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Enzymatic resolution of (*RS*)-2-arylpropionic acid thioesters by *Candida rugosa* lipase-catalyzed thiotransesterification or hydrolysis in organic solvents

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Abstract

An enzymatic resolution process was developed to produce (*S*)-naproxen ester, (*S*)-naproxen or (*S*)-ibuprofen from the corresponding racemic thioesters by using lipase-catalyzed thiotransesterification or hydrolysis in organic solvents. Enzyme activity is greatly enhanced when activated naproxen thioesters containing an electron-withdrawing group are the substrates. Unlike other lipases, *Candida rugosa* lipase may discern the sulfur moiety of the thioesters, and yields lower enzyme activity when compared to the corresponding oxygen-containing analogues. Enzyme performances were further compared under various conditions, i.e. different combinations of reaction type (thiotransesterification or hydrolysis), solvent (isooctane or cyclohexane), substrate (naproxen or ibuprofen thioesters) and lipase sources.

1. Introduction

Hydrolytic enzymes such as esterases, proteases and, in particular, lipases have received much attention because of their effectiveness in regio- and enantioselective hydrolysis, esterification and transesterification of organic acids, alcohols, amines, amides and esters in the presence of organic solvents.[1,2] However, in the literature, very few studies deal with enzymatic transformations involving thioacids and thioesters. Zaks and Klibanov, for instance, employed a porcine pancreatic lipase in the thiotransesterification of methyl butyrate and butanethiol in various organic solvents and found rates similar in magnitude to transesterification involving alcohols with the same enzyme.[3] Caussette et al. have successfully carried out the esterification of oleic acid and butanethiol catalyzed by an immobilized *Mucor miehei* lipase in n-hexane, in which a low affinity of butanethiol to the enzyme was observed.[4] Sproull et al. also considered the esterification of butanol and various thioacids with the same enzyme in

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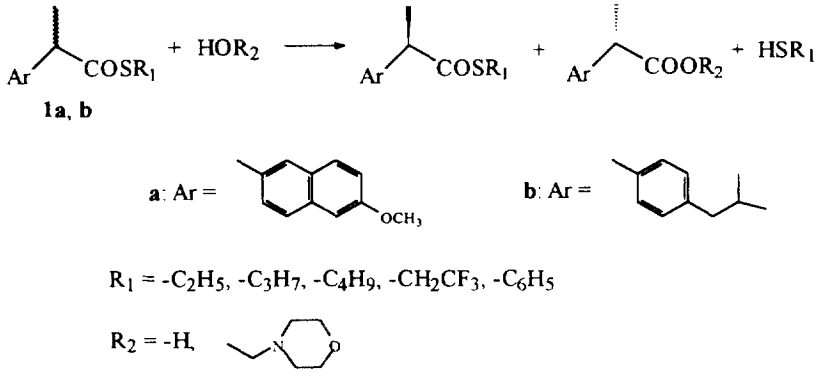
a chloroform:hexane (25:75, v/v) mixture and found rates much faster than those for the corresponding carboxylic acids.[5]

In another study, Bianchi and Cesti considered the lipase-catalyzed thiotransesterification of mercapto esters such as (*RS*)-3-(acetylthio)-2-methylpropionic acid methyl ester with propanol.[6] They discovered that porcine pancreatic lipase and Lipase P (Amano) have good enantioselectivity for the (*R*)-enantiomer and obtained higher reaction rates by using the thio substrates instead of the corresponding oxa-containing analogues. In the resolution of (*RS*)-2-octanol using (*S*)-ethyl thiooctanoate as an acyl donor, *Candida antarctica* lipase was found to have high enantioselectivity for the (*R*)-enantiomer.[7] Recently, Tan et al. have employed Lipase P-30 (Amano) and trioctylamine as the catalysts for hydrolysis and racemization, respectively, in the dynamic resolution of (*RS*)-2-(phenylthio)propionic acid ethyl thioester.[8] In their report, both a higher yield and higher enantiomeric excess for the desired enantiomer than a normal resolution without racemization were obtained.

2-Arylpropionic acids (i.e. profens), an important class of non-steroidal anti-inflammatory drugs, have their pharmacological activity mainly on the (*S*)-enantiomer.[9] Considerable efforts have been made on the lipase-catalyzed enantioselective resolution of (*S*)-profens or their ester prodrugs.[10] However, as far as we know, there are still no reports of using profen thioesters as the starting material in the previous resolution processes. Therefore, as shown in Scheme 1, with naproxen thioester **1a** and ibuprofen thioester **1b** as two representative members of this class, an enzymatic resolution process was developed by using *Candida rugosa* lipase-catalyzed thiotransesterification or hydrolysis in organic solvents.



Scheme 1.

2. Results and discussion

2.1. Naproxen thioester selection in thiotransesterification

With 4-morpholine ethanol as an acyl acceptor and Lipase MY (Meito Sangyo) as the biocatalyst in isooctane, Table 1 shows the time–course variations of the enantiomeric excesses for the substrate (ee_S), the main product of ester (ee_{PM}) and the byproduct of acid (ee_{PN}), as well as the conversion for the substrate (X_t), the yields for the main product of ester (X_M) and the byproduct of acid (X_N) for various naproxen thioesters. Compared with the enzyme performance in the enantioselective esterification of racemic naproxen with ethanol, n-propanol or butanol,[10e] very low conversions of X_t and hence the enzyme activity were obtained when ethyl, n-propyl and n-butyl thioester were employed as the substrate in thiotransesterification. When activated thioesters containing an electron-withdrawing moiety such as

Table 1

Time courses of Lipase MY mediated thiotransesterification of naproxen thioesters						
R_1		ethyl	n-propyl	n-butyl	phenyl	trifluoroethyl
105 (h)	ee_s (%)	1.1	0.5	0.6	53.8	38.1
	X_t (%)	2.0	0.4	0.9	36.7	28.6
	e_{PN} (%)				88.6	77.7
	X_N (%)				3.3	3.1
	ee_{PM} (%)	> 99	> 99	> 99	98.2	93.2
	X_M (%)	2.0	0.4	0.9	33.4	25.6
198 (h)	ee_s (%)	1.9	0.6	1.3	64.5	47.7
	X_t (%)	2.8	0.6	1.6	41.2	33.4
	ee_{PN} (%)				88.8	87.6
	X_N (%)				4.1	3.5
	ee_{PM} (%)	> 99	> 99	> 99	97.8	91.5
	X_M (%)	2.8	0.6	1.6	37.1	29.9

Conditions: 3 mM racemic naproxen thioester, 10 mM 4-morpholine ethanol and 20 mg ml^{-1} Lipase MY in isooctane at 37 °C.

phenyl or trifluoroethyl group were used, great improvements in the substrate conversion were found (Table 1). These imply that, unlike other lipases,[3,5,6] the oxygen hole for acyl binding in the active site of *Candida rugosa* lipase may have difficulty in accommodating the large sulfur atom due to steric and/or electrostatic effects and hence reduces the enzyme activity. This unfavorable factor is relaxed by introducing an electron-withdrawing moiety in the thioesters. A detailed analysis of the yields (X_M and X_N) and enantiomeric excesses for the product (ee_{PM} and ee_{PN}) for both activated thioesters indicates that this enzyme has higher enantioselectivity in thiotransesterification and is more favorable when using phenyl thioester as the substrate. However, this benefit is offset by its lower saturated solubility of 20.4 mM (compared with 290 mM for trifluoroethyl thioester) in isooctane at 37°C and the higher boiling point of 169°C for thiophenol (35°C for trifluoroethanethiol). In order to decrease the hydrolysis side-reaction, a further study on controlling a suitable water content of the crude lipase is needed.

The time–course variations of ee_S, ee_{PM}, ee_{PN}, X_t, X_M and X_N when racemic naproxen trifluoroethyl ester was employed as the substrate are shown in Table 2. Comparing the results in both tables by considering the applied enzyme concentration on the reaction rate, we conclude that Lipase MY has higher enantioselectivity and activity when using the ester substrate instead of the corresponding sulfur-containing analogue. This result indeed provides further strong evidence to support the above elucidation of the lower enzyme activity of *Candida rugosa* lipase for thioesters.

By further inspecting the time–course yields in Table 2, a maximum of X_N=12.4% at 49 h for the hydrolysis side-reaction was found. According to the Ping Pong kinetic mechanism as shown in Scheme 2, the acyl donor ($RCOOR_1$) at first liberates trifluoroethanol (HOR_1) in the acylation step. Then, the nucleophilic alcohol (HOR_2) competes with water to form ester ($RCOOR_2$) and acid (RCOOH), respectively, in the deacylation step. Since trifluoroethanol is a good leaving group, one may regard transesterification and hydrolysis to be irreversible. However, with the addition of an excess of 4-morpholine ethanol and the formation of naproxen, a reversible lipase-catalyzed esterification between the acid and the alcohol occurs to decrease X_N and enhances X_M. Finally, an equilibrium may exist among water, 4-morpholine ethanol, naproxen and naproxen ester product. Therefore, further studies on finding

Table 2

Time courses of lipase mediated transesterification of naproxen trifluoroethyl ester			
Enzyme sources		Lipase MY (Meito Sangyo)	*C. rugosa* lipase (Sigma)
11 (h)	ee_s (%)	27.7	23.2
	X_t (%)	22.8	21.5
	ee_{PN} (%)	91.8	84.7
	X_N (%)	11.5	8.9
	ee_{PM} (%)	95.4	92.4
	X_M (%)	11.3	12.6
49 (h)	ee_s (%)	58.4	50.5
	X_t (%)	37.9	37.2
	ee_{PN} (%)	77.7	68.7
	X_N (%)	12.4	11.2
	ee_{PM} (%)	95.1	92.4
	X_M (%)	25.5	26.0
96 (h)	ee_s (%)	69.0	65.0
	X_t (%)	43.0	44.7
	ee_{PN} (%)	69.4	53.1
	X_N (%)	10.3	10.7
	ee_{PM} (%)	95.1	90.9
	X_M (%)	32.7	34.0

Conditions: 2 mM racemic naproxen trifluoroethyl ester, 15 mM 4-morpholine ethanol and 5 mg ml^{-1} lipase in isooctane at 37 °C.

optimal conditions such as best reaction time, substrate concentrations and water content are needed in order to obtain the highest X_M and ee_{PM}.

$$E + RCOOR_1 \xrightarrow{-HOR_1} ES^* \xleftrightarrow{+HOR_2} E + RCOOR_2$$

$$E + RCOOR_1 \xrightarrow{-HOR_1} ES^* \xleftrightarrow{+H_2O} E + RCOOH$$

$$E + RCOOH \xleftrightarrow{-H_2O} ES^* \xleftrightarrow{+HOR_2} E + RCOOR_2$$

Scheme 2.

When another *Candida rugosa* lipase purchased from Sigma was employed as the biocatalyst, very similar enzyme performances for both lipases were obtained (Table 2). Therefore, we employed this lipase preparation in the enantioselective thiotransesterification for both activated thioesters and presented the time–course variations of ee_{PM} and X_t in Fig. 1(A) and 1(B). As expected, both enzymes have very similar performances at a given thioester and organic solvent. However, increasing the solvent hydrophilicity by replacing isooctane with cyclohexane results in the decrease of X_t for a given thioester and enzyme preparation. This effect is especially evident for naproxen phenyl thioester [Fig. 1(A)], although no explanations were given.

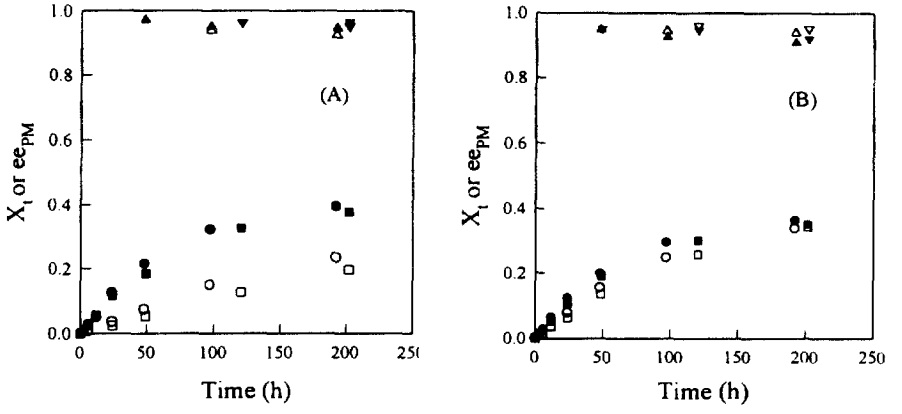


Fig. 1. Effects of solvent, lipase preparation, phenyl (A) and trifluoroethyl (B) thioesters on time–course variations of ee_{PM} and X_t for 3 mM thioester, 10 mM 4-morpholine ethanol and 5 mg ml^{-1} lipase at 37°C. In isooctane: for Lipase MY, ee_{PM} (▼) and X_t (■); for *C. rugosa* lipase, ee_{PM} (▲) and X_t (•). In cyclohexane: for Lipase MY, ee_{PM} (▽) and X_t (□); for *C. rugosa* lipase, ee_{PM} (△) and X_t (◦)

Table 3

Time courses of Lipase MY mediated hydrolysis of profens thioesters in isooctane

Substrates			naproxen phenyl thioester	naproxen trifluoroethyl thioester	ibuprofen phenyl thioester
100 (h)	ee_P	(%)	78.2	88.0	77.9
	X_t	(%)	15.8	14.4	27.4 (96 h)
194 (h)	ee_P	(%)	75.3	75.5	66.6
	X_t	(%)	20.7	18.7	37.8 (240 h)

Conditions: for naproxen thioesters: 3 mM substrate, 20 mg ml^{-1} enzyme; for ibuprofen thioester: 1.2 mM substrate, 50 mg ml^{-1} enzyme at 37 °C.

2.2. *Enantioselective hydrolysis for activated profen thio esters*

By using Lipase MY as the biocatalyst, water contained in the crude enzyme (6.4%, w/w) and the activated thioesters as substrates in isooctane, the time–course variations of the enantiomeric excess for naproxen (ee_P) and the conversion for the thioester (X_t) are demonstrated in Table 3. After comparing the results with those of thiotransesterification in Table 1, we conclude that Lipase MY has lower enzyme enantioselectivity and activity for hydrolysis. Moreover, very little difference on the enzyme performance was found when either phenyl thioester or trifluoroethyl thioester was the substrate. This result is also valid when ibuprofen phenyl thioester is the substrate if enzyme performances for both phenyl thioesters are compared by considering the effect of lipase concentration on enzyme activity (Table 3).

3. Conclusions

An enzymatic resolution process was developed to produce (*S*)-naproxen esters from racemic naproxen thioesters by using *Candida rugosa* lipase-catalyzed thiotransesterification in organic solvents. Enzyme activity is greatly enhanced when activated thioesters containing an electron-withdrawing group are the substrate. Unlike other lipases, *Candida rugosa* lipase may discern the sulfur moiety of thioesters, and

Table 4

Retention time and capacity factor in HPLC		
Compounds	retention time (min)	capacity factor
2-nitrotoluene	5.2	0.68
(R)- and (S)-naproxen	17.0 and 18.8	4.5 and 5.1
(R)- and (S)-naproxen-4-morpholinoethyl ester	30.0 and 34.5	8.7 and 10.1
(R)- and (S)-naproxen-trifluoroethyl ester	6.7 and 7.2	1.2 and 1.3
(R)- and (S)-naproxen-ethyl thioester	5.6 and 6.3	0.81and 1.0
(R)- and (S)-naproxen-n-propyl thioester	5.3 and 5.9	0.71and 0.90
(R)- and (S)-naproxen-n-butyl thioester	5.1 and 5.6	0.65and 0.81
(R)- and (S)-naproxen-phenyl thioester	8.1 and 8.8	1.6 and 1.8
(R)- and (S)-naproxen-trifluoroethyl thioester	6.4 and 7.5	1.1 and 1.4
racemic flurbiprofen	27.9	8.0
(R)- and (S)-ibuprofen-phenyl thioester	18.7 and 14.3	5.0 and 3.6

yields lower enzyme activity when compared with the result for the corresponding oxygen-containing analogues. Lipase MY has been found to have higher enzyme enantioselectivity and activity when thiotransesterification but not hydrolysis in isooctane was carried out. The enzymatic process was also extended to obtain (*S*)-naproxen or (*S*)-ibuprofen by hydrolyzing the corresponding racemic activated thioesters in isooctane, in which similar enzyme performances were found regardless of the source of thioesters.

4. Experimental

4.1. General remarks on analytical procedure

The thiotransesterification and hydrolysis for naproxen thioesters or transesterification for naproxen ester was monitored by HPLC using a chiral column (Chiralcel OD, Daicel Chemical Industries) capable of separating the internal standard of 2-nitrotoluene, (*R*)- and (*S*)-naproxen as well as their derivatives with retention time and capacity factor shown in Table 4. The mobile phase was a mixture of n-hexane:isopropanol:acetic acid (97:3:1, v/v) at a flow rate of 1.0 ml min^{-1}. UV detection at 270 nm was for quantification at the column temperature of 25°C. For monitoring the hydrolysis of ibuprofen phenyl thioester, the same HPLC was employed except that a chiral column (Chiralcel OJ, Daicel Chemical Industries), an internal standard of racemic flurbiprofen and the composition of the mobile phase (n-hexane:isopropanol:acetic acid=100:0.4:0.4) were changed. The retention time and capacity factor for (*R*)- and (*S*)-phenyl thioesters were also tabulated in Table 4.

4.2. General procedure for the synthesis of racemic naproxen thioesters[11]

To 25 ml of ice-cooled 1,2-dimethoxyethane were added 200 mM of racemic naproxen, 600 mM of anhydrous pyridine, 300 mM of phenyl dichlorophosphate and 320 mM of thiol, which were reacted at room temperature for 16 h with stirring. The resulting solution was added to 50 ml of ice-cooled NaOH solution (1 M), and then 70 ml of chloroform for extraction. The organic layer was separated

Table 5

^{1}H NMR ($CDCl_3$) data for various racemic thioesters and ester
(RS)-naproxen-trifluoroethyl ester δ 1.64 (3H, t), 3.92 (3H, s), 3.94-4.03 (1H, q), 4.28-4.63 (2H, m), 7.13-7.18 (2H, q), 7.37-7.42 (1H, q), 7.67-7.78 (3H, m)
(RS)-naproxen-ethyl thioester δ 1.16-1.23 (3H, t), 1.59-1.62 (3H, d), 2.78-2.90 (2H, m), 3.92 (3H, s), 3.96-4.06 (1H, m), 7.12-7.18 (2H, t), 7.37-7.42 (1H, q), 7.69-7.74 (3H, d)
(RS)-naproxen-n-propyl thioester δ 0.88-0.95 (3H, t), 1.45-1.59 (2H, m), 1.60-1.62 (3H, d), 2.77-2.89 (2H, m), 3.90 (3H, s), 3.91-4.07 (1H, m), 7.12-7.17 (2H, t), 7.37-7.42 (1H, q), 7.69-7.74 (3H, q)
(RS)-naproxen-n-butyl thioester δ 0.87-0.92 (3H, t), 1.29-1.40 (2H, m), 1.45-1.59 (2H, m), 1.60-1.67 (3H, m), 2.74-2.95 (2H, m), 3.91 (3H, s), 3.98-4.08 (1H, m), 7.12-7.19 (2H, t), 7.39-7.44 (1H, q), 7.70-7.51 (3H, t)
(RS)-naproxen-phenyl thioester δ 1.63-1.67 (3H, t), 3.92 (3H, s), 4.03-4.19 (1H, m), 7.14-7.19 (2H, t), 7.31-7.39 (5H, m), 7.41-7.46 (1H, q), 7.72-7.76 (3H, q)
(RS)-naproxen-trifluoroethyl thioester δ 1.63-1.67 (3H, t), 3.36-3.70 (2H, m), 3.92 (3H, s), 4.01-4.12 (1H, m), 7.14-7.20 (2H, q), 7.30-7.38 (1H, m), 7.69-7.75 (3H, t)
(RS)-ibuprofen-phenyl thioester δ 0.89 (3H, s), 0.92 (3H, s), 1.54-1.58 (3H, d), 1.79-1.93 (1H, m), 2.45-2.48 (2H, d), 3.91-4.02 (1H, m), 7.01-7.14 (2H, d), 7.23-7.27 (2H, d), 7.33-7.34 (5H, d)

and washed twice with 50 ml of 1 M NaOH solution and twice with 50 ml of saturated NaCl solution, dried over $MgSO_4$, filtered and concentrated under vacuum. The resulting oil was purified by silica gel liquid chromatography with the mobile phase of n-hexane:ethyl acetate (5:1, v/v), and concentrated in vacuum. The desired racemic naproxen thioesters were confirmed from the retention time in HPLC. ^{1}H NMR spectra were also recorded at 200 MHz on a Bruker AC-200 spectrometer in deuteriochloroform solutions with tetramethylsilane as an internal standard. Chemical shifts are reported in ppm (from TMS) in Table 5. When peak multiplicities are reported, the following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

4.3. *General procedure for the synthesis of racemic naproxen trifluoroethyl ester*[12]

To 20 ml of benzene were added 15 mmol of racemic naproxen and 27 mmol of thionyl chloride, which were heated to 80–90°C with reflux for 1.5 h. The resulting solution was concentrated under vacuum, and 30 ml of benzene, 15 mmol of anhydrous pyridine and 27 mmol of trifluoroethanol were added. The mixture was refluxed at 80–90°C for 4 h. The reaction mixture was cooled to room temperature and extracted four times with 50 ml of NaOH solution (1 M) and twice with 100 ml of deionized water. The organic layer was separated and dried over $MgSO_4$, centrifuged and concentrated under vacuum. The resulting oil was purified by silica gel liquid chromatography with the mobile phase of n-hexane:ethyl acetate (5:1), and concentrated under vacuum. The desired racemic naproxen trifluoroethyl esters, with ^{1}H NMR ($CDCl_3$) data shown in Table 5, were confirmed from the retention time in HPLC.

4.4. General procedure for the thiotransesterification of racemic naproxen thioesters

To 15 ml of isooctane were added 3 mM of racemic naproxen thioester, 10 mM of 4-morpholine ethanol and 20 mg ml^{-1} of Lipase MY (Meito Sangyo), which were reacted with stirring at 37°C. The sample was injected onto the above HPLC system at different time intervals. The active naproxen thioester was determined from the yield and the enantiomeric excess for the ester product. Similar experiments were performed with activated thioesters as the substrate and *Candida rugosa* lipase (Sigma) in cyclohexane (or isooctane) to study effects of lipase sources, solvent hydrophilicity and type of activated thioester on enzyme performances.

4.5. General procedure for the transesterification of racemic naproxen trifluoroethyl ester

To 15 ml of isooctane were added 2 mM of racemic naproxen trifluoroethyl ester, 15 mM of 4-morpholine ethanol and 5 mg ml^{-1} of Lipase MY or *C. rugosa* lipase, which were reacted with stirring at 37°C. The sample was injected onto the above HPLC system at different time intervals, from which yields and the enantiomeric excesses for the ester and acid products were calculated.

4.6. General procedure for the hydrolysis of racemic naproxen thioesters

To 15 ml of isooctane were added 20 mg ml^{-1} of Lipase MY and 3 mM of activated naproxen thioesters, which were reacted with stirring at 37°C. The sample was injected onto the above HPLC system at different time intervals, from which the yield and the enantiomeric excess for the acid product were calculated.

4.7. General procedure for the synthesis of racemic ibuprofen phenyl thioester[11]

To 10 ml of ice-cooled 1,2-dimethoxyethane were added 200 mM of racemic ibuprofen, 600 mM of anhydrous pyridine, 300 mM of phenyl dichlorophosphate and 400 mM thiol, which were heated with stirring to room temperature over 12 h. To the resulting solution was added 20 ml of ice-cooled NaOH solution (1 M), and then 25 ml of chloroform for extraction. The organic layer was separated and washed twice with 20 ml of 1 M NaOH solution and twice with 25 ml of saturated NaCl solution, dried over $MgSO_4$, filtered and concentrated under vacuum. The resulting oil was purified by silica gel liquid chromatography with the mobile phase of n-hexane:ethyl acetate (50:3, v/v), and concentrated under vacuum. The desired racemic ibuprofen phenyl thioester, with ^1H NMR ($CDCl_3$) data shown in Table 5, was confirmed from the retention time in HPLC.

4.8. General procedure for the hydrolysis of ibuprofen phenyl thioester

To 20 ml of isooctane were added 50 mg ml^{-1} of Lipase MY and 1.2 mM of ibuprofen phenyl thioester, which were reacted with stirring at 37°C. The sample was injected onto the above HPLC system at different time intervals, from which the yield and the enantiomeric excess for the acid product were calculated.

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