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# Hydrolysis of New Phthalimide-Derived Esters Catalyzed by Immobilized Lipase

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## Abstract

The last step of the production of four phthalimide-derived acids, designed to act as antiasthma drugs, was performed by enzymatic hydrolysis of the respective methyl or ethyl esters. The esters 4-ethyl-[2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)]-phenoxyacetic methyl ester (PHT-MET), 4-ethyl-[2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)]-phenoxyacetic ethyl ester, 4-(1, 3-dioxo-1,3-dihydro-2-isoindoylyl)-phenoxyacetic ethyl ester, and 2-(1,3-dioxo-1, 3-dihydro-2-isoindoylyl)-phenoxyacetic ethyl ester were hydrolyzed by immobilized lipase. The enzymatic reaction could be used only to produce the desired 4-substituted compounds. The best result that was found to hydrolysis of PHT-MET, and, therefore, that ester was selected for optimization experiments in a three-phase system. Reactions were performed with solid biocatalyst (Lipozyme® RM IM), organic solvent phase (ethyl acetate), and aqueous phase (saturated $Na_2CO_3$ solution). To optimize the reaction conditions, an experimental design optimization procedure was used. The variables studied were the amount of enzyme, the temperature, and the volume of the aqueous solution. Time course experiments were then performed for different initial enzyme concentrations (0.5, 0.9, and 1.4 $U_H$/mL of solvent). The optimized reaction conditions found were 20 mg of Lipozyme (0.9 $U_H/mL_{solvent}$) and 5.0 mL of $Na_2CO_{3(sat)}$ at 40°C for 6 h.

**Index Entries:** Immobilized lipase; asthma drug; phthalimide compounds; organic media; biocatalysis.

## Introduction

Asthma is a chronic inflammatory disease that is normally associated with clinical symptoms, such as dyspnea, coughing, sneezing, chest tightness,

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and narrowing of the bronchia. Those symptoms result from physiologic processes including edema, influx and activation of inflammatory cells, hypersecretion of mucus and contraction of the bronchial tubes, followed by muscle hypertrophy and hyperplasia. A further effect is bronchi spasm, one of the main characteristics of the reversible obstruction of the respiratory tract *(1,2)*.

In general, modern asthma treatments involve the use of drugs, such as eosinophilia inhibitors, cell adhesion blockers, phosphodiesterase, and tumor necrosis factor inhibitors, and antileukotrienes, which combat stages of the inflammatory process of this disease. The last three drugs have been found to be the most promising, because they can be used to treat chronic asthma with the least side effects *(3)*.

The planning, synthesis, and pharmacologic evaluation of potential new prototype antiinflammatory and antiasthmatic agents involve the investigation of new derivatives from families of phthalimide compounds. The derivatives proposed by Lima *(4)* include acids such as 4-ethyl-[2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)]-phenoxy acetic acid, designed to act as a leukotriene D4 receptor antagonist. However, this prototype drug needs the chemoselective hydrolysis of esters as precursors for its synthesis. The difficulty of achieving high yields through chemoselective hydrolysis using conventional organic chemistry has led to a new approach using lipases as biocatalyst for the hydrolysis of these prototype esters.

Lipases (glycerol ester hydrolases; EC 3.1.1.3) catalyze the hydrolysis of long-chain acyl glycerol esters. Not only do they act on these substrates, but they also are capable of catalyzing hydrolysis and synthesis reactions in ester groups of different compounds. In particular, lipases are a class of enzyme that is of great importance to the research of biocatalysis applied to traditional organic chemistry. The good stability of lipases in the presence of organic solvents makes them appropriate for use in intermediate stages of conventional chemical processes and in the catalysis of chemical reactions involving substrates that are insoluble in aqueous media.

One example of the economic importance of lipases in drug production processes is the production of diltiazen hydrochloride, which is useful as a coronary vasodilator. Since 1993, Tanabe Seiaku S.A. has used enzymatic resolution to produce 50 t per year. of intermediate chiral, which is subsequently converted into diltiazem *(5,6)*.

Many different studies have investigated the development of the stereoselectivity of lipases, involving different aryl propionic acids as target substrates, such as (*R*,*S*)-ibuprofen, ketoprofen, flurbiprofen, and naproxen *(6–12)*.

The aim of the present study was to evaluate the chemoselective hydrolysis, catalyzed by immobilized lipase, of different potential prototype antiasthmatic agents from the phthalimide ester family.

## Materials and Methods

### *Enzyme and Substrates*

Lipozyme® RM IM (commercial lipase preparation of *Rhizomucor miehei*, immobilized in an anion-exchange resin) was kindly donated by Novozyme (Denmark). Acetonitrile was high-performance liquid chromatography grade, and all the other reagents and solvents were analytical grade.

4-Ethyl-[2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)]-phenoxyacetic methyl ester (PHT-MET), 4-ethyl-[2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)]-phenoxyacetic ethyl ester (PHT-ET), 4-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)-phenoxyacetic ethyl ester (*p*-PHT-ET), and 2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)-phenoxyacetic ethyl ester (*o*-PHT-ET) were produced by LASSBio/UFRJ (Brazil) and were used as substrates. Table 1 shows their routes of synthesis. 4-Ethyl-[2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)]-phenoxy acetic acid (LASSBio 482), used as a standard for HPLC, was provided by LASSBio/UFRJ. The structures of the substrates for hydrolysis were confirmed by nucleur magnetic resonance of the hydrogen (NMR $^1H$).

### *Hydrolysis Reaction*

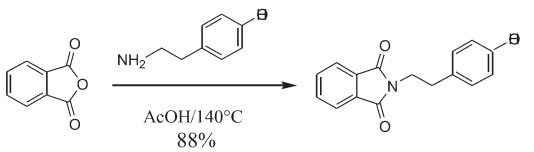
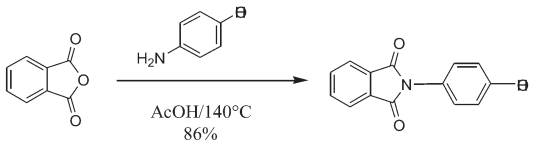
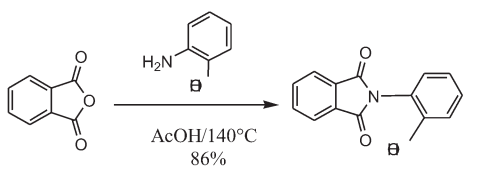
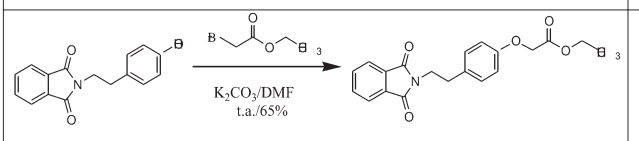
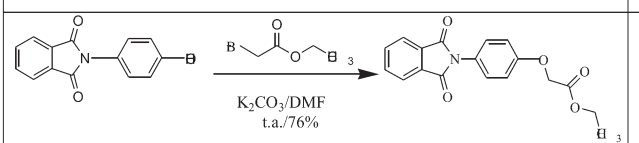
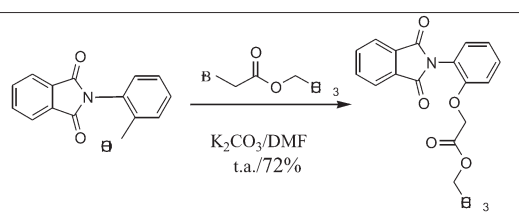
#### Hydrolysis of Different Substrates in a Two-Phase System

Hydrolysis experiments of different substrates (Table 2) were performed in a two-phase system in closed-batch stirred reactors using 10 mg of each substrate (PHT-ET, PHT-MET, *p*-PHT-ET, *o*-PHT-ET), 2.5 mL of ethyl acetate or acetone, and 20 mg of Lipozyme RM IM (0.94 $U_H$/mL) at 40°C for 4 h. The reaction was stopped by removing the enzyme from the reaction medium. The organic phase (containing the substrate and product) was rotaevaporated and analyzed by HPLC.

#### Hydrolysis of PHT-MET in a Three-Phase System

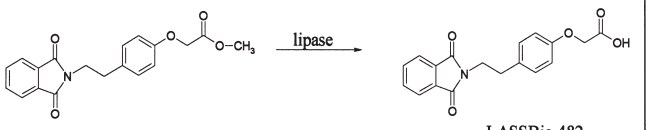
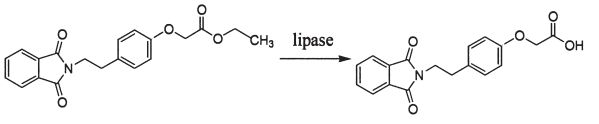
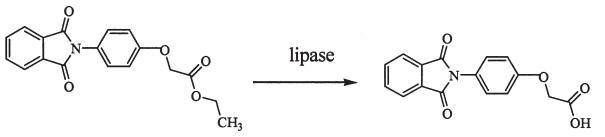
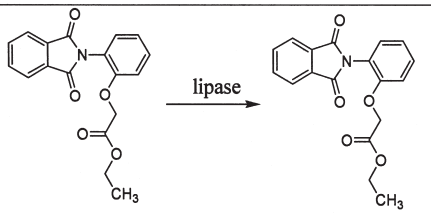
Experiments were performed in a three-phase reaction system comprising a solid biocatalyst (immobilized *R. miehei* lipase, Lipozyme RM IM), an organic phase (ethyl acetate), and an aqueous phase (saturated $Na_2CO_3$ solution). The main reason for choosing this three-phase reaction system was to provide water for the hydrolysis reaction while shifting the reaction equilibrium toward the main product by extracting it from the organic phase. The reaction was stopped by removing the enzyme from the reaction medium. The organic and aqueous phases were separated after decantation. The product (LASSBio 482) was recovered from the aqueous phase by adding HCl solution (1:2) until reaching pH 3.0, and was extracted with ethyl acetate. A control experiment with no enzyme was performed using 10 mg of PHT-MET, 2.5 mL of ethyl acetate, and 2.5 mL of saturated $Na_2CO_3$ at 40°C.

Table 1
Routes of Synthesis for Obtaining Phthalimide-Derived Ester Substrates[a]

| Reaction | Product characteristics |
|---|---|
|   | 2-(4-Hydroxyphenethyl) -1,3-isoindoleinodione ($C_{16}H_{13}NO_3$), MM=267g/mol, white powder, mp.:230-232°C |
|   | 2-(4-Hydroxyphenyl)-1,3-isoindoleinodione ($C_{14}H_9NO_3$), MM=239g/mol, yellow powder, mp.:300°C. |
|   | 2-(2-Hydroxyphenyl)-1,3-isoindoleinodione ($C_{14}H_9NO_3$), MM=239g/mol, rose crystals, mp.:224-226°C. |
|   PHT-ET | 4-ethyl-(2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl))-phenoxyacetic ethyl ester ($C_{20}H_{19}NO_5$), MM=353g/mol, white crystals, mp.: 105-107°C |
|   *p*-PHT-ET | 4-(1,3-dioxo-1,3-dihydro-2-isoindoylyl)-phenoxyacetic ethyl ester ($C_{18}H_{15}NO_5$), MM=325g/mol, yellow powder, mp.: 131-134°C |
|   *o*-PHT-ET | 2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl))-phenoxyacetic acid ethyl ester , MM=325 g/mol, rose powder, mp.: 137-138°C |

[a] DMF, dimenthylformamide; t.a., room temperature; MM, molecular mass; mp, melt point.

Table 2
Enzymatic Hydrolysis of Phthalimide-Derived Ester Substrates[a]

| Reaction | Conversion |
|---|---|
|   | 50% |
|   | 20% |
|   | 20% |
|   | Not hydrolysed. |

[a]Hydrolysis conditions were: 10 mg of substrate, 20 mg of Lipozyme RM IM, and 2.5 mL of ethyl acetate.

The product and substrate present in the organic phase and organic extract from the aqueous phase were quantified by HPLC analysis. Some samples were analyzed by NMR $^1H$ in order to verify whether the phthalimide group of the molecule, which is responsible for the biologic activity, was preserved.

## *Time Course Experiments*

Time course hydrolysis experiments were performed using 10 mg of PHT-MET; 2.5 mL of ethyl acetate; 2.5 mL of saturated aqueous $Na_2CO_3$; and 10, 20, and 30 mg of Lipozyme RM IM. The reaction times were 0.5, 1, 2, 4, 6, 8, and 15 h. The reactions were halted by removing the enzyme, and the product was extracted as described in the previous section.

## Analytical Methods

### NMR $^1$H

NMR $^1$H spectra were recorded using a Varian XI-200 or Brucker DPX-200 at 200 MHz. The samples were dissolved in a deuterated solvent (dimethyl sulfoxide [DMSO] $d_6$), and tetramethylsilane was used as an internal reference. NMR $^1$H of LASSBio 482 (200 MHz; DMSO $d_6$); δ: 2.86 (t; J = 7.28 Hz; $R[CO]_2NCH_2C\underline{H}_2R$); 3.78 (t; J = 7.28 Hz; $R[CO]_2NCH_2C\underline{H}_2R$); 4.62 (s; $ArOC\underline{H}_2CO_2H$); 6.82 (d; J = 8.52 Hz; H3′ and H5′); 7.12 (d; J = 8.52 Hz; H2′ and H6′); 7.83 (s; H5-H6 and H4-H7) ppm. NMR $^1$H of PHT-MET (200 MHz; $CDCl_3$): δ: 2.92 (t; J = 7.64 Hz; $R[CO]_2NCH_2C\underline{H}_2R$); 3.79 (s, $ArOCH_2CO_2C\underline{H}_3$); 3.88 (t; J = 7.64 Hz; $R[CO]_2NC\underline{H}_2CH_2R$); 4.60 (s; $ArOC\underline{H}_2CO_2CH_3$); 6.82 (d; J = 8.47 Hz; H3′ and H5′); 7.12 (d; J = 8.42 Hz; H2′ and H6′); 7.69 (m; H5 and H6); 7.81 (m; H4 and H7) ppm. NMR $^1$H of PHT-ET (200 MHz; $CDCl_3$): δ: 1.29 (t; J = 7.14 Hz; $RCO_2CH_2C\underline{H}_3$); 2.93 (t; J = 7.51 Hz; $R[CO]_2NCH_2C\underline{H}_2R$); 3.88 (t; J = 7.51 Hz; $R[CO]_2NCH_2C\underline{H}_2R$); 4.28 (q; J = 7.14 Hz; $RCO_2C\underline{H}_2CH_3$); 6.84 (d; J = 8.70 Hz; H3′ and H5′); 7.18 (d; J = 8.61 Hz; H2′ and H6′); 7.72 (m; H5 and H6); 7.83 (m; H4 and H7) ppm. NMR $^1$H of *o*-PHT-ET (200 MHz; $CDCl_3$): δ: 1.15 (t; J = 7.14 Hz; $ROCH_2CO_2CH_2C\underline{H}_3$); 4.15 (q; J = 7.14 Hz; $ROCH_2CO_2C\underline{H}_2CH_3$); 4.62 (s; $ROCH_2CO_2CH_2CH_3$); 6.97 (dd; J = 8.33 and 1.10 Hz; H3′); 7.15 (dt; J = 7.60 and 7.60 Hz; H5′); 7.32 (dd; J = 7.78 and 1.74 Hz; H6′); 7.42 (dt; J = 8.30 and 7.51 Hz; H4′); 7.78 (m; H5 and H6); 7.95 (m; H4 and H7) ppm.

### High-Performance Liquid Chromatography

The reaction products were quantified in a Waters HPLC system consisting of a 510 pump, a 486 UV/Visible detector, and a reverse-phase column (Novapak C18). The products were detected at 280 nm. The mobile phase was composed of acetonitrile, $H_2O$, and acetic acid (50:50:1), and the flow rate was 0.4 mL/min. Prior to HPLC analysis, samples of the organic and aqueous phases were dried, dissolved in acetonitrile, and filtrated through C18 cartridges (Pr-Cola). All analyses were performed at 25°C.

The concentrations of the products and substrates present in the samples were quantified by multiplying the peak area by a factor obtained from the calibration curves made with the standard solutions, with concentrations ranging from 0.06 to 0.30 m*M*.

Conversion (*X*) was calculated using Eq. 1:

$$X = \frac{[P]_{f.org} + [P]_{f.aq}}{([S]_{f.org} + [S]_{f.aq})_i} \quad (1)$$

in which [*P*] and [*S*] are the molar concentrations of the product and substrate, respectively; and *f.org*, *f.aq*, and *i* are the organic phase, aqueous phase, and initial reaction time, respectively.

#### Enzyme Activity Assay

Enzyme activity was essentially assayed as proposed for soluble lipase by Freire et al. *(13)*. Immobilized enzyme (0.1 g) was used in each reactor, which contained 19.0 mL of olive oil emulsion (5.0% [w/v]); 0.1 *M* sodium phosphate buffer, pH 7.0; and arabic gum solution (5.0% [w/v]). The reaction was stopped by adding 20 mL of acetone:ethanol solution (1:1) after the specified time (5, 10, 15, and 20 min). Control reactions were performed without the addition of enzyme.

Titration with 0.05 *N* NaOH was performed using a pH-Stat Mettler DL 50.

One unit of lipase activity was defined as the amount of enzyme that produced the equivalent of 1 μmol of fatty acid/min ($U_H$) under the assay conditions.

### *Data Processing*

Data from the time course experiments were simulated through a semi-empirical model (Eq. 2), whose parameters were statistically estimated using the least-squares procedure available in Statistica® 5.0 *(14)*:

$$\begin{aligned} X &= 1 - \exp\{K_1 \times E \times [1 - \exp(K_2 \times t)]\} \\ K_1 &= -\exp\left[-1.885 - 5639 \times \left(\frac{1}{313} - \frac{1}{T}\right)\right] \\ K_2 &= -\exp\left[-1.905 + 28873 \times \left(\frac{1}{313} - \frac{1}{T}\right)\right] \end{aligned} \quad (2)$$

in which $X$ is conversion; and the independent process operation conditions are $E$, $T$, and $t$, representing the amount of enzyme (mg), temperature (K), and time (h), respectively. The curves in Fig. 2 represent the simulation results.

## Results and Discussion

### *Activity of Lipozyme RM IM*

The activity of Lipozyme RM IM was repeatedly assayed under initial reaction rate conditions and was determined as 118 ± 16 $U_H$/g.

### *Hydrolysis of Different Substrates in a Two-Phase System*

#### Effect of Solvent on Enzymatic Hydrolysis

The use of hydrophobic solvents is often considered favorable for lipase activity in an organic medium, especially when the solubility of the substrate presents good correlation with Log *P*, which is defined as the logarithm of the partition coefficient of a substance in a two-phase water: 1-octanol standard system *(7,15,16)*. It is generally considered that the most suitable solvents for catalysis by lipases are those whose Log *P* is >2 *(8)*.

Preliminary experiments for the hydrolysis of PHT-ET were performed using the following solvents: DMSO, hexane, acetone, ethyl acetate, and chloroform. The reactions were followed by HPLC. The low solubility of substrates (*see* Table 2) in nonpolar solvents, such as hexane, led to a very low conversion. On the other hand, high hydrophilic solvents, such as DMSO (Log *P*: –1.3), despite their good solubility properties, also provided a very low conversion, probably owing to enzyme inactivation. Although ethyl acetate and acetone were not usually used as solvents for enzymatic catalysis by lipases, they presented good conversion results. This could be attributed to the good solubility of the substrate, making it more accessible, and to the high stability of the biocatalyst. It was found that the enzymatic activity recovery was 87 and 100% for acetone and ethyl acetate, respectively. When chloroform was used as solvent, the substrate presented good solubility, but there was a lower recovery of enzymatic activity. For this reason, the solvents that were selected as the most suitable for the hydrolysis reaction were ethyl acetate and acetone.

The enzymatic hydrolysis of the substrates PHT-ET, *p*-PHT-ET, and *o*-PHT-ET using acetone and ethyl acetate as solvent showed conversion of 11.6 and 20.0% for *p*-PHT-ET and 38.0 and 20.0% for PHT-ET, respectively. No conversion of the *o*-PHT-ET substrate was observed. Thus, of the solvents investigated, ethyl acetate was selected as the best solvent to study enzymatic hydrolysis. It is important to note that although small quantities of acetic acid might be formed owing to the hydrolysis of acetate, it has no effect on lipase activity *(17)*.

### Effect of Side-Chain Position

Table 2 shows the reactions of enzymatic hydrolysis for different phthalimide esters with different configurations concerning position of the ester (*ortho* or *para*) and the presence or absence of a carbon chain between the phthalimide and alcoxy-ester group. It was found that enzyme hydrolysis took place for the substrates with the alcoxy-ester group in the *para* position: PHT-MET, PHT-ET, and *p*-PHT-ET. These results showed that the reaction was independent of the size of the carbon chain between the phthalimide and alcoxy-ester group.

It was also observed that *o*-PHT-ET substrate did not react, probably because there was a steric hindrance avoiding fitting of the alcoxy-ester group in the hydrophobic pocket of the enzyme. In addition, the methyl ester (PHT-MET) was more reactive than the ethyl ester (PHT-ET), which may be attributed to the alcoxy-ester chain size. Therefore, PHT-MET was chosen as the substrate for the following experiments.

## *Hydrolysis in a Three-Phase System*

The reactions took place in the organic phase because both the substrate (PHT-MET) and its hydrolysis product (LASSBio 482) presented a

Table 3
Experimental Design Matrix of Enzymatic Hydrolysis of PHT-MET Catalyzed by Lipozyme RM IM[a]

| Enzyme (mg) | Temperature (°C) | $Na_2CO_{3(sat)}$ (mL) | Conversion (%) |
|---|---|---|---|
| 5.0 | 40 | 0.00 | 18 |
| 10.0 | 35 | 1.25 | 22 |
| 10.0 | 35 | 1.25 | 31 |
| 10.0 | 35 | 1.25 | 12 |
| 15.0 | 40 | 2.50 | 84 |
| 15.0 | 30 | 0.00 | 12 |
| 20.0 | 50 | 2.50 | 86 |
| 20.0 | 40 | 5.00 | 88 |
| 35.0 | 45 | 3.75 | 97 |
| 35.0 | 45 | 3.75 | 83 |
| 35.0 | 45 | 3.75 | 90 |
| 50.0 | 50 | 5.00 | 92 |
| 50.0 | 40 | 2.50 | 90 |

[a]The amount of substrate was 10 mg of PHT-MET and solvent was 2.5 mL of ethyl acetate.

low degree of solubility in water. However, the presence of some water in the reaction medium was necessary to bring about the hydrolysis reaction and enhance enzyme activity *(15,16,18)*.

Thus, a three-phase reaction system was studied for the hydrolysis reactions with different enzyme concentration, temperature, and volume of saturated $Na_2CO_3$ (Table 3). The use of the three-phase system led to a significant increase in substrate conversion, which reached 97% in 4 h. The NMR $^1H$ spectrum of the reaction product (Fig. 1) shows that the phthalimide ring was preserved. The use of the saturated aqueous $Na_2CO_3$ solution provided water for the hydrolysis reaction and simultaneously displaced the reaction equilibrium toward product formation. In an alkaline medium, acid LASSBio 482 is in salt form, which makes it much more soluble and allows this product to be extracted from the organic phase to the aqueous phase.

Although a higher reactor temperature could have increased reaction rates, it could also cause enzyme denaturation, substrate degradation, solvent volatilization, and higher energy costs. Thus, 40°C was chosen as the temperature for time course experiments. The complete statistical analysis of hydrolysis results and discussion on the significance of parameters are described elsewhere *(14)*. The results obtained for the hydrolysis of these esters using a three-phase system were encouraging, leading to the development of a more in-depth study to optimize the system.

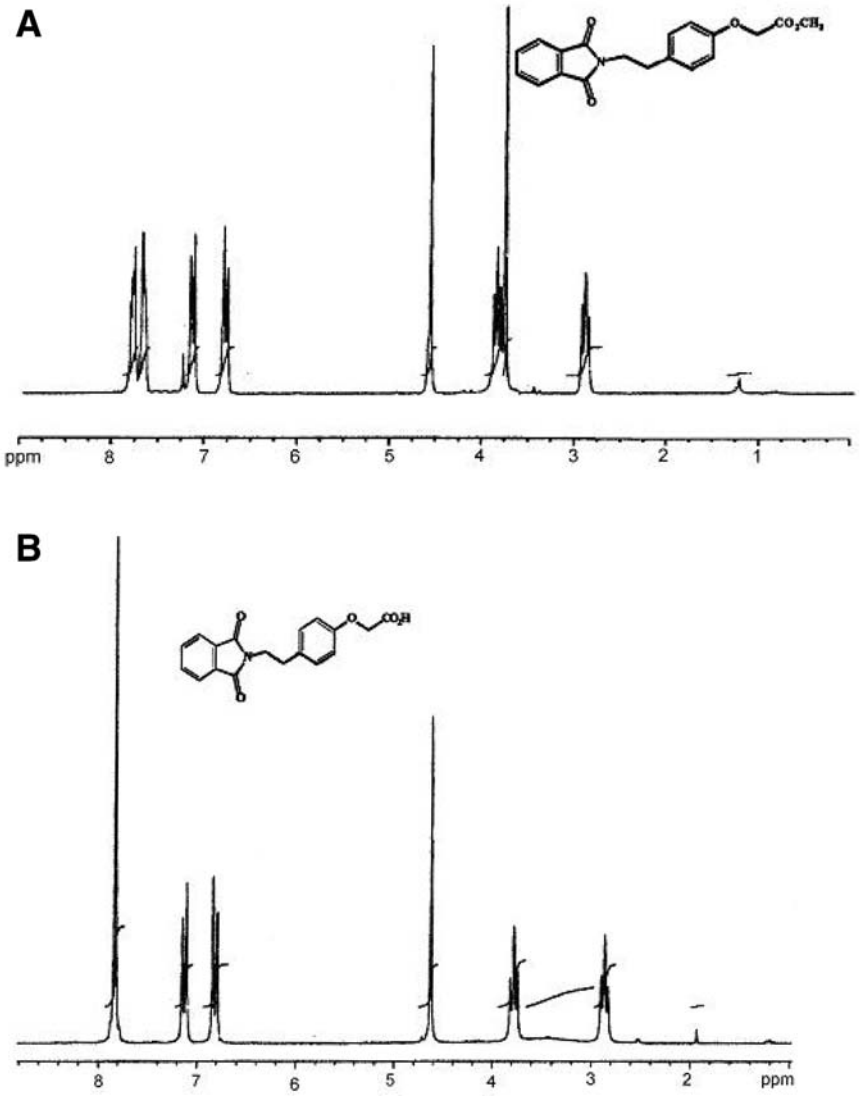



**Fig. 1.** NMR $^1$H spectra of **(A)** PHT-MET and **(B)** product of enzymatic hydrolysis, showing hydrolysis selectivity to ester bond.

## *Time Course Experiments*

Time course experiments for PHT-MET hydrolysis were performed under optimal conditions for ester hydrolysis ($V_{Na_2CO_3}$ = 5.0 mL, $T$ = 40°C) previously reported *(14)* with three different enzyme concentrations (10, 20, and 30 mg of Lipozyme, corresponding to 0.5, 0.9, and 1.4 U/mL$_{solvent}$), respectively. Statistical analysis of the experiments showed that the volume of the aqueous phase was not a very significant process parameter *(14)*. However, 5.0 mL of $Na_2CO_{3(sat)}$ solution was employed because it does not add costs to the process and would facilitate substrate/product

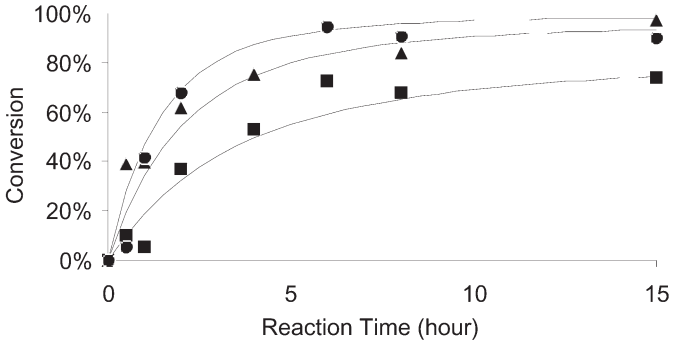



**Fig. 2.** Batch hydrolytic experiments with different biocatalyst concentrations: 10 (■), 20 (▲), and 30 mg (●) of enzyme. The reaction conditions were as follows: 10 mg of ester, 2.5 mL of ethyl acetate, 5.0 mL of $Na_2CO_{3(sat)}$, and temperature of 40°C. The values (symbols) are the mean of experimentally determined data, and the curves are the best-fitting curves for the semiempirical rate equation (Eq. 2) to the experimental data.

separation. Figure 2 shows the conversion achieved in enzymatic hydrolysis experiments up to 15 h. It was noticed (Fig. 2) that when using >20 mg of Lipozyme RM IM (0.9 $U_H/mL_{solvent}$), there was no significant increase in the productivity of the system after 15 h for this substrate concentration.

## Conclusion

The experimental results showed that the proposed enzymatic hydrolysis was a highly promising technique for the chemoselective production of new antiasthmatic prototype drugs. The reactions proceeded well in those cases in which *para*-substituted phenoxy groups were present and not for the *ortho*-substituted substrate. The latter case occurs probably because there is a steric hindrance, avoiding fitting of the alcoxy-ester group in the hydrophobic pocket of the enzyme. Experimental conversion of up to 98% using a three-phase system was achieved under optimum reaction conditions (20 mg of Lipozyme [0.9 $U_H/mL_{solvent}$], 5.0 mL of $Na_2CO_{3\ (sat)}$, a-t 40°C, and batch reaction time of 6 h).

## Acknowledgments

We thank LASSBio/UFRJ for providing substrates and supporting this work. We also thank Novozyme for donating Lipozyme RM IM. This work was supported by FUJB, FAPERJ, and CAPES.

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