

African Journal of Biotechnology Vol. 8 (21), pp. 5958-5963, 2 November, 2009
Available online at <http://www.academicjournals.org/AJB>
ISSN 1684-5315 © 2009 Academic Journals

Full Length Research Paper

Enzymatic hydrolysis of esters from 2-carboxy-6-methoxy-2,3-dihydrobenzofuran acid

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Accepted 21 September, 2009

In this work we describe the results of the enzymatic hydrolysis of esters from 2-carboxy-6-methoxy-2,3-dihydrobenzofuran acid using *Candida antarctica* lipase supported on acrylic resin, with enantiomeric excesses ranging between 80 to 99%.

Key words: 2-Carboxy-6-methoxy-2,3-dihydrobenzofuran acid, *Candida antarctica* lipase, enantioselective hydrolysis.

INTRODUCTION

The 2, 3-dihydrobenzofuran system is the core of a large number of important compounds which are present in nature or have been obtained through synthesis (Pieters et al., 1999). These compounds have a variety of significant pharmacological activities, some of which are cytotoxic and can be found in both roots and leaves of plants (Tsai et al., 1998). Other 2,3-dihydrobenzofuran derivatives are used in the treatment of diabetic retinopathy and arthritis (Apers et al., 2002); others are used in the therapy for arteriosclerosis, liver diseases and in the treatment of vascular diseases of the brain (Kueth et al., 2005). Some synthetic dihydrobenzofurans have even proved to be better antioxidants than vitamin E (Nicholas et al., 2006; Park et al., 2001). On other hand, some compounds with the 2, 3-dihydrobenzofuran system have been tested in the production of liquid crystal displays in television sets of 25 inches or larger (Bremer and Lietzau, 2005).

Enantiomerically pure 2, 3-dihydrobenzofuran acids have been obtained by resolution through the corresponding diastereoisomeric salts using chiral amines like

phenylethylamine and amphetamines (Harada et al., 1968; Tovar-Miranda et al., 1999). This last procedure is very tedious because the methodology requires firstly the salt to be a solid; then this salt must be recrystallized several times to reach a constant optical rotation. After this, the salt must be treated with HCl to recover the enantiomerically pure acid. The present study describes the results when supported on acrylic resin Lipase B from *Candida antarctica* is used in the enzymatic resolution of a substantially flat structure as 2,3-dihydrobenzofuran acid that is the core of a large number of compounds showing important pharmacological activities, which by other methods are very difficult to carry out.

MATERIALS AND METHODS

Chemicals

The solvents, dioxane (D), *ter*-butyl alcohol (tB) and methanol were purchased from Sigma-Aldrich and used without any treatment, at least it is indicated. The esters were purified on column chromatography using silica gel 60 (0.040 - 0.063 mm) purchased from Merck. *p*-Toluenesulfonic acid and anhydrous sodium sulfate were obtained from JT Baker. Lipase B from *C. antarctica*, Novozym was obtained from Novo Nordisk Co. and employed without any further treatment.

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Analytical

The reaction products were identified by Nuclear Magnetic Resonance, ^1H and ^{13}C NMR measurements were performed on a Varian Mercury Plus 300BB using deuterated chloroform (CDCl_3) solutions containing TMS as internal standard and compared with literature reports (Tovar-Miranda et al., 1999). Enantiomeric excesses were determined as the corresponding methyl esters by High Performance Liquid Chromatography (HPLC) using a Chiracel-OD column (250 mm length \times 4.6 mm i.d.) with a DAD detector and a solvent system (mobile phase) of hexane/isopropyl alcohol (90:10); the flow rate was kept at 0.8 ml/min during the analysis. The optical rotation was determined in a Perkin-Elmer polarimeter, Mod. 341 and compared with those previously described (Tovar-Miranda et al., 1999). TLC were performed on aluminium sheets (5 \times 5 cm) silica gel 60 F_{254} from Merck, eluted with hexane-ethyl acetate (80:20) and detected with UV-lamp at 254 nm.

Typical procedure for the enzymatic hydrolysis

To a solution of 100 mg of ester in 2 ml of dioxane-*ter*-butanol (9:1), were added 50 mg of *C. antarctica* lipase B supported on acrylic resin. The reaction mixture was stirred at 180 or 300 rpm at room temperature. The reaction progress was followed by Thin Layer Chromatography (TLC). After 60 min the reaction mixture was filtered through a cotton plug and the enzyme washed with dichloromethane. The organic layer was washed twice with 5% NaOH (15 ml), followed by water (2 \times 15 ml) and dried with anhydrous sodium sulfate and evaporated to yield the corresponding ester as a colorless liquid. The acid was recovered from the aqueous phase by acidification with HCl followed by extraction with ethyl ether. The organic layer was washed with water until the pH was near 7, dried with anhydrous sodium sulfate and evaporated to give a brown solid. The obtained acid was esterified by refluxing with methanol and *p*-toluenesulfonic acid for 2 h, to yield the corresponding methyl ester which was purified by column chromatography using hexane-ethyl acetate (90:10) for its chromatographic analysis.

General procedure for preparative experiments

In an Erlenmeyer flask were dissolved 700 mg of **5d** or **5e** in 14 ml of D-tB (9:1) and 350 mg of acrylic supported enzyme were added. The reaction mixture was stirred at room temperature and the progress of the reaction was monitored by TLC. Then the reaction mixture was filtered through a cotton plug and rinsed with dichloromethane. The organic layer was washed with 5% NaOH (2 \times 20 ml), followed by water (2 \times 20 ml). The solvent was dried and evaporated to yield 314 mg (45%) of unreacted ester. The aqueous layers were combined and acidified with HCl and extracted with ethyl ether; the organic layer was washed with water (2 \times 20 ml) and brine (2 \times 20 ml), dried with anhydrous Na_2SO_4 and evaporated to give 297 mg (54%) of the corresponding acid as a brown solid.

RESULTS AND DISCUSSION

The acid **5** was prepared following the procedure shown in Figure 1, which has been described in the literature (Fuson et al., 1944; Witiak et al., 1971), starting with the umbeliferone **2**. For this study, the esters (**6a-g**) for the enzymatic resolution were obtained through the reaction

of **5** with the corresponding alcohols: methyl, ethyl, isopropyl, *n*-butyl, isobutyl, benzyl and heptyl, in the presence of *p*-toluenesulfonic acid.

From a previous work (Villanueva-Lendecky et al., 2000), dioxane (D), *ter*-butanol (tB) or mixtures thereof were chosen as solvent for carrying out the enzymatic reactions. It was also decided to use the ester **6b** to determine the solvent or solvents mixture that could give the best enantioselectivity by the enzymatic hydrolysis. Dioxane was initially used and then, in an attempt to improve the enantioselectivity, the following mixtures of dioxane (D) and *ter*-Butanol (tB) were tested; 9:1, 7:3, 1:1, 3:7 and 1:9. These reactions were carried out at room temperature; for these experiments a 2:1 ratio substrate/*C. antarctica* lipase (Novozym)(w/w) were used. Based on these results, it was possible to establish that the mixture of dioxane-*ter*-butanol 9:1 (D-tB 9:1) was the most adequate for evaluating the behavior of the other esters during the enzymatic reaction, in order to determine the effect of the chain length on the resolution process. The absolute configuration of the obtained acid was determined by the comparison of the optical rotation of the methyl esters with reported data (Tovar-Miranda et al., 1999).

Interestingly, the hydrolysis took place in an apparent absence of water, because no water was added to the reaction medium and the solvents were supposed to be pure. It is worth mentioning that the solvents used in these reactions were taken directly from the bottle without any additional purification; consequently they may have contained some water which could be the responsible for the hydrolytic reaction. The enzyme used was the fraction B of *C. antarctica* lipase (CAL B) and it was also used without any additional treatment. These facts prompted us to carry out an experiment using anhydrous solvents; dioxane was dried from sodium-benzophenone, while *ter*-butanol was distilled from sodium. Under equivalent reaction conditions, but under a nitrogen atmosphere, the ethyl ester gave an enantiomeric excess (ee) of 59%.

To determine the influence of the amount of water on the enantioselectivity of the enzymatic hydrolysis, known amounts water were added to the anhydrous solvents and the enantioselectivity was determined by using the ethyl ester as substrate because it showed the greatest enantioselectivity in an anhydrous mixture of D:tB (9:1). The quantities of added water were 2.5, 5.0 and 7.5% (v/v) and the results were 50, 74 and 59% ee respectively. It was clear that some amount of water (around 5%; v/v) was necessary for the adequate activity of the enzyme.

In other experiments, the influence of the ester chain on enantioselectivity was studied. Consequently the hydrolysis of these all esters (**6a-g**) was carried at room temperature, but the enantioselectivity of the reaction was also assayed at low temperature, specifically at 0°C. Table 1 summarizes the results of these experiments.

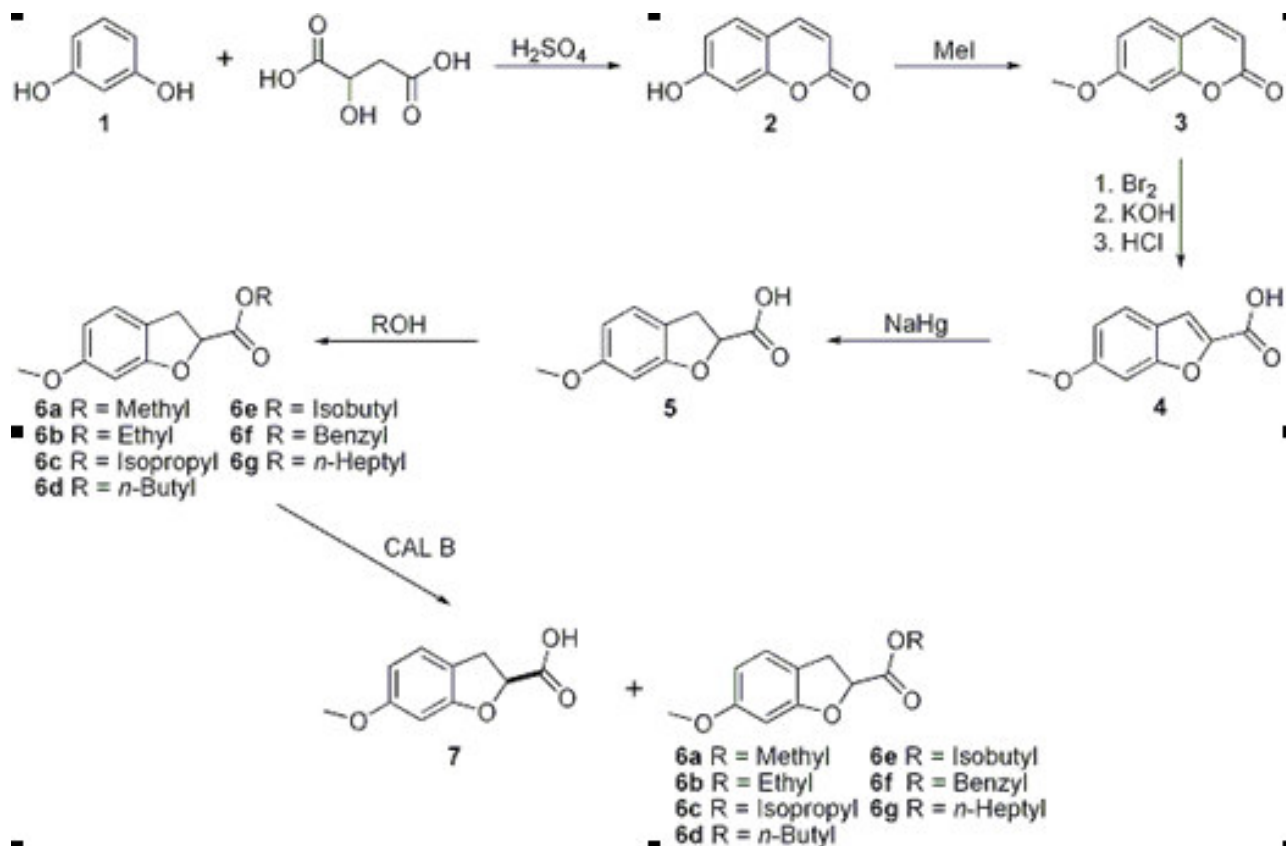


Figure 1. Synthesis and enzymatic hydrolysis of substrates 6a - g.

Table 1. Enzymatic hydrolysis of the esters^a at room temperature and 0 °C.

Compound	Solvent	ee (ester) ^c	ee (acid) ^d	Conversion (%)	E ^e
6a ^b	D- <i>t</i> B 9:1	65.9	27.0	27.2	3
6b ^b	D- <i>t</i> B 9:1	66.6	75.5	45.1	14
6c ^b	D- <i>t</i> B 9:1	10.7	43.8	14.6	3
6d ^b	D- <i>t</i> B 9:1	48.8	83.7	52.6	18
6e ^b	D- <i>t</i> B 9:1	7.0	88.8	34.9	18
6f ^b	D- <i>t</i> B 9:1	9.7	33.2	20.9	2
6g ^b	D- <i>t</i> B 9:1	41.3	78.9	38.5	12
6a ^f	D- <i>t</i> B 9:1	63.6	28.8	57.2	3
6b ^f	D- <i>t</i> B 9:1	64.9	72.1	48.3	11
6c ^f	D- <i>t</i> B 9:1	11.6	40.4	17.5	3
6d ^f	D- <i>t</i> B 9:1	44.7	79.4	41.1	13
6e ^f	D- <i>t</i> B 9:1	16.1	83.0	35.2	12
6f ^f	D- <i>t</i> B 9:1	12.8	46.6	25.9	3
6g ^f	D- <i>t</i> B 9:1	42.4	77.8	36.0	12

^aReaction cond.: 100 mg of ester and 50 mg of enzyme; magnetic stirring, 1 h.

^bReaction carried out at room temperature.

^cDetermined by HPLC.

^dOptical purity of the acid was determined as the methyl ester by HPLC.

^eE values of the isolated acid 7; calculated as methyl ester using the program "Selectivity" by K. Faber and H. Hoening, <http://borgc185.kfunigraz.ac.at>.

^fReaction carried out at 0 °C.

Table 2. Enzymatic hydrolysis^a at room temperature and 0°C.

Compound	Solvent	ee (ester) ^c	ee (acid) ^d	Conversion (%)	E ^e
6a ^b	D- <i>t</i> B 1:9	71.5	24.8	59.1	3
6b ^b	D- <i>t</i> B 1:9	75.3	21.5	64.1	3
6c ^b	D- <i>t</i> B 1:9	10.8	12.2	35.7	1
6d ^b	D- <i>t</i> B 1:9	71.5	41.3	54.5	5
6e ^b	D- <i>t</i> B 1:9	1.7	53.2	49.9	3
6f ^b	D- <i>t</i> B 1:9	59.2	21.3	57.1	3
6g ^b	D- <i>t</i> B 1:9	69.2	41.0	53.6	5
6a ^f	D- <i>t</i> B 1:9	71.8	18.5	61.3	3
6b ^f	D- <i>t</i> B 1:9	74.8	31.4	56.4	4
6c ^f	D- <i>t</i> B 1:9	15.9	17.4	40.5	2
6d ^f	D- <i>t</i> B 1:9	68.8	48.2	48.0	6
6e ^f	D- <i>t</i> B 1:9	2.3	52.6	48.5	3
6f ^f	D- <i>t</i> B 1:9	57.4	15.1	57.2	2
6g ^f	D- <i>t</i> B 1:9	62.0	33.1	52.6	4

^aReaction cond.:100 mg of ester and 50 mg of enzyme; magnetic stirring, 1 h.

^bReaction carried out at room temperature.

^cDetermined by HPLC.

^dOptical purity of the acid was determined as the methyl ester by HPLC.

^eE values of the isolated acid **7**; calculated as methyl ester.

^fReaction carried out at 0°C.

From the Table 1 it can be established that the best results were obtained with the *n*-butyl (**6d**) and *isobutyl* (**6e**) esters, which showed 83.7 and 88.8% ee respectively, for the acid, at room temperature in D-*t*B (9:1). Similar enantioselectivity was observed when the reaction took place at 0°C, the acid being produced in 79.4 and 83.0% of ee, respectively. Without a doubt, this optical purity can be rise to values higher than 90% ee through just one recrystallization of the obtained acid. A direct relationship between the enantioselectivity and the kind and length of the ester chain also seems to exist, since the heptyl ester also showed acceptable values (79 and 78% of ee), both at room temperature and at 0°C, respectively. So, these results show that the reaction and the enantioselectivity are better with linear chain esters.

The effect of mixture of solvents was re-evaluated, but this time involving all esters. The experiments were carried out inverting the ratio of solvents to D-*t*B 1:9. The reactions were carried out once again at room temperature and at 0°C and the results are detailed in Table 2.

The enzyme in this solvent mixture (D-*t*B 1:9) behaved in a similar way at both room temperature and 0°C. Under these conditions, the enzyme showed greater selectivity toward short-chain esters (**6a** and **6b**, 72 and 75% ee, respectively). It was also observed that when the mixture D-*t*B (1:9) was used, the conversions were greater than with D-*t*B (9:1), comparing Tables 1 and 2. From the results it can also be established that for esters **6c** and **6f** the hydrolysis did not show good enantioselectivity under any of the reaction conditions studied (temperature

and solvents); this may be due to steric effect.

The ethyl ester (**6b**) was the derivative that showed the best enantioselectivity of all the esters in this study under any of the reaction conditions. In addition, there appears to be a solvent effect on **6b**, because when D-*t*B 1:9 was used, the ester was recovered in an acceptable ee, 75.3 and 74.8% of ee, at room temperature and 0°C, respectively (Table 2). In contrast, when D-*t*B 9:1 was used, the remaining ester **6b**, at room temperature and 0°C (Table 1), gave 66.6 and 64.9 % ee and the produced acid showed the greatest optical purity, 75.5 and 72.1% of ee (Table 1). This could be interpreted as an effect of the bulkiness of the alcohol on the active site of the enzyme during the transition state. On the other hand, conversion and yields differ in each solvent mixture, being highest in D-*t*B 9:1; this suggests that the reaction time becomes very important and that sometimes an hour of reaction is too long for this experiment.

Interesting results were observed when the length of the hydrocarbonated chain increased, as in the case of **6d**. The experiments indicated that the solvent intervened again in the selectivity, because in D-*t*B 1:9 the remaining ester was isolated in good selectivity, 71.5% of ee (Table 2), at room temperature. When the solvent was changed (D-*t*B 9:1), the selectivity changed too and the acid acquired the greatest enantiomeric excess, with value of 83.7% of ee (Table 1), for the reaction at room temperature, whereas for the reaction at 0°C, the acid was isolated in 79.4% of ee (Table 1).

From these results, it becomes evident that there is a

Table 3. Enzymatic hydrolysis of esters^a **6d** and **6e** at room temperature with stirring at 180 and 300 rpm.

Entry	Ester	Time (min)	Conversión (%)	ee (ester)	ee (acid) ^b	E ^d
1 ^c	6d	30	27.3	41.8	88.0	23
2 ^c	6d	60	19.6	64.4	89.9	36
3 ^c	6d	90	21.3	7.8	85.6	14
4 ^c	6d	120	31.9	58.6	74.8	12
5 ^c	6d	150	30.3	59.7	68.8	10
6c	6e	30	11.6	16.5	92.5	32
7 ^c	6e	60	26.9	5.6	90.9	22
8 ^c	6e	90	19.5	46.1	83.5	18
9 ^c	6e	120	22.7	25.0	98.7	254
10 ^c	6e	150	22.6	21.4	84.2	14
11 ^d	6d	30	38.5	43.4	83.0	16
12 ^d	6d	60	40.5	51.4	74.8	11
13 ^d	6d	90	43.0	66.0	73.3	12
14 ^d	6d	120	44.5	59.2	62.5	8
15 ^d	6d	150	49.0	64.7	54.3	6
16d	6e	30	29.6	27.1	85.4	16
17 ^d	6e	60	36.9	4.6	84.3	12
18 ^d	6e	90	40.0	17.3	97.1	77
19 ^d	6e	120	43.1	16.1	78.2	10
20 ^d	6e	150	46.5	2.4	85.0	12

^aReaction cond.:100 mg of ester and 50 mg of enzyme.^bOptical purity of acid was determined as methyl ester.^cStirred at 180 rpm.^dStirred at 300 rpm.^eE values of the isolated acid **7**; calculated as methyl ester.

combined effect between the structure of the ester alkyl chain and the reaction solvent, for example, the ester **6a**, being the smallest, did not suffer any influence from the solvent or the temperature of reaction; on the contrary, ester **6f** showed a substantial improvement in enantioselectivity due to an increase in conversion, when the 9:1 solvent mixture changed to 1:9 (Tables 1 and 2).

According to the obtained results, **6d** and **6e** were the derivatives for which the enzyme showed greater enantioselectivity regarding the produced acid, **7**. Therefore it was decided to carry out experiments to determine the reaction time to get the maximum optical purity of the hydrolysis product. These experiments were carried out at two speeds of stirring, 180 and 300 rpm, in order to also evaluate the effect of agitation on the reaction.

From the results of the reaction conducted at 180 rpm (Table 3), it was observed that **6d** at 60 min. (entry 2) gave the isolated acid in good optical purity (89.9% of ee, E = 36), while the remaining isolated ester in this experiment showed an acceptable ee (64.4%). In the case of **6e**, truly amazing results were obtained because the acid was isolated with an optical purity of 98.7% of ee, with an E = 254, after 120 min of reaction (entry 9); as seen in

Table 3, the good enantioselectivity was observed from the first minutes of the reaction onward.

The results of the reactions taking place at the higher speed of stirring, 300 rpm are also shown in Table 3. The optical purities of the isolated acids were good but slightly lower than those obtained at 180 rpm; an example of this was the hydrolysis of **6d**, which had a good enantioselectivity after 30 min (entry 11, Table 3). It is also noticeable that, in general, the conversions were almost duplicated in the reactions at higher stirring speed. Similarly, in the case of ester **6e** (entry 18), 90 min was the reaction time required to obtain the product of hydrolysis in its best optical purity, 97% of ee, with an E = 77. From these results seems possible that the speed of agitation was not decisive in regard to the enantioselectivity, but it did influence the rate of the hydrolysis reaction. Undoubtedly, the size of the alcohol chain was more important and the results indicated the ester **6e** as the derivative with the best stereoselectivity.

Preparative scale experiments were carried out to determine the influence of the enzyme and substrate concentration on the enantioselectivity of the reaction with esters **6d** and **6e**; the results of these experiments

Table 4. Effect of reaction concentration on the enzymatic hydrolysis of esters **6d** and **6e**^a.

Entry	Ester	Subst. conc. (mg/ml)	Conversion (%)	ee (%) ester	ee (%) ^e acid	E ^f
1	6d ^b	50	53.6	58.9	49.9	5
2	6d ^c	25	45.0	74.0	64.3	10
3	6d ^d	100	43.8	38.9	41.6	4
4	6e ^b	50	52.3	95.3	64.3	16
5	6e ^c	25	40.0	89.3	48.2	8
6	6e ^d	100	45.1	91.7	76.5	24

^aReaction: Subst/enzyme 2:1(w/w) at 25 °C, at 180 rpm, in D-rB (9:1), for 4 h.

^bReaction with 700 mg of ester.

^cDiluted reaction with 100 mg of ester.

^dConcentrated reaction with 100 mg of ester.

^eOptical purity of the acid was determined as the methyl ester by HPLC.

^fE values of the isolated acid **7**; calculated as methyl ester.

are summarized in Table 4. Diluted reactions (entries 2 and 5) as well as concentrated ones (entries 3 and 6) were carried out. The best conditions resulted in the concentrated reaction with the ester **6e** (entry 6), both products of the reaction showed the high enantioselectivity (91.7 and 76.5% ee). It is worth to mention that in entry 4, the isolated remaining ester showed with an excellent optical purity, 95% of ee. The isolated acids from the preparative reactions were recrystallized and then esterified to determine their optical purity; thus, the acid from **6d** increased its ee from 49.9 to 71.2%, whereas the acid from **6e** increased from 64.3 to 82.3%; these increases in optical purity were obtained with just one recrystallization.

Conclusion

The best selectivity was observed for the *n*-butyl and isobutyl esters at both temperatures room and 0 °C; in both cases the acid showed the highest optical purity, although the methyl and ethyl esters showed an acceptable selectivity at both temperatures. It was found that the composition of the solvent mixture had a slight influence on the selectivity, especially for the short-chain esters. As previously noted, **6b** was the ester that most constantly showed high selectivity under all reaction conditions; it was observed that **6d** and to a lesser extent **6g**, showed even higher selectivity, but only with certain solvent mixtures. Although, for this case the recovered esters had the highest optical purity. The experiments also suggest that the speed of agitation does not significantly influence the enantioselectivity of the enzyme. When the substrate concentration in the reaction was changed, only for the **6d** and **6e** derivatives, **6e** presented the best behavior in all cases; this apparently indicates that the concentration of the reaction is not important, even during preparative experiments. Interestingly, the compound with the highest optical purity

in these preparative reactions was the isolated ester; whereas in the experiments carried out with 100 mg, it was the isolated acid that showed the highest optical purity, under the same conditions.

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