacetate **3a**

^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 7.36–7.10 (m, 5H); 4.22–3.99 (m, 2H); 3.66–3.43 (m, 2H); 2.93–2.58 (m+br s, 3H); 2.22–1.99 (s+m, 4H). HPLC: Chiralcel OD, hexane:2-propanol 95:5, flow rate 1mL/min.

4.4. Monoacetate **3b**

^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 7.40–7.19 (m, 5H); 4.37 (d, 2H); 3.83 (d, 2H); 3.23–3.05 (q, 4H); 2.16 (br s, 1H); 2.04 (s, 1H). HPLC: Chiralcel OB, hexane:2-propanol 85:15, flow rate 0.8 mL/min.

4.5. Monoacetate **3c**

^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 4.07 (d, 2H); 3.52 (br d, 2H); 2.58 (br s, 1H); 2.16–1.89 (m+s, 4H); 0.95 (d, 3H).

4.6. Synthesis of the benzoate derivative **4**

To a solution of 0.1 mmol of monoacetate **3c** in 2 mL of anhydrous CH_2Cl_2, 1.2 equiv. of Et_3N and 1.2 equiv. of benzoyl chloride were added at 0°C. The reaction was followed by GC. Upon disappearence of the substrate, the mixture was worked up with 10% HCl, 10% $NaHCO_3$ and saturated NaCl. The organic phase was dried on Na_2SO_4 and evaporated under reduced pressure. The residue was characterized by GC and HPLC analysis and by measuring the optical rotation.[22] ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 8.22–8.0 (m, 2H); 7.64–7.40 (m, 3H); 4.28 (d, 2H); 4.11 (d, 2H); 2.48–2.11 (m, 1H); 2.05 (s, 3H); 1.09 (d, 3H). HPLC: Chiralcel OD, hexane:2-propanol 99:1, flow rate 0.5 mL/min.

4.7. Synthesis of the lipase-based HPLC stationary phase

The silica gel (Kromasil 200 Å, 5μm, Eka Nobel, Sweden) was heated for 16 h at 180°C under vacuum. Distilled (3-glycidoxypropyl)trimethoxysilane (1.9 g) in 5 mL of dry toluene was added dropwise to 5 g of activated silica in 60 mL of dry toluene. The mixture was refluxed for 12 h. After filtration the product was washed twice with dry toluene and acetone. The derivatized silica was then dried for 15 h at 80°C under vacuum. Epoxysilica (1 g) was slurry packed into a 50×4.6 mm i.d. HPLC column under conventional conditions, using acetone as pumping solvent. The column was washed with acetone and dried at 80°C under helium. After equilibration of the column with the coupling solution, i.e. 0.05 M KH_2PO_4, pH=7.5, containing 2 M ammonium sulfate, the coupling solution (35 mL) containing 100 mg of PCL was applied to the column at 0.5 mL/min. The column was back flushed every 30 minutes until the solution was exhausted. The column was disconnected, plugged and allowed to stand in the refrigerator all night. The column was then washed with 100 mL of 0.05 M KH_2PO_4, pH=6, and the remaining epoxide groups blocked with a solution (30 mL) of glycine 1 M in 0.05 M KH_2PO_4, pH=7, and finally equilibrated with a solution (30 mL) of 0.05 M KH_2PO_4, pH=6.

4.8. General procedure for enzymatic hydrolysis

Procedure A: Diacetate **2a**–**c** (0.16 mmol) was dissolved in 0.5 mL of *n*-propanol and added to a suspension of 100 mg of lipase in 29.5 mL of 100 mM phosphate buffer (pH 7). The mixture was magnetically stirred at room temperature and the reaction course was followed by HPLC analysis for diacetates **2a**–**b** and by GC analysis for diacetate **2c**. The reaction was interrupted by filtration of the

enzyme: the filtrate was extracted with Et_2O, dried on Na_2SO_4 and evaporated under reduced pressure. The residue was characterized by GC or HPLC analysis.

Procedure B: The reaction mixture was prepared as in procedure A, except lipase, and it was continuously eluted through the lipase column. The reaction was followed as indicated above.

Upon completion of the reaction, the column was flushed with clean buffer and re-used.

4.9. General procedure for enzymatic esterification

Procedure A: To a solution of 2.5 mmol of **1a**–**c** in 8 mL of vinyl acetate, 100 mg of lipase were added. The suspension was magnetically stirred at room temperature and the reaction course was followed by HPLC analysis for **2a**–**b** and by GC analysis for **1c**. The reaction was interrupted by filtration of the enzyme and the filtrate was evaporated under reduced pressure. The residue was characterized by GC or HPLC analysis.

Procedure B: The reaction mixture was prepared as in procedure A, except lipase, and it was continuously eluted through the lipase column. The reaction was followed as indicated above.

Upon completion of the reaction, the column was flushed with clean vinyl acetate and re-used.

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