applied sciences

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Biocatalytic Insights for The Synthesis of New Potential Prodrugs: Design of two Ibuprofen Derivatives

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Abstract: Due to its effectiveness, ibuprofen is one of the most popular anti-inflammatory drugs worldwide. However, the poor water solubility of this active ingredient severely limits its spectrum of pharmaceutical formulations (and often results in severe adverse effects due to high administered doses). To overcome these limitations, in this work, we enzymatically synthesized more hydrophilic derivatives of ibuprofen through its covalent attachment to two biobased polyalcohols: erythritol and glycerol. Herein, we report the optimized reaction conditions to produce an IBU–erythritol ester (82% ± 4% of conversion) by using *Candida antarctica* lipase B (CalB). Furthermore, we also report the enantioselective *solventless* esterification of (*S*)-ibuprofen with glycerol (83% ± 5% of conversion), exploiting immobilized *Rhizomucor miehei* lipase as a biocatalyst. The full NMR characterizations of the prodrug esters were performed via ^{1}H, ^{13}C-NMR, DEPT, COSY, HSQC, and HMBC-NMR. The approach reported in this work can be extended to a large variety of poorly water-soluble active pharmaceutical ingredients (APIs).

Keywords: ibuprofen; NSAID; erythritol; glycerol; esterification; lipase; prodrug; derivative

Citation: Zappaterra, F.; Presini, F.; Venturi, V.; Lerin, L.A.; Giovannini, P.P.; Costa, S. Biocatalytic Insights for The Synthesis of New Potential Prodrugs: Design of two Ibuprofen Derivatives. *Appl. Sci.* **2023**, *13*, 9852. https://doi.org/10.3390/app13179852

Academic Editor: Lidia Feliu

Received: 30 June 2023
Revised: 25 August 2023
Accepted: 29 August 2023
Published: 31 August 2023

1. Introduction

The anti-inflammatory market size is continuously growing. The estimated size of the global market for anti-inflammatory therapeutics was USD 99.6 billion in 2021, with a projected increase to USD 127.5 billion by 2030. This market is expected to register a compound annual growth rate (CAGR) of 4.5% from 2022 to 2030. Among these compounds, ibuprofen (IBU) is the second most prescribed nonsteroidal anti-inflammatory drug (NSAID), representing 27.8% of the market in 2020. Ibuprofen is an active pharmaceutical ingredient (API) commonly used to produce drugs that function as NSAIDs, which are derivatives of propionic acid. NSAIDs like ibuprofen are often used to relieve pain, reduce inflammation, and help against fever. In 1961, Dr. Adams and John Nicholson patented ibuprofen as 2-(4-isobutylphenyl) propionic acid. Since then, ibuprofen has gained widespread popularity and is now one of the most commonly used NSAIDs globally. In 2020, the worldwide market for the ibuprofen active pharmaceutical ingredient (API) was valued at USD 606 million, and it is expected to reach USD 877.9 million by the end of 2027, with a CAGR of 5.3% during the period of 2021–2027.

Despite the clear growth of the market for ibuprofen, the development of 2-(4-isobutylphenyl) propionic acid-based derivatives is not increasing at the same ratio, disregarding the needs of most of the chronic diseases for which ibuprofen is highly administrated [1,2]. Indeed, even if the usefulness of ibuprofen is clear, its chronic use faces several well-reported side effects. Prolonged use of ibuprofen can lead to serious and undesirable

contra-indications, including damage to the gastric mucosa, such as stomach ulcers, bleeding, and perforation. These disadvantages are mostly due to the limited bioavailability of ibuprofen, which is the result of its very low solubility in water (only 21 mg/L) [3] and results in serious adverse effects when it is administered at high doses in the currently available solid dosage forms. Due to this aspect, ibuprofen ranks among the poorly water-soluble drugs, characterized by dissolution-limited oral bioavailability [4]. In particular, the carboxylic acid group of ibuprofen limits its solubility in acidic media (such as in the stomach) [5]. The contact of ibuprofen's carboxylic group with the gastric mucosa leads to damage (through a combination of local irritation and the inhibition of the cytoprotective function of prostaglandins) [6]. To overcome these limitations, we planned to design ibuprofen prodrugs masking the carboxylic acid of ibuprofen and enhancing its water solubility. A prodrug is a derivative of an active pharmaceutical ingredient obtained through modifications, sometimes chemical, involving the covalent attachment of molecules that, as in the case of this article, may lead to the increased water solubility of the native active compound. Among the generally reported characteristics of prodrugs, they should readily revert to the native drug form once inside the body. For this reason, prodrugs listed as "ester prodrugs" find significant utility, given the abundant presence of lipases and esterases in our bodies [7]. In this regard, the literature reports studies on the esterification of racemic ibuprofen with glycerol. Toledo et al. published a study on the diffusion of ibuprofen into the active site of *Candida antarctica* lipase type B (CalB) [8], as well as investigations into the esterification at the molecular level through concentration-modulated infrared spectroscopy [9]. Ravelo et al. reported the kinetic model of the esterification of racemic ibuprofen with CalB in *solventless* [10] and biphasic [11] systems, both for immobilized [12] and free [13] biocatalysts. Moving from viscous glycerol towards more hydrophilic polyols, Zappaterra et al. reported the enzymatic esterification of *rac*-ibuprofen with sorbitol [14] and xylitol [2]. The esterification of ibuprofen masks its carboxylic functional group, limiting the side effects of the active ingredient. In this study, we report the study of the esterification of ibuprofen with erythritol.

Erythritol is a highly water-soluble polyol and noncariogenic sweetener that has been used safely in many countries due to its inability to be metabolized by cariogenic organisms. Erythritol has been a component of the human diet for millennia, occurring naturally in various fruits, like pears, melons, and grapes, as well as in mushrooms and foods produced via fermentation, such as cheese, soy sauce, and wine. Erythritol is widely used in the food and beverage industry as a sweetener and to enhance taste and texture. It functions as a flavor enhancer, humectant, formulation aid, nutritive sweetener, stabilizer, thickener, sequestrant, and texturizer. Compared to other polyols, it is preferred due to its minimal or absent glycemic response, making it a safe choice for diabetics. Indeed, erythritol is primarily used as a bulk sweetener in various food products, including confectioneries, chewing gum, bakery items, and beverages, due to its non-impact on glucose and insulin levels. Its molecular structure bears similarities to mannitol, a well-established antioxidant. Given the role of oxidative damage in the development and progression of diabetic complications, the potential antioxidant activity of erythritol has been investigated in both in vitro and in vivo studies [15].

The current literature indicates that erythritol is easily absorbed, does not undergo systemic metabolism, and is rapidly excreted without undergoing any changes in the urine [16]. Yokozawa et al. [17] conducted a study on the effects of erythritol on glucose metabolism and oxidative stress in diabetic rats. The results showed that erythritol had a positive impact on lowering the serum glucose levels by up to 15% at the highest dose, as well as in hepatic and renal tissues. Erythritol was also found to be an effective hydroxyl radical scavenger and inhibitor of diazo compound-induced erythrocyte damage in vitro. In diabetic rats, erythritol consumption did not affect the endothelial function and was accompanied by the presence of erythritol metabolites with antioxidant properties in the urine. These findings suggest that erythritol acts as an antioxidant in vivo and may help protect against vascular damage induced by hyperglycemia. Unlike other polyols that

often cause digestive issues, erythritol is highly tolerated by the body. Research indicates that at typical consumption levels, erythritol does not cause a laxative effect. Its small molecular size plays a crucial role in its high level of digestive tolerance, allowing it to be rapidly absorbed into the upper digestive tract. As a result, only minimal amounts reach the lower digestive tract, where the degradation of polyols and osmotic effects typically lead to gastric discomfort.

For the synthesis of the ibuprofen esters, we decided to embark on the path of chemical modifications mediated by pathways with biological origins, also called biotransformation. These biochemical transformations can be achieved by exploiting whole cells (bacteria [18] or fungi [19]), their lysate, or isolated enzymes [20]. The latter allows for the synthesis of prodrugs through straightforward synthetic pathways, characterized as cheaper, less energy-consuming, and more selective compared to the canonical chemical modifications. Thus, among the reactions that can be carried out with these biotransformative processes, esterification reactions represent green methods for the synthesis of active ingredient derivatives. For this purpose, lipases, specifically triacylglycerol hydrolases (EC 3.1.1.3), are extensively utilized for biocatalytic esterification reactions. They act as the main biocatalysts in enzymatic esterification processes. Indeed, since the 1990s, lipases have facilitated researchers and industries in the synthesis of ester bonds under mild reaction conditions, with no need for co-factors, while displaying stereo-specificities towards a wide range of substrates. Nowadays, green processes are the key step to achieve goals with a controlled and aware environmental impact. Moreover, the exploitation of green chemistry can also provide new ways to achieve goals that cannot be achieved with the traditional pathways. By exploiting enzymes, is it possible to attain products with specificity and purity that cannot be reached with chemical synthesis [21]. This article reports the study of a biocatalytic approach for producing prodrugs enhanced in water solubility. With this aim, ibuprofen was chosen as the target active principle, and erythritol as the hydrophilization moiety. Along with the study, the enantioselective esterification of ibuprofen with glycerol is also reported.

2. Materials and Methods

2.1. Materials

Lipozyme® 435 (immobilized lipase B from *C. antarctica*; 9000 U/g) was purchased from Novozymes A/S (Frederiksberg, Denmark). Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*; 10,000 U/g) and Novozym 51032 (free lipase from *Aspergillus oryzae*; 15,000 U/g) were kindly supplied by Novozymes A/S (Frederiksberg, Denmark). Free *Candida rugosa* lipase powder was kindly supplied by Amano Enzyme USA Co., Ltd., (Madeline Lane, Elgin, IL, USA; 500 U/g). Ibuprofen sodium salt, racemic (≥98% pure), meso-erythritol (≥98% pure), silica gel (60 Å, 70–230 mesh, 63–200 µm), and *d6*-DMSO degree 99.8% were purchased from Sigma–Aldrich (Buchs, Switzerland). The (*S*)-ibuprofen (>99% pure) was purchased from Fluka (Ronkonkoma, NY, USA). Glycerol and all solvents, including 2-methylbutan-2-ol (2M2B), were of ACS grade, purchased from Sigma–Aldrich (Buchs, Switzerland), and underwent no pretreatment. NMR spectra were recorded with a 400 MHz Varian Gemini spectrometer (Varian, Palo Alto, CA, USA).

2.2. Computational Analysis

The crystal structures of CaLB and *Rhizomucor miehei* lipase (RM) were downloaded from the Protein Data Bank (PDB) database (PDB IDs: 1TCA for CALB and 4TGL for RM). Substrate molecules were generated with ChemDraw and converted to 3D by using Avogadro software 1.2.0 (https://avogadro.cc, accessed on 1 February 2023) [22]. Both lipases were prepared using PyMOL software 2.5.4 (https://pymol.org/2/, accessed on 15 January 2023) [23] by eliminating all water molecules, heteroatoms, any co-crystallized solvent, and substrates from the structure. Before docking, the molecules were further processed for the minimization step using the "dock prep" tool in Chimera software 1.16 (https://www.cgl.ucsf.edu/chimera/, accessed on 15 January 2023) and by adding

polar hydrogens and partial charges to the lipase structures. The Autodock Vina tool [24] integrated into Chimera software [25] was used for the docking. The grid box was set by using the center of mass calculated in PyMol software, based on the catalytic triads of the enzymes (Ser105, Asp 187, and His 224 for CalB [8], and Ser 144, Asp 203, and His 257 for RM [26,27]) and by considering that the ligand's extended conformations fit well in the grid box (25 × 25 × 25 Å). The best candidates were visualized and further analyzed (distances, interactions, and surface hydrophobicities) using the Chimera software.

2.3. Biocatalytic Synthesis of the Ibuprofen Esters **3a**/**3b** and **6**

(a) *Preparation of ibuprofen acid from its sodium salt:* In a flask, 5 g of sodium ibuprofen salt was dissolved in 50 mL of water under agitation (100 rpm) at 50 °C. The solution was then acidified to pH 1 with 37% HCl. The contents of the flask were transferred to a separatory funnel, and 50 mL of toluene was added. After agitation and phase separation, the organic phase containing ibuprofen was separated. A second extraction was performed by adding another 50 mL of toluene to the aqueous phase. The organic phases were combined and dried with anhydrous Na_2SO_4. The sodium sulfate was filtered, and the solvent was evaporated using a rotary evaporator. This yielded 3 g of ibuprofen;

(b) *Reaction conditions:* The reactions were performed in 20 mL vials with screw caps, which were placed in an oil bath on a magnetic stirrer with temperature control of the bath. After the enzymatic stability assessment, the effect of the esterification parameter was tested (attempts to achieve a euthetic mixture of ibuprofen and erythritol with a melting point below 100 °C failed).

Erythritol **2** (1, 3.5, or 6 equivalents) was added to a 147 mM solution of IBU **1** in 2-methylbutan-2-ol (2M2B) (5 mL). The biocatalyst amount was varied from 20 to 60% *w*/*w* with respect to ibuprofen. The reaction was warmed at a set of temperatures (50, 70, or 90 °C). The reaction was kept at the tested temperature and 400 RPM, initially for 24 h (subsequently studied until 144 h). For further molar ratio investigations, 3 equivalents of **1** were added to a 147 mM solution of erythritol in 2M2B (5 mL). In this case, the biocatalyst amount was 20% *w*/*w* with respect to **1**. For the synthesis of **6a**/**6b**, 40% *w*/*w* of biocatalyst with respect to the mass of **1** was added to a 66 mM solution of **1** in 3 mL of **5**. The conversion was determined via ^{1}HNMR at 400 MHz after the identification from the ratio of the signal integrals of the CH_3 protons of the esterified **1** (δ = 1.36, 1.34, and 1.35 ppm for **3**, **4**, and **6**, respectively) with respect to the corresponding signals of the unreacted **1** (δ = 1.32 ppm). Data are expressed as mean ± SD of triplicate determinations obtained from three independent experiments. Lipase activity assay was based on the protocol reported by Zappaterra et al. [14].

2.4. Thin-Layer Chromatography (TLC)

TLC analyses were employed to assess the progress of the reaction. A total of 100 µL of the reaction mixtures was diluted 1/10 in MeOH and analyzed on TLC plates (TLC Silica gel 60, 5 × 10 cm, Merck, Berlin, Germany). Chromatography was performed with a mobile phase containing ethyl acetate/hexane/acetic acid (60:35:5 (*v*/*v*/*v*)). After complete elution, the thin layer was dried, and the separation results were acquired via UV spectroscopy (λ = 254 nm) followed by chemical derivatization with a phosphomolybdic acid solution. The esters of ibuprofen resulted in retention factors (Rfs) of 0.4 for the erythritol ester and 0.6 for the glycerol ester (while the Rf for the acid ibuprofen was 0.85).

2.5. Purification and Spectroscopic Characterization of the Prodrugs

The reaction products were separated via glass column chromatography. The column was prepared with silica gel using a mixture of ethyl acetate/hexane/acetic acid/MeCN (50:25:8:17 (*v*/*v*/*v*/*v*)) as the elution solvent. The fractions containing the product of interest were combined, the solvent was evaporated using a rotary evaporator, and the residue was analyzed via NMR (400 MHz Varian spectrometer; Varian, Palo Alto, CA, USA).

For chromatographic purification of the glyceric prodrug, a preliminary extraction step was necessary to eliminate the excess unreacted glycerol. The whole initial sample was washed 3 times with 10 mL of toluene to remove the excess unreacted glycerol and extract the ester into the organic solvent. The solvent was removed with a rotary evaporator, and the purification of the glyceric ester was reached via glass column chromatography (product yields: **3**, 75%; **4**, 50%; and **6**, 63%). The physical state of the monoesters was oily, while the diester of erythritol was solid. NMR showed the following ^{1}H- and ^{13}C-NMR spectra (acquired with 400 MHz Varian Gemini spectrometer; Varian, Palo Alto, CA, USA); all the spectra are provided as Supplementary Materials. NMR spectra were recorded for DMSO-d6 solutions at room temperature. Chemical shifts are given in parts per million from Me4Si as internal standard. Coupling constants (J) are reported in hertz (Hz). Patterns are indicated as follows: s: singlet; d: doublet; dd: double doublet; t: triplet; q: quadruplet; m: multiplet:

*IBU-erythritol ester (**3a/3b**; (2S,3R)-2,3,4-trihydroxybutyl (R)-2-(4-isobutylphenyl)propanoate* and *(2R,3S)-2,3,4-trihydroxybutyl (R)-2-(4-isobutylphenyl)propanoate)*: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.18 (dd, *J* = 8.1, 1.9 Hz, 2 H); 7.08 (dd, *J* = 8.2, 2.0 Hz, 2 H); 4.23 (dd, *J* = 11.3, 2.9 Hz); 4.09 (dd, *J* = 11.3, 2.8 Hz); 3.99 (dd, *J* = 11.3, 7.2 Hz); 3.87 (dd, *J* = 11.3, 7.0 Hz); 3.71 (q, *J* = 7.5 Hz, 1 H); 3.51 (m, 2 H); 3.33 (m, 2 H); 2.39 (d, *J* = 7.1 Hz, 2 H); 1.81 (m, 1 H); 1.36 (dd, *J* = 7.1, 1.9 Hz, 3 H); 0.83 (d, *J* = 6.6 Hz, 6 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.53; 140.13; 138.41; 129.45 (2 C); 127.57 (2 C); 72.75; 69.83; 67.03; 63.42; 44.71; 44.66; 30.03; 22.64 (2 C); 19.20.

*1,3-diacylated IBU-erythritol ester (**4**; three possible configurations: (2R,3S)-2,3-dihydroxybutane-1,4-diyl (2R,2′R)-bis(2-(4-isobutylphenyl)propanoate),* (2R,3S)-2,3-dihydroxybutane-1,4-diyl (2S,2′S)-*bis(2-(4-isobutylphenyl)propanoate)*, and *(2R,3S)-2,3-dihydroxy-4-(((R)-2-(4-isobutylphenyl)propanoyl)oxy)butyl (S)-2-(4-isobutylphenyl)propanoate)*: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.16 (dd, *J* = 8.0, 2.0 4 H); 7.06 (dd, *J* = 8.1, 2.1 Hz, 4 H); 4.18 (dd, *J* = 11.2, 1.2 Hz, 1 H); 4.05 (dd, *J* = 11.3, 2.7 Hz, 1 H); 3.97 (m, 1 H); 3.86 (m, 1 H); 3.70 (m, 2 H); 3.51 (m, 1 H); 2.37 (d, *J* = 7.2 Hz, 5 H); 1.76 (m, 2 H); 1.34 (m, 6 H); 0.82 (d, *J* = 6.7 Hz, 12 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.47; 140.14; 138.37; 129.46; 127.57; 69.66; 66.61; 66.49; 53,75; 44.66; 30.03; 22.62; 19.15.

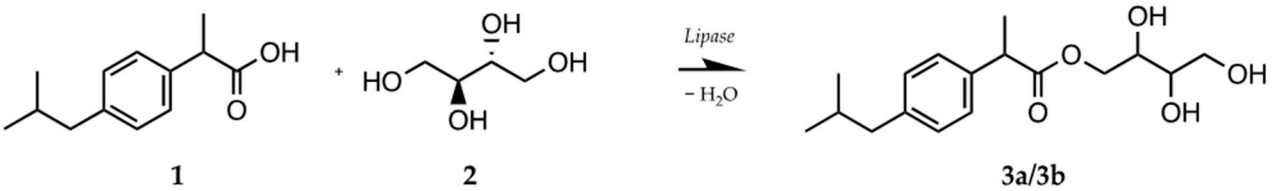
*IBU-glycerol ester (**6a/6b**; (S)-2,3-dihydroxypropyl (S)-2-(4-isobutylphenyl)propanoate* and *(R)-2,3-dihydroxypropyl (S)-2-(4-isobutylphenyl)propanoate)*: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.17 (dd, *J* = 8.0, 1.8 Hz, 2 H); 7.08 (dd, *J* = 8.1, 1.9 Hz, 2 H); 4.04 (dd, *J* = 11.1, 4.5 Hz); 3.94 (m, 1 H); 3.84 (dd, *J* = 11.1, 6.2 Hz); 3.72 (q, *J* = 7.2 Hz, 1 H); 3.56 (m, 1 H); 3.25 (m, 2 H); 2.39 (d, *J* = 7.2 Hz, 2 H); 1.78 (hept, *J* = 6.8 Hz, 1 H); 1.35 (d, *J* = 7.2 Hz, 3 H); 0.83 (d, *J* = 6.6 Hz, 5 H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 174.39; 140.17; 138.36; 129.48 (2 C); 127.53 (2 C); 69.68; 66.40; 62.98; 44.64; 44.42; 30.03; 22.63 (2 C); 19.10.

3. Results and Discussion

3.1. Screening of Free and Immobilized Lipases for the Synthesis of Ibuprofen (IBU)–Erythritol Ester

The methodology developed in this study focuses on the functionalization of ibuprofen (indicated as IBU) to obtain hydrophilic prodrugs, embracing the principles of green chemistry. The process reported in this study deviates from the esterification of ibuprofen previously reported (for instance, the *solventless* reaction with glycerol or those in biphasic systems involving the ester of ibuprofen with sorbitol). Herein, both the polyalcohol and ibuprofen acid were in solid form and studied in a monophasic reaction medium. Thus, it was necessary to use a solvent able to solubilize both substrates, characterized as different in polarity and, thus, hydrophilic/hydrophobic profile (the logP of ibuprofen is 3.97, while that of erythritol is −2.29). This work demonstrates that erythritol has a detrimental effect on the esterification capacity of the exploited lipases, and that the effect of the solvent on the enzymatic activity is not negligible. The work here reported provides an important integration into the already vast literature concerning processes biocatalyzed by lipases, extending the knowledge even beyond the widespread *solventless* reactions (which see the use of an alcohol as both solvent and reagent) and the intrinsically limited biphasic reactions

(which, due to the presence of water, cannot achieve high yields). The limitations of this approach are underlined, and for each one, we provide experimental evidence to support the hypotheses. For this purpose, bioreactors were employed, harnessing biocatalysts with molecular mechanisms capable of producing the desired prodrugs under environmentally sustainable conditions. The herein studied approach can, in principle, be extended to a variety of poorly water-soluble APIs. Scheme 1 shows the esterification of ibuprofen with erythritol for the synthesis of the desired prodrug (IBU–erythritol ester).



Scheme 1. Lipase-catalyzed esterification of (**1**) ibuprofen racemate and (**2**) erythritol to attain (**3a**/**3b**) IBU–erythritol ester.

The lipase could, in principle, produce a mixture of four possible stereoisomers of the monoester **3** (**3a**–**3d**, Figure 1). In this study, the stereoisomeric outcome was predicted through a computational approach.

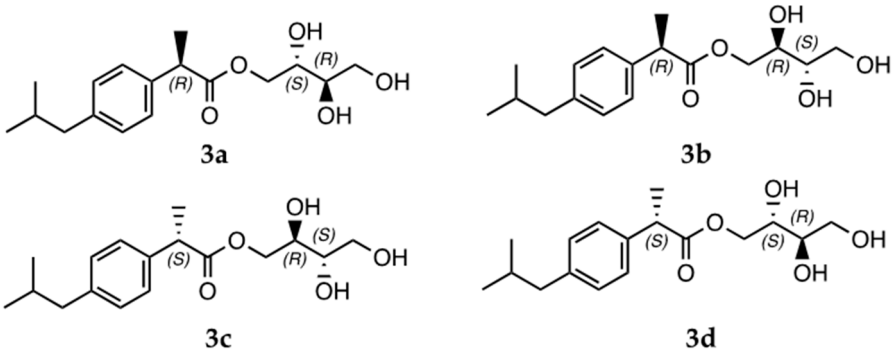


Figure 1. Stereochemistries of the four possible reaction products. Enantiomeric pairs: (**3a**)/(**3c**), (**3b**)/(**3d**); diastereomeric pairs: (**3a**)/(**3b**), (**3c**)/(**3d**), (**3b**)/(**3c**), (**3a**)/(**3d**).

In the rational design of the prodrug **3**, the polyol moiety represents both a hydrophilicity enhancer (due to its high water solubility) as well as a potential functional improving agent (for its antioxidant properties) for a dualistic IBU derivative. To promote this esterification reaction, four lipases were tested as biocatalysts: *Candida antarctica* lipase B, *Rhizomucor miehei* lipase, *Candida rugosa* lipase, and *Aspergillus oryzae* lipase, starting from the commercially available immobilized form of *Candida antarctica* lipase type B Lipozyme® 435 (hereafter indicated as L435). Although water/organic solvent systems can be exploited for biocatalyzed reactions, in this case, the presence of water needed to be avoided to shift the esterification–hydrolysis equilibrium [14]. As we previously demonstrated, *t*-amyl alcohol 2-methylbutan-2-ol (2M2B) was a suitable solvent able to solubilize both the substrates of this study, and it was used to create a homogeneous reaction mixture [2]. It is important to highlight that, to obtain the IBU–erythritol ester **3**, the biocatalyzed route needs to agree with the green chemistry principles, as it allows for the selective esterification of the primary hydroxyl groups of erythritol [28] without the need for protection–deprotection strategies, which strongly decrease the process atom and step economies. Furthermore, this reaction design, which employs the direct esterification of IBU as acid instead of its ester derivatives, produces water, avoiding the formation of organic coproducts (alcohols, such as methanol). Due to its two primary hydroxyl groups, erythritol can be potentially mono- or di-esterified by the biocatalyst. Because a high number of hydroxyls results in a more hydrophilic derivative, this study investigated the reaction conditions able to tune

the lipase behavior to obtain the IBU–erythritol ester **3** minimizing the formation of the diester byproduct **4** (Figure 2).

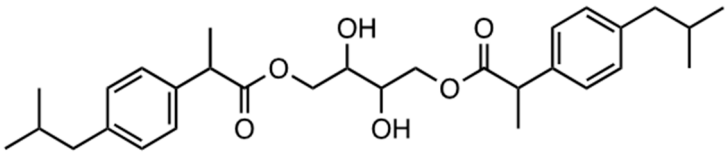


Figure 2. Undesired byproduct **4** resulting from the 1,3-diacylation of erythritol catalyzed by lipases (the three possible configurations of product **4** are schematically represented in the Supplementary Material on page 25, Figure S24).

Ibuprofen has two enantiomeric forms by virtue of its chiral center on C2. Even though the therapeutic activity of ibuprofen is mainly attributable to the (*S*)-enantiomer, it has been reported that, in the human body, (*R*)-ibuprofen undergoes a metabolic inversion of configuration [29]. The racemic mixture of ibuprofen remains the predominant form used in clinical settings. This can be attributed, in part, to historical factors, as the racemic mixture was the initial form of ibuprofen to be developed and approved for use. Furthermore, the racemic mixture is generally well tolerated and efficacious for most individuals, and, in many cases, limited clinical benefits have been observed with the administration of the enantiopure (*S*)-ibuprofen [29]. For these reasons, in this work, ibuprofen was initially used as a racemate.

3.2. Computational Analysis for the Evaluation of the CalB Stereoselectivity

Although the broad substrate specificity and the stereoselectivity of lipases are some of the characteristics that decide the synthetic success of these catalysts, the enantioselectivity of L435 can restrict its activity towards a preferred enantiomer of ibuprofen racemate [30]. The enantioselectivity of this enzyme can be described by using computational analyses (docking) able to assess the most stable poses of the two enantiomers of ibuprofen in the active site of the enzyme, highlighting the most favored for the catalysis. The (*R*)- and (*S*)-enantiomers of ibuprofen were docked into the catalytic site of L435 using AutoDock Vina, based on a Monte Carlo search and rapid gradient-optimization conformational search coupled with a simple scoring function for free-energy estimation [31]. The Near Attack Conformation (NAC) [32] is the assumption to identify the "productive" pose, namely, that which is able to produce the attack of the catalytic serine (Ser105) to the carbon atom of the acyl group of the ligands (the (*R*)- and (*S*)-ibuprofen, in this case). The results of the docking experiments are reported in Table 1.

Table 1. AutoDock Vina data relative to the docking free energies (E) computed only for the poses corresponding to the lowest energy values. The distances (*d*) and angles (ϑ) were calculated by taking as a reference the C atom of the ibuprofen carboxyl group.

Lipase	Ligand	E (kcal/mol)	*d* (Å)	ϑ (°)
CalB	(*R*)-ibuprofen	−6.7	3.21	67.7
	(*S*)-ibuprofen	−6.2	3.97	101.8

The lower the energy (E), the more stable the pose of the enantiomer, and the more favored the catalysis. To be considered as a productive pose, the acyl carbon of ibuprofen must have a maximum distance (*d*) to the serin oxygen (Ser105 of CalB) of 3.2 Å. Furthermore, the orientation of the acyl carbon of the ligands towards the serine oxygen (ϑ) must be such so as not to exceed a 90° angle. Based on these assumptions, the data shown in Table 1 display how the (*R*)-enantiomer of ibuprofen is the energetically favored substrate for the esterification with L435.

Figure 3 shows the best poses of the two enantiomers of ibuprofen in the active site of the enzyme. Based on these data, molecules **3a** and **3b** of Figure 1 are the expected products.

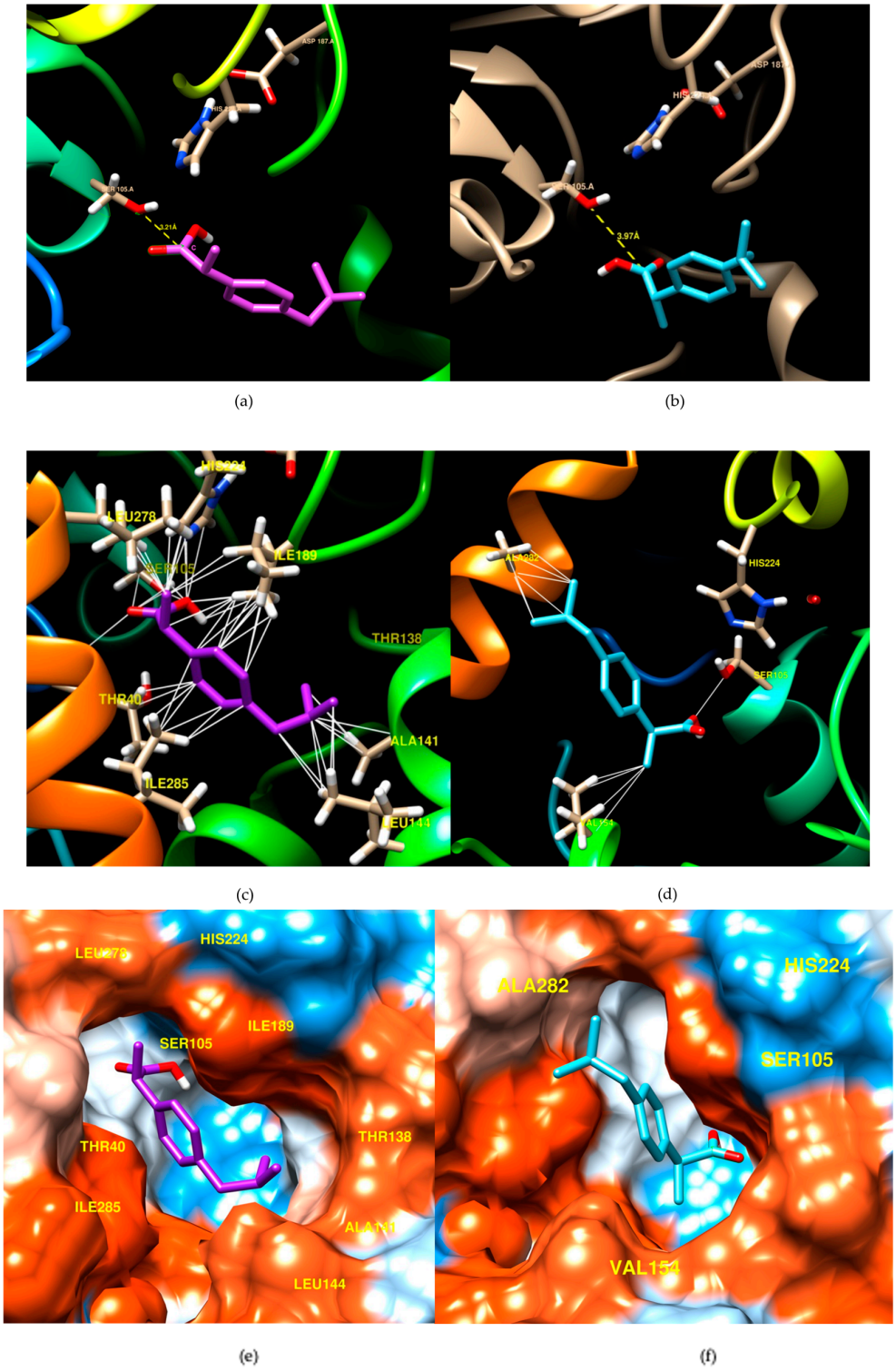


Figure 3. AutoDock Vina representation of the best poses of (*R*)-ibuprofen (**left**, purple) and (*S*)-ibuprofen (**right**, blue) in the active site of CalB (PDB ID: 1TCA; catalytic triad: Ser105, His224, and Asp187). (**a**,**b**) *d* values computed for the distance between the acyl carbon of ibuprofen and serin oxygen of CalB. (**c**,**d**) Residues in the active site involved in binding interactions with ibuprofen. (**e**,**f**) Kyte–Doolittle hydrophobicity scale representation of the active site of CalB with docked ibuprofen (blue, hydrophobic regions; red, hydrophilic regions).

3.3. Experimental Validation of the Computational Study

We move to the experimental reaction design using an initial 1:1 molar ratio between *rac*-**1** and **2** in order to favor the formation of the above monoesters **3**, testing different amounts of biocatalyst and the effect of three different temperatures. The % conversion is calculated from the ratio between the esterified and free ibuprofen detected via ^{1}H-MNR analysis (as shown in Figure 4a; the results of the reactions are shown in Figure 4b).

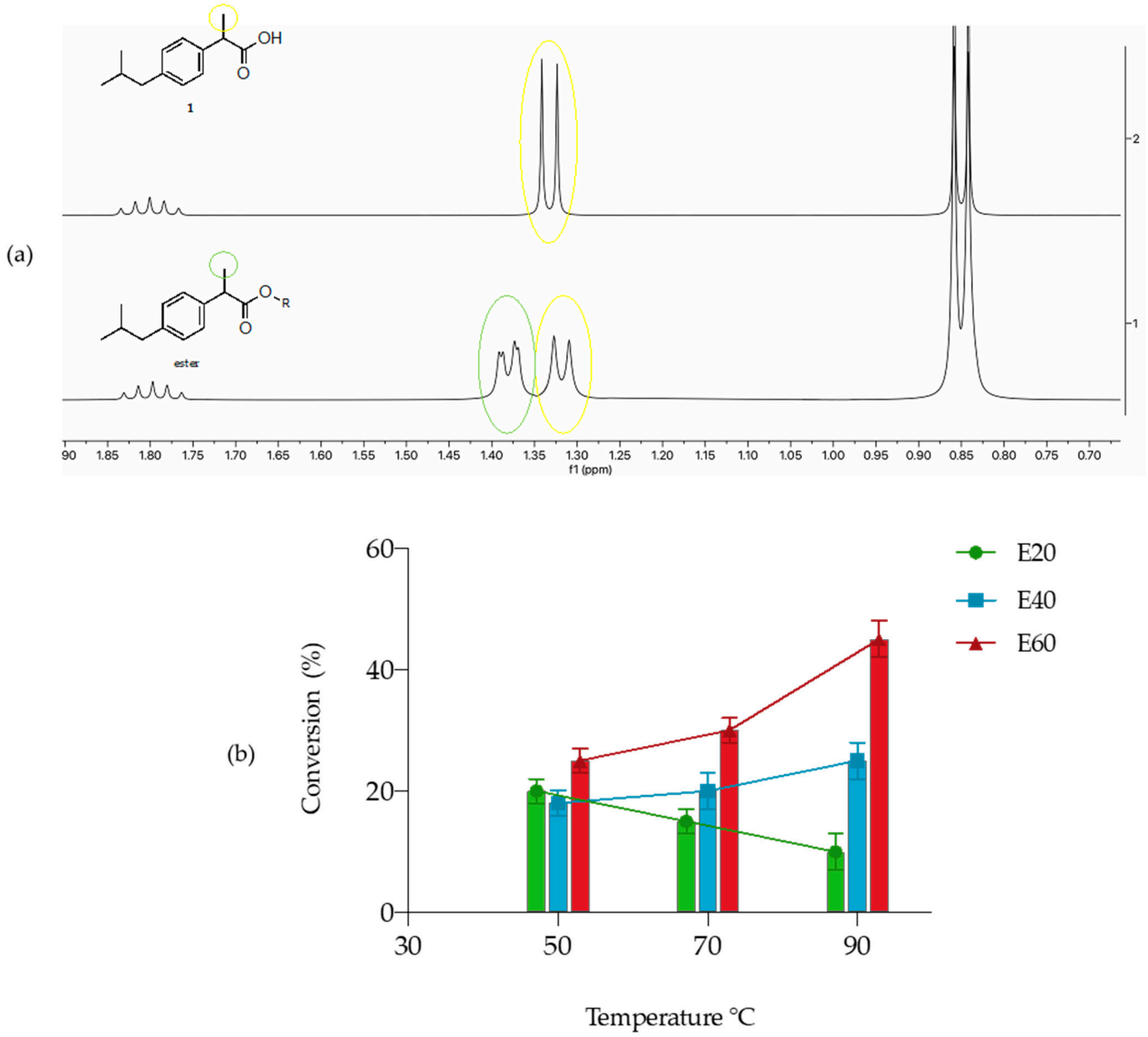


Figure 4. (**a**) Proton NMR (region from 0.7 to 1.85 ppm) of **1** (**top**) and the raw reaction of esterification of **1** with **2** to attain **3** (**bottom**). (**b**) E20, E40, and E60 indicate the reaction with different w/w percentages of the enzyme with respect to ibuprofen (20, 40, and 60%, respectively). Reaction condition: 1:1 **1** to **3** molar ratio, 400 rpm, 24 h reaction time.

Figure 4a demonstrates the application of NMR to calculate the conversion of **1** by using the shift of the CH_3 proton signal from the starting **1** (1.32 ppm) to the products **3** (1.38 ppm). L435 is known to be able to reach high working temperatures. For this reason, we tested three temperatures: 50 °C, 70 °C, and 90 °C, with a fixed reaction time of 24 h. Data shown in Figure 4b suggest that, in the presence of 2M2B as the solvent, at the lower enzyme amount, inactivation phenomena negatively affected the conversion at the higher temperatures. With an enzyme concentration of 20%, the conversion of the API decreased with the increase in temperature. Raising the enzyme concentration to

40% (E40), the amount of biocatalyst compensates for the inactivation phenomena, with a resulting increase in the conversion for the higher temperatures. This trend is even more evident by charging the reactor with 60% of the enzyme (E60). Corresponding blank experiments performed under the same conditions in the absence of the catalyst did not afford any products. Temperature also affects the solubilization of the substrates, thus implying that higher temperatures result in improved mass transfer. However, as the temperature increases, the active site may become more flexible, potentially leading to a reduction in enzyme selectivity and an increase in the formation of byproducts, such as polyacylated erythritols. Indeed, at 90 °C, with the enzyme concentration of 60%, the reaction afforded about 10% of the 1,4 diacylated derivative **4**. Both product **3** and the byproduct **4** were isolated via column chromatography and characterized via NMR analyses (^{1}H-, ^{13}C-, DEPT-, COSY-, HSQC-, and HMBC-NMR; see Supplementary Materials), which confirmed that the monoester product is an equimolar mixture of diastereoisomers (**3a** and **3b**, Figure 2), as deductible by the double set of signals attributable to the protons of the esterified hydroxymethylene group of erythritol in the region between 4.3 and 3.8 ppm of the ^{1}H-NMR spectrum (Figure 5). The solubility of a compound in aqueous-based media is a crucial property. Solubility directly influences a compound's bioavailability, affecting its absorption, and serves to validate the in vitro assay data obtained. It is generally reported that compounds with solubilities greater than 60 µg/mL may have good absorption potential [33]. The water solubility of product **3** based on the turbidimetric method reported by Lipinski et al. [34] was 463 µg/mL, which was 22 times better than the acid ibuprofen.

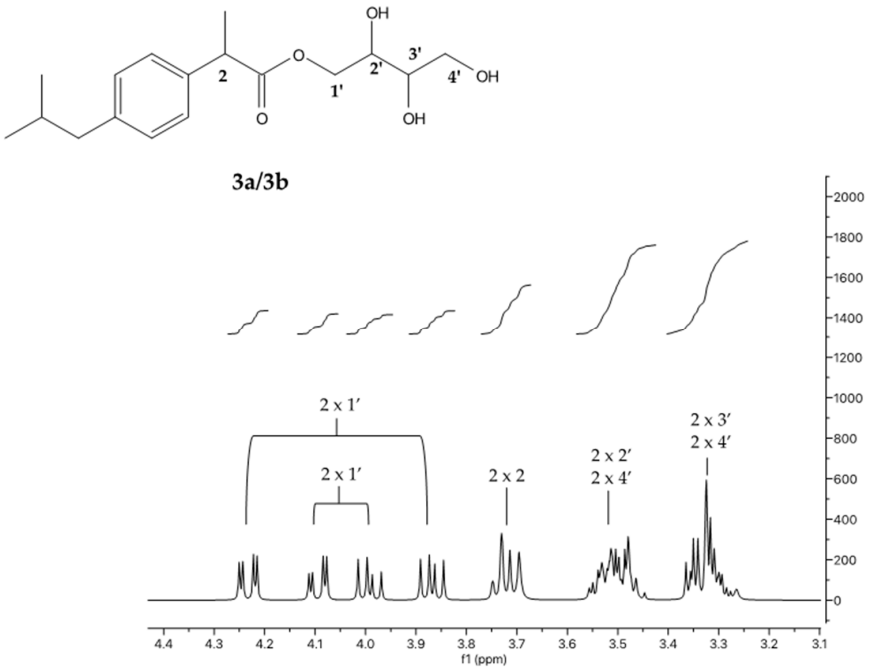


Figure 5. Proton NMR of the equimolar mixture of diastereoisomers **3a**/**3b**.

In the attempt to maintain a high production of monoesters **3a** and **3b**, minimizing the diacylation of erythritol, we investigated the effect of different substrate molar ratios by raising the amount of polyol with respect to ibuprofen. The results are shown in Figure 6.

In both the temperatures tested (50 and 90 °C: the lowest and the highest considered in this study), raising the alcohol molar concentration led to a decrease in the conversion. Although a protective effect on the biocatalyst has been reported for certain polyalcohols, such as glycerol (mainly attributed to its viscosity) [35], reactions with polyols solid at room temperature, such as erythritol, can be challenging. In fact, it was reported that some polyhydric alcohols can reduce the lipase activity [36], and erythritol seems to be no exception. Polyhydric alcohols can interfere with the water necessary for maintaining

the catalytically active conformation of the enzyme. The scientific literature discusses how three key characteristics of polyalcohols, the molecular size, hydrophobicity, and solubility, impact the enzyme's efficiency. The binding energy, which is influenced by the size of the polyhydroxylated molecule, plays a crucial role in activating the enzyme when the substrate binds to the active site. A low binding energy results in a suboptimal three-dimensional conformation of the lipase, leading to a slower reaction rate. Additionally, the enzyme's exposure to alcoholic functions increases with the solubility of the polyhydroxylated substrate in water, thereby promoting denaturation and subsequent inactivation. Furthermore, the influence of the reaction solvent on the distribution of water, understood as the quantity of water in the aqueous layer surrounding the catalyst, must be highlighted. Indeed, it is generally reported that solvents with logP values lower than 2 are less suitable for biocatalytic purposes [37]. In our case, the use of high volumes of 2M2B for the solubilization of the erythritol excess was detrimental to the enzyme activity. For these reasons, we moved to test reactions performed with a higher amount of racemic ibuprofen with respect to erythritol (molar ratio: 3:1). The reaction course was monitored for 6 days. Data are shown in Figure 7.

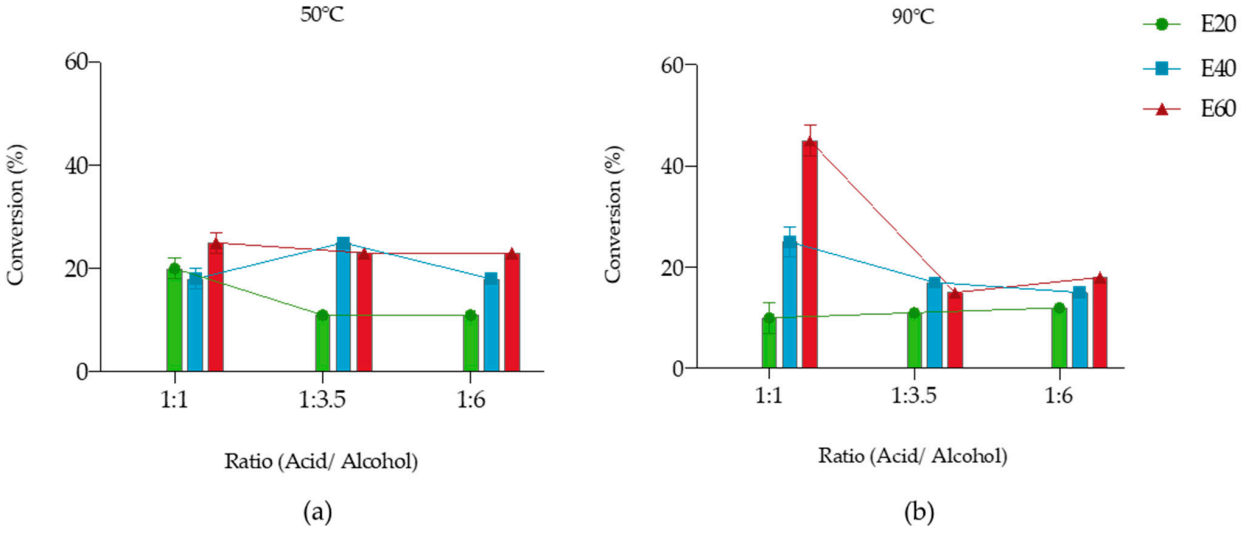


Figure 6. Conversion (%) for the reactions conducted with acid/alcohol molar ratios of 1:1, 1:3.5, and 1:6; and 20, 40, and 60% of the enzyme for 24 h at temperatures of 50 °C (**a**) and 90 °C (**b**).

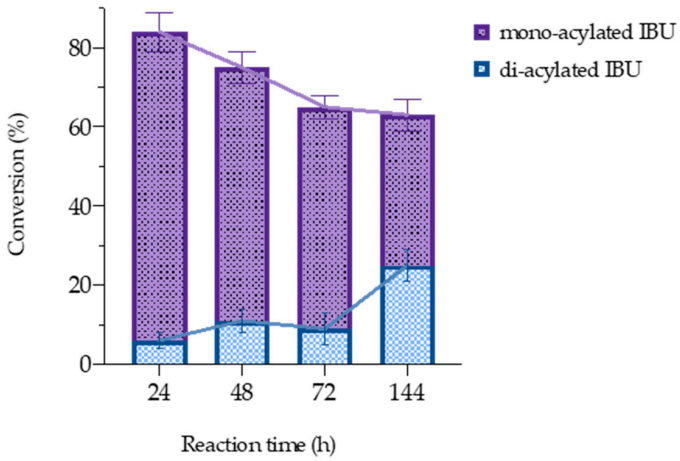
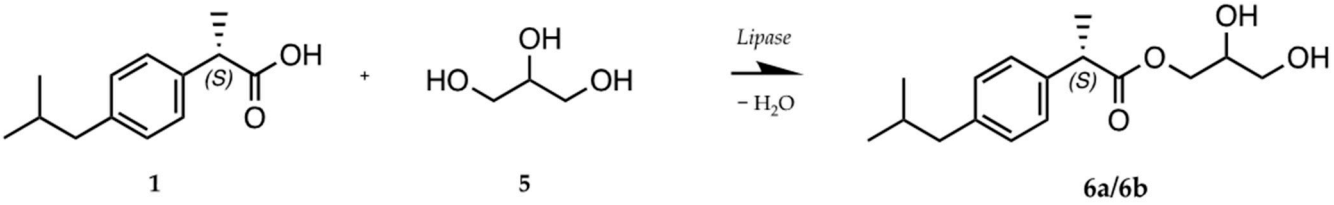


Figure 7. Conversion (%) of erythritol to an equimolar mixture of mono-acylated IBU–erythritol ester **3a-3b** (purple) and 1,3 di-acylated ester **4** (blue) as a function of the reaction time. Reaction condition: 20% (*w*/*w* with respect to **1**) L435, **1**/**2** molar ratio of 3:1, 50 °C, 400 RPM.

It was evident that the ibuprofen excess afforded high erythritol conversion. The low temperature of 50 °C was chosen in order to increase the selectivity, avoiding the formation of the undesired 1,4-bis ester **4** [10,35]. Taking into account the preference of L435 for the (*R*)-enantiomer of ibuprofen, the 3:1 molar ratio provided enough substrate for the complete conversion of erythritol into the derivatives **3a** and **3b**, limiting at the same time the bis-acylation of erythritol, which, however, increased at longer reaction times. After the first 24 h, a progressive lowering of the conversion was also observed, probably due to the increasing amount of water in the reaction medium. Tests conducted at higher enzyme concentrations led to increased diester concentrations (data not shown), which were not favorable for the objectives of our study. With the enzyme amount used for the reaction reported in Figure 7, we achieved significant amounts of the monoester in comparison to the diester, aided by the lower temperature. Furthermore, due to the excess of ibuprofen over erythritol, the adverse effects of the polyol on the enzyme were mitigated, enabling high conversions at low enzyme concentrations. The reaction performed under the same conditions on the pure (*S*)-enantiomer of ibuprofen reached a 12% conversion after 6 days, confirming *C. antarctica* lipase B (L435 in this case) as an (*R*)-selective enzyme towards ibuprofen. In virtue of the above results, we moved to investigate the esterification of the (*S*)-enantiomer of ibuprofen by testing biocatalysts alternative to the *C. antarctica* lipase B. Based on the literature, we chose the immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) [38], a free *Candida rugosa* lipase [39], and a free *Aspergillus oryzae* lipase (Novozym 51032). The reactions were conducted using a 1:1 ibuprofen/erythritol molar ratio and the same amount of enzymatic units employed for the reaction catalyzed by L435, at the optimal temperature reported for each of the enzymes involved (40 °C, 35 °C, and 70 °C for Lipozyme RM IM, *C. rugosa* lipase, and Novozym 51032, respectively). The reactions performed with Novozym 51032 reached a 5% conversion in 24 h, while neither the reaction performed with the immobilized RM IM nor that for the free *C. rugosa* lipase afforded the desired products, testifying to how the esterification of ibuprofen with the erythritol polyol in 2M2B can be challenging for most of the enzymes. In particular, the free *C. rugosa* lipase showed a visible instability (precipitation) once added to the reaction mixture.

3.4. Lipozyme RM IM-Catalyzed Synthesis of (S)-IBU–Glycerol Ester

Due to the herein reported limitations imposed by 2M2B and erythritol for the esterification of ibuprofen, we decided to move towards *solventless* strategies involving glycerol as a hydrophilizer for the preparation of an (*S*)-ibuprofen prodrug with increased hydrophilicity compared to the lead compound (Scheme 2).



Scheme 2. Lipase-catalyzed esterification of (**1**) (*S*)-ibuprofen with (**5**) glycerol to attain (**6a**/**6b**) (*S*)-IBU–glycerol ester.

Similar to what was previously reported for the reaction between *rac*-**1** and **2** (Scheme 1), the reaction in Scheme 2 can also yield two different ester configurations between (*S*)-**1** and **5**, resulting in **6a**/**6b** (Figure 8).

Glycerol showed the ability to completely dissolve ibuprofen without the need for cosolvents. The reaction with free *C. rugosa* lipase was unsuccessful, leading to no ester production after 24 h. The *C. rugosa* enzyme is an interfacial enzyme reported as strongly influenced by the presence of an interfacial area for its shift to an active conformation [40]. In this case, as the *solventless* reaction medium was a monophasic system with limited interfacial surface between substrates, probably the enzyme did not expose the active site

via lid movement, not reaching its catalytically active form. The lipase from *Aspergillus oryzae* showed moderate activity in the esterification of **1** with **5**, reaching a conversion of 23% after 24 h. Instead, the reaction catalyzed by RM IM achieved a conversion of 83 ± 5% after 24 h, without traces of the diglyceride, confirming glycerol as a great reagent for the *solventless* esterification of (*S*)-ibuprofen. Additional advantages of using glycerol lie in its safety, liquid nature at room temperature, viscosity, high boiling point, and low vapor pressure. Glycerol is a coproduct of the biodiesel industry, and its availability has grown enormously in recent years, lowering its price. By virtue of this, its uses have multiplied, extending beyond its uses in the food, cosmetic, and pharmaceutical fields, such as the enzymatic synthesis of esters and polyesters [41]. Compared to the above-reported esterification with erythritol, where the use of a reaction solvent was mandatory, in this *solventless* synthesis, glycerol acts not only as a substrate but also as a protein stabilizer, shifting the native structure of the catalyst to a more compact state [42]. This helps prevent the loss of enzymatic activity, enhances the thermal unfolding temperature, and prevents irreversible protein aggregation. Consequently, the utilization of this highly versatile short polyalcohol provides greater flexibility in the experimental procedures of enzymatic syntheses. To verify the role of 2M2B in the failure of the RM IM-catalyzed esterification of ibuprofen with erythritol, we repeated the reaction of (*S*)-ibuprofen with glycerol by adding 2M2B in the same amount of glycerol (glycerol was miscible to 2M2B). Under this condition, the conversion dropped to 31 ± 5%, thereby testifying to the negative effect of 2M2B on RM IM activity. Figure 9 shows the diagnostic signals in the ^{1}H-NMR spectrum of compound **6a**/**6b**, which is composed of an equimolar mixture of diastereoisomers because of the prochiral nature of glycerol (the complete NMR characterization is reported in the Supplementary Materials).

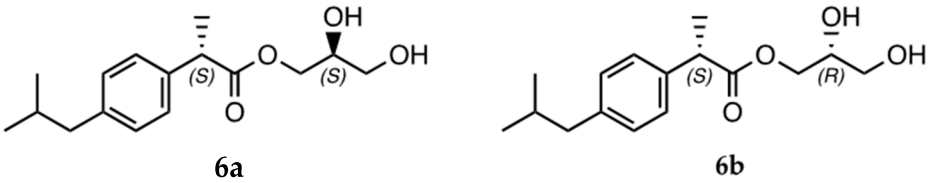


Figure 8. Stereochemistries of the two possible reaction products **6a** and **6b**.

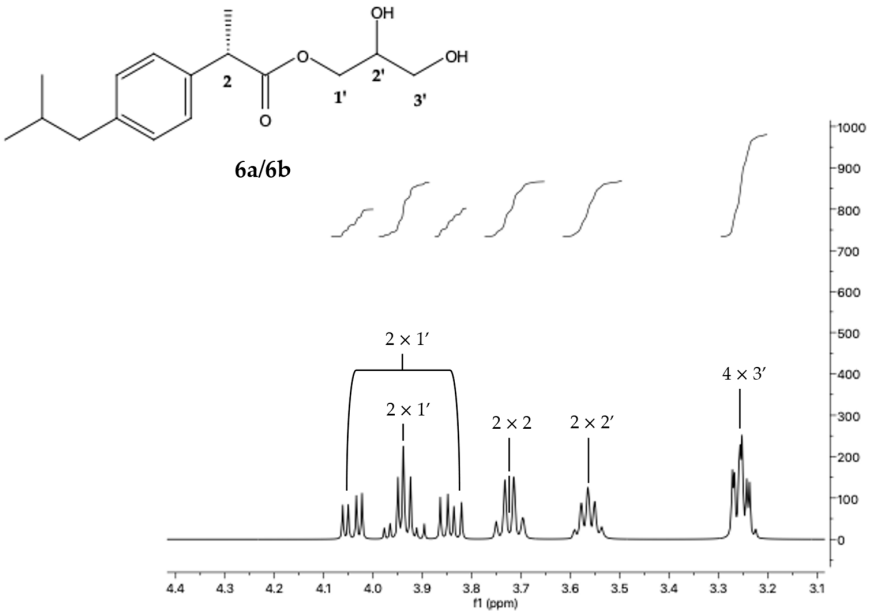
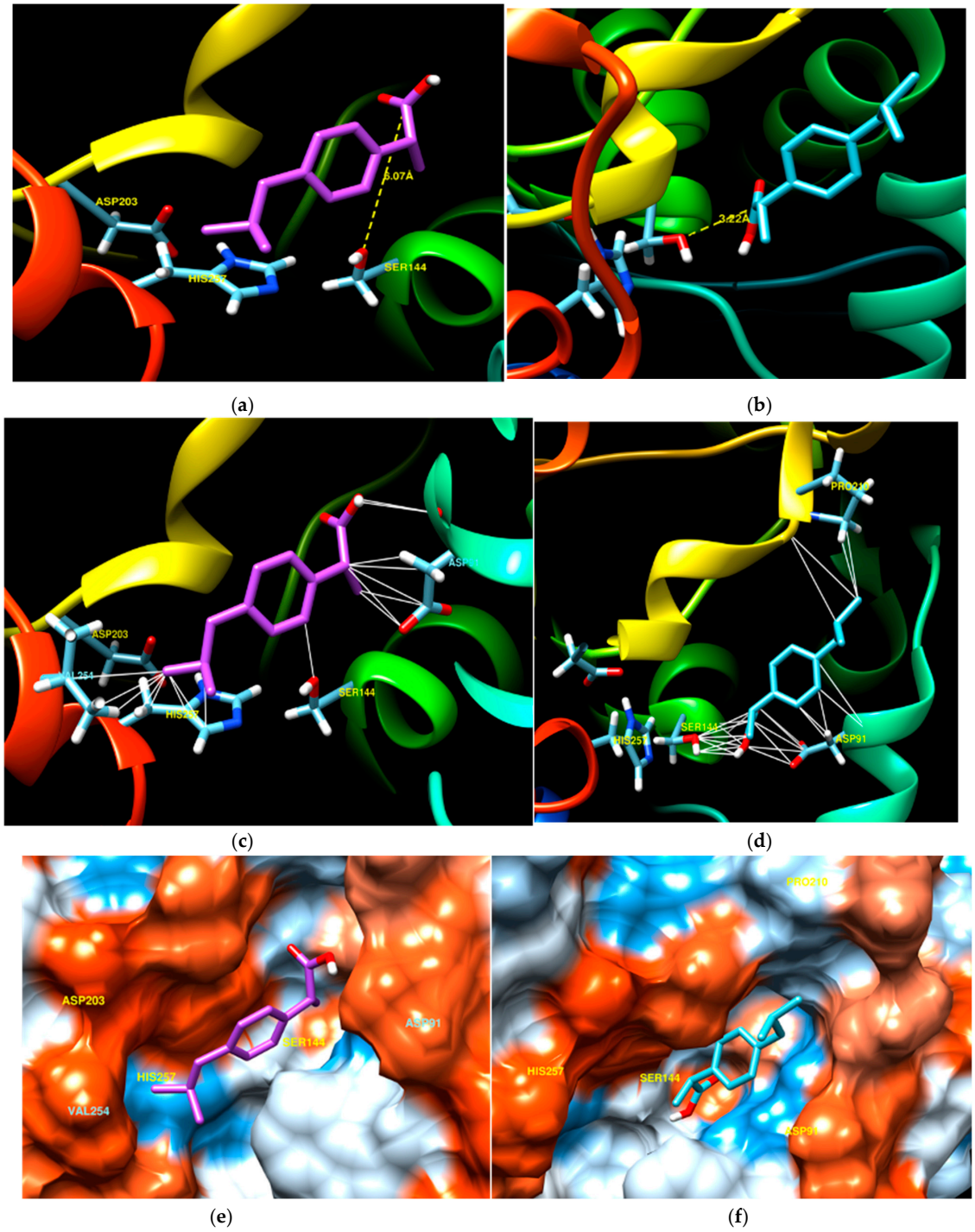


Figure 9. Diagnostic region of the ^{1}H-NMR spectrum of the equimolar diastereomeric mixture of compound **6a**/**6b**.

The docking of **1** to the active site of the *Rhizomucor miehei* lipase confirmed the preference of the enzyme towards the (*S*)-enantiomer of **1** (Figure 10). Indeed, the distance between the acyl carbon of 1 and the serin144 of the catalytic triad of RM lipase is 6.07 Å for (*R*)-**1**, compared to the 3.22 Å for (*S*)-**1** (energy (kcal/mol): -6.5 for (*R*)-1 and -6.7 for (*S*)-**1**).



(**a**) (**b**) (**c**) (**d**) (**e**) (**f**)

Figure 10. AutoDock Vina representation of the best poses of (*R*)-ibuprofen (**left**, purple) and (*S*)-ibuprofen (**right**, blue) in the active site of *Rhizomucor miehei* lipase (PDB ID: 4TGL; catalytic triad: Ser144, His 257, and Asp 203). (**a**,**b**) *d* values computed for the distance between the acyl carbon of ibuprofen and serin oxygen of RM. (**c**,**d**) residues in the active site involved in binding interactions with ibuprofen. (**e**,**f**) Kyte–Doolittle hydrophobicity scale representation of the active site of RM with docked ibuprofen (blue, hydrophobic regions; red, hydrophilic regions).

While previous research has explored ibuprofen–glycerol ester synthesis, our study introduces a novel catalytic approach using *Rhizomucor miehei* lipase, yielding significant ibuprofen conversion rates. Unlike the widely used *Candida antarctica* lipase B, RM IM displays distinct properties. To the best of our knowledge, this is the first article to report the selective esterification of (*S*)-ibuprofen with glycerol using the RM IM enzyme employing a molecular docking approach, assessing solvent effects, and fully characterizing the reaction product, thereby filling the gap left by previous literature. Our overarching objective is crafting selective (*S*)-enantiomer esters, yielding highly potent prodrugs with superior water solubilities compared to their glyceric counterparts.

4. Conclusions

Biocatalysis can provide effective protocols for the rational design of API derivatives. The NSAID market is vast but needs new prodrugs that are able to overcome the limits of time-honored traditional drugs. This work presents strategies aligning the production of more hydrophilic ibuprofen derivatives with the requirements of the enzymes utilized as biocatalysts for their synthesis. *Candida antarctica* lipase B and *Rhizomucor miehei* lipase were demonstrated as suitable biocatalysts for the enantioselective esterification of (*R*)- and (*S*)-ibuprofen, respectively. The esterification of erythritol with ibuprofen using an immobilized *Candida antarctica* lipase B (Lipozyme® 435) as the catalyst and *t*-amyl alcohol as the solvent confirms what was predicted by the computational study regarding the stereo preference of the enzyme for the (*R*)-ibuprofen. The fine-tuning of the reaction conditions allowed for the obtainment of the expected ibuprofen–erythritol ester **3** (Scheme 1) with a good ibuprofen conversion (83%) as an equimolar mixture of the diastereoisomers **3a** and **3b** (Figure 1). This new product, as well as the bis-ester **4** formed as a side product under non-optimized conditions, were fully characterized through ^{1}H and ^{13}C-NMR analyses by using two-dimensional homo- and heterocorrelated techniques. In particular, the IBU–erythritol ester was found to exhibit a 22-fold increase in water solubility, establishing erythritol as a suitable polyol for synthesizing water-soluble ibuprofen derivatives. Further studies will focus on other aspects highlighted for the "ideal" prodrug, such as characterizing the physicochemical properties of the ester and testing its stability in acidic media (e.g., utilizing simulated gastrointestinal fluid). Additionally, antioxidant tests will be conducted on the IBU–erythritol ester, considering erythritol's antioxidant properties. Finally, the use of glycerol in place of erythritol provides an alternative route to produce the more hydrophilic (*S*)-ibuprofen derivative **6a**/**6b**, valorizing this low-cost, biobased polyol in the pharmaceutical field. To the best of our knowledge, this is the first report to employ *Rhizomucor miehei* lipase for the enantioselective synthesis of the ibuprofen monoglyceride ester **6a**/**6b**. Also, this compound was fully characterized, closing a gap in the current literature.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app13179852/s1, Figure S1: ^{1}H-NMR of erythritol ester of ibuprofen (**3a**/**3b**); Figure S2: ^{13}C-NMR of erythritol ester of ibuprofen (**3a**/**3b**); Figure S3: DEPT-NMR of erythritol ester of ibuprofen (**3a**/**3b**); Figure S4: COSY-NMR of erythritol ester of ibuprofen (**3a**/**3b**); Figure S5: HSQC-NMR of erythritol ester of ibuprofen (**3a**/**3b**); Figure S6: Zoom of the HSQC-NMR of erythritol ester of ibuprofen (**3a**/**3b**); Figure S7: HMBC-NMR of erythritol ester of ibuprofen (**3a**/**3b**); Figure S8: ^{1}H-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S9: Zoom of the ^{1}H-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S10: ^{13}C-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S11: DEPT-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S12: COSY-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S13: Zoom of the COSY-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S14: HSQC-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S15: Zoom of the HSQC-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S16: HMBC-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S17: Zoom of the HMBC-NMR of glycerol ester of ibuprofen (**6a**/**6b**); Figure S18: ^{1}H-NMR of the diacylated erythritol ester of ibuprofen (**4**); Figure S19: ^{13}C-NMR of the diacylated erythritol ester of ibuprofen (**4**); Figure S20: DEPT-NMR of the diacylated erythritol ester of ibuprofen (**4**); Figure S21: COSY-NMR of the diacylated erythritol ester of ibuprofen (**4**); Figure S22: HSQC-NMR of

the diacylated erythritol ester of ibuprofen (**4**); Figure S23: HMBC-NMR of the diacylated erythritol ester of ibuprofen (**4**); Figure S24: Representation of the three possible configurations of product **4** (**4a**, **4b**, and **4c**).

Author Contributions: F.Z.: Conceptualization, investigation, methodology, visualization, software, writing—original draft, writing—review and editing; F.P.: investigation; V.V.: investigation; L.A.L.: data curation, formal analysis, resources; P.P.G.: supervision, project administration, funding acquisition, writing—review and editing; S.C.: conceptualization, validation, resources, project administration. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Recovery and Resilience Plan (NRRP), Mission 04 Component 2 Investment 1.5—NextGenerationEU, Call for tender n. 3277 dated 30 December 2021. Award Number: 0001052 dated 23 June 2022.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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