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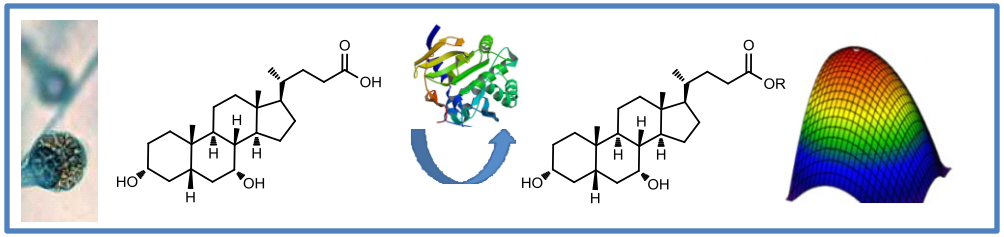
Graphical Abstract (for review)



Highlights

- Chenodeoxycholic acid esters were prepared by an enzymatic approach.
- The heterologous *Rhizopus oryzae* lipase, immobilized on Octadecyl Sepabeads was the best biocatalyst.
- The influence of various reactions parameters was studied.
- Conversion of chenodeoxycholic acid was optimized using the response surface methodology and a central composite rotatable design.

Enzyme-catalyzed preparation of chenodeoxycholic esters by an immobilized heterologous *Rhizopus oryzae* lipase

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Keywords: chenodeoxycholic acid, esterification, lipase, immobilization.

Abstract

A lipase-catalyzed preparation of ethyl and stearyl esters of chenodeoxycholic acid is described. Stearyl chenodeoxycholate is a new product and both bile acid esters were prepared through an enzymatic approach for the first time. The heterologous *Rhizopus oryzae* lipase, immobilized on two different supports proved to be an efficient catalyst, even more active than CAL B, in the esterification reaction using a complex substrate such as a bile acid. The immobilization of the enzyme on Octadecyl Sepabeads at pH 7 and 25ºC was the best choice to catalyze the esterification reaction. The influence of various reaction parameters, such as nature of the alcohol, alcohol:substrate ratio, enzyme:substrate ratio, solvent and temperature, was evaluated. Using the response surface methodology and a central composite rotatable design, the conversion of stearyl chenodeoxycholate was optimized by means of the study of the effect of enzyme:substrate ratio and alcohol:substrate ratio. The value 20 for ratios (E/S) and (A/S) were predicted as the optimal values to reach the maximum conversion. However, including economic aspects these ratios can be reduced up to 15. The well known advantages of biocatalysis and the activity shown by the immobilized heterologous lipase make the reported procedure a convenient way to prepare chenodeoxycholic esters.

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Introduction

Bile acids biosynthesis is the main pathway of cholesterol catabolism and consequently plays an important role in its regulation [1]. In digestion, bile acids are important for the solubilization of cholesterol in the gallbladder and for stimulating the absorption of cholesterol, fat-soluble vitamins and lipids from the intestine [2]. As regulatory molecules, bile acids activate specific nuclear receptors and cell signaling pathways in cells in the liver and gastrointestinal tract and regulate serum triglyceride level in humans and rodents [3-5]. As bile acids are non toxic, they are widely used in drug formulations as excipients and can influence gastrointestinal solubility and chemical or enzymatic stability of drugs [6-8]. Moreover, they can modify and optimize drug unfavourable physico-chemical properties acting as absorption modifiers [9]. In this way, bile acid derivatives are also used as skin penetration enhancers in the formulation of transdermal delivery systems for drugs that are otherwise not sufficiently skin-permeable [10]. Regarding their application in organic synthesis, bile acid-based compounds can control the stereochemical course of a given reaction by discrimination of chiral and achiral guests and enantioselective recognition [11].
In human, primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from hepatic cholesterol: CA is hydroxylated at positions C-3, C-7 and C-12, whereas the hydroxyl group in C-12 is absent in CDCA [12].
Bile acids exhibit a hydrophobic side (β) and a hydrophilic side (α). This structure is responsible for their amphipathic nature and, consequently, in water, bile acids self-associate to form multimers above a critical concentration. The characterization of the crystal structure of bile acids by X-ray resolution showed that hydrogen bonding plays a key role in these structures, forming different network patterns [13-14], In the network observed at the CDCA crystal each hydrogen bond site participates in the formation of two hydrogen bonds with two other molecules and, consequently, each CDCA molecule is interconnected with six molecules [15]. These networks can form inclusion compounds, such as polymers including CDCA as the host [16].
Various interesting therapeutic properties of CDCA were reported such as its use in the dissolution of cholesterol gallstones [17] and more recently its action in promoting the release of fibroblast growth factors and gut peptide secretion [18].
In the case of CDCA derivatives in C-3, C-7 or C-24, they lacked the supramolecular structure

of the CDCA and, therefore, have different physicochemical and biological properties.

In reports describing the synthesis and evaluation of their biological activities, it was observed that CDCA derivatives showed to be potential active ingredients in pharmaceuticals. For example novel chenodeoxycholic acid-verticinone esters exhibited antitussive and *in vitro* antitumor activity [19,20] and some unsymmetric CDCA bis-thiocarbazone derivatives showed excellent antibacterial activity against *Bacilllus subtilis* and *Staphylococcus aureus* [21].

CDCA esters are also important as intermediates in the synthesis of related steroid compounds. In the synthesis of 6-ethyl chenodeoxycholic acid, a farnesoid X receptor agonist, it was not possible to achieve the preparation of silyl enol ether without the previous protection of the carboxyl function at C-24 as a benzyl ester [22]. It was also necessary to use a methyl ester of CDCA as raw material in the synthesis of 3-bromo and 3-azido CDCA [23] and ethyl chenodeoxycholate was used as intermediate in the synthesis of chenodiol from stigmasterol [24].

So far, CDCA esters were prepared by traditional chemical procedures. Methyl ester was prepared using acetyl chloride and methanol a 0°C [25], methanol and p-toluensulfonic acid or methanesulfonic acid [21] and also applying these conditions under microwave irradiation [26]. Benzyl esters were prepared by treatment of CDCA with benzyl bromide and Cs_2CO_3 at 150°C during 24 h [22]. These chemical methods involve reagents which are not friendly to the environment such as acid chlorides and sulfonic acids and high temperatures.

Biocatalysis proved to be a good alternative to the synthesis of organic compounds through a Green Chemistry approach. Enzymes and whole cells of microorganisms show interesting advantages, such biodegradability and working under mild reaction conditions [27]. Moreover, they are capable of accepting a wide array of substrates and catalyze reactions in a chemo- and regioselelective way, carrying out different chemical transformations without the need for tedious protection and deprotection steps in compounds with several functional groups [28]. Over the last years, biocatalysis using lipases in non-aqueous media has been widely used in synthesis of pharmaceuticals catalyzing several synthetic reactions such as esterification, transesterification, aminolysis, polymerization, etc. [29-32]. Enzymes are also well-known by its high enantioselective behavior and this property formed the basis for their widespread use for the synthesis of enantiomerically pure compounds [33,34]. Studies carried out in our laboratory on the esterification and transesterification of multiple substrates have

shown that lipases are useful in the synthesis of biologically active compounds, particularly steroids derivatives [35,36]. We have also used whole cells in the biotransformation of α- and β-ketoesters and the steroid drospirenone [37]. Regarding the application of whole cells to bile acids, the biotransformation of CDCA by *Geobacillus stearothermophilus*, the use of *Acinetobacter calcoaceticus lwoff*ii in the chemoenzymatic synthesis of ursodeoxycholic acid and the 7α-hydroxy epimerization of CDCA by *Xanthomonas maltophilia*, were reported [38]. In the field of lipases, acylation of chenodeoxycholic acid methyl ester with trichloethyl butanoate and fatty acid esters was performed using lipases from *Candida cylindracea* and *Candida antarctica* respectively [39]. As above mentioned, in the chemical acylation of bile acids it was also necessary the previous esterification of carboxyl group in C-24 to carry out the acylation successfully.

The extracellular sn-1,3 regioselective lipase from *Rhizopus oryzae* (ROL) has been found an interesting catalyst in esterification processes to produce fatty acid esters and enantiomeric resolution [40].

However, lipases exhibit a complex catalytic mechanism which is even modified when they are used in organic solvent [41]. Therefore, for a best performance in esterification reaction, a good stability and catalytic activity is mandatory. In this case it has been demonstrated that the open conformation of a lipase is the most stable and active form. The use of strategies to fix the open conformation of the lipase has been possible by the immobilization on hydrophobic matrixes *via* oriented interfacial activation mechanism (Figure 1) [42].

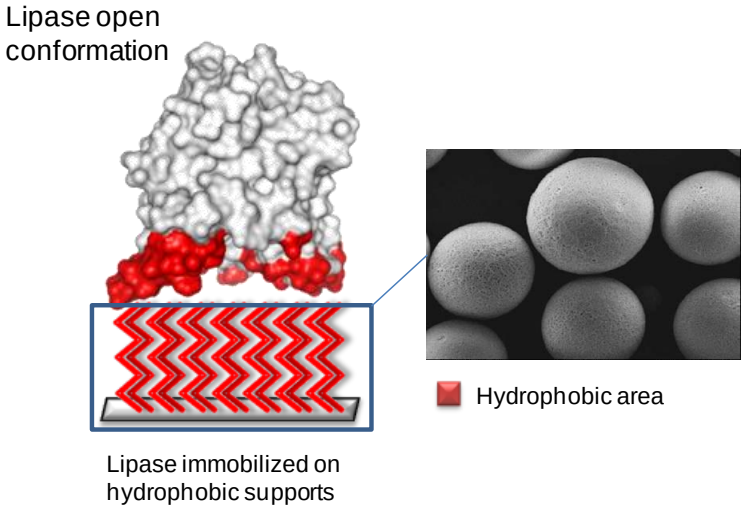
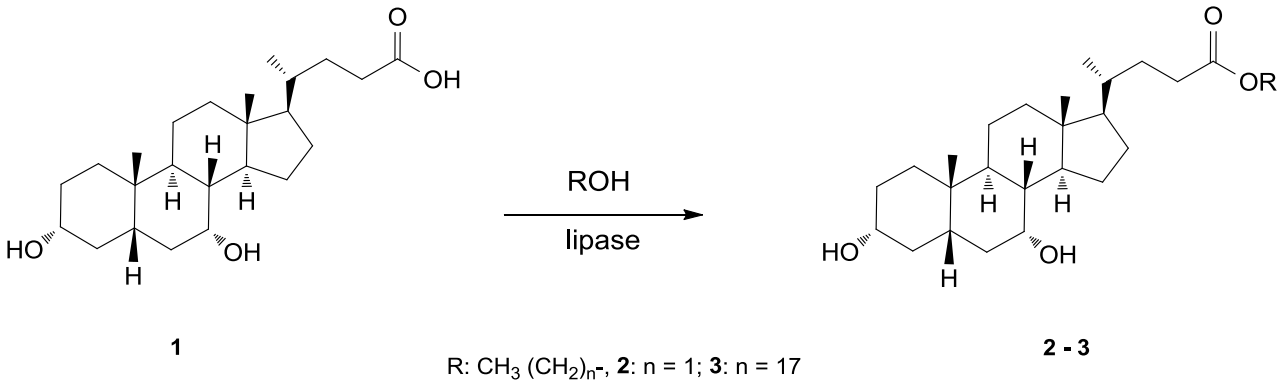


Figure 1. Oriented immobilized lipases on hydrophobic supports.

In these cases the use of resins such as Sepabeads or Lewatit represents an advantage because they can be used in different organic solvents without any swelling effect [42, 43].

In the last decade, ROL has been cloned and expressed successfully in the cell factory *Pichia pastoris* under different promoters, and operational strategies [44]. The recombinant ROL was more than 40-fold-higher than commercial native ROL, with no esterase contamination [45]. It has also immobilized on different supports to increase its stability and used as biocatalyst in a wide range of biotransformations: structured lipids as production of low caloric triacylglycerols and human milk fats substitutes, flavours as pine-apple aroma, biodiesel production and steroids derivatives [46].

Therefore, as far as we know, here we present for the first time the enzymatic esterification of a bile acid: chenodeoxycholic acid (**1**) (Scheme 1) catalyzed by immobilized commercial *Candida antarctica* lipase (CAL B) and recombinant *Rhizopus oryzae* lipase (ROL). The optimization of the process by using different interfacial activated immobilized preparations of ROL has been performed. Moreover, after fixing some operational parameters, the optimal conditions of enzyme: substrate ratio and alcohol: substrate ratio have been determined by a central composite rotatable experiment design using the response surface methodology, for the best immobilized derivative.



1 **2 - 3**

R: $CH_3(CH_2)_n$-, **2**: n = 1; **3**: n = 17

Scheme 1. Enzyme-catalyzed esterification of chenodeoxycholic acid.

2. Experimental

2.1. General

Chenodeoxycholic acid, p-nitrophenylbutyrate (pNPB) and solvents used in this work were of analytical grade and purchased from Sigma-Aldrich. *Candida antarctica* lipase B (CAL B): Novozym 435 (7400 PLU/g) was a generous gift of Novozymes Spain. Octadecyl-Sepabeads was purchased from Resindion rsl and Lewatit VP OC1600 was purchased from Bayer .The immobilized enzymes were dried overnight in vacuum drying oven before use (0.1 kPa, 30 ^{o}C). Enzyme: substrate ratio (E/S): enzyme amount (CAL B, ROL 1 and ROL 2) in mg/substrate amount in mg. Alcohol: substrate ratio (A/S): alcohol amount in mg/ substrate amount in mg. Enzyme specific activity (micromoles/mg lipase x h): ROL 1: 0.62; ROL 2: 0.17; CAL B: 0.10. Enzymatic reaction was carried out on Innova 4000 digital incubator shaker, New Brunswick Scientific Co. at 200 rpm. To monitor the reaction progress aliquots were withdrawn and analyzed by TLC performed on commercial 0.2 mm aluminum-coated silica gel plates (F254) and visualized by 254 nm UV or immersion in an aqueous solution of $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.04 M), $Ce(SO_4)_2$ (0.003 M) in concentrated H_2SO_4 (10%). % Conversion was determined by gas chromatography on a Thermo Focus GC chromatograph equipped with a flame ionization detector and a using HP-17 column (10 m x 0.53 mm ID, 0.25 thickness; Agilent Corporation, USA). Nitrogen was the carrier gas. Both injector and detector temperatures were set at 250 and 300 °C respectively. Column temperature was programmed from10 to 200 °C at a rate of 10 °C/min and stable at 200 °C for 1 min, then at a rate of 15 °C/min up to 290 °C stable at this temperature for 10 min, then at a rate of 8 8C/min up to 230 8C/min and stable at 230 8C for 35 min. Retention times: chenodeoxycholic acid (**1**) 13.5 min; ethyl chenodeoxycholate (**2**): 14.3 min, stearyl chenodeoxycholate (**3**): 15.2 min. Melting point were determined on a Fisher Johns apparatus and are uncorrected. Elemental analysis was carried out with a CE-440 Elemental Analyzer. Proton and carbon NMR spectra were carried out on a Bruker AM-500 (500MHz for ^{1}H and 125.1 for ^{13}C) in $CDCl_3$. Chemical shifts (δ) are reported in ppm downfield from TMS as the internal standard. Coupling constant (J) values are given in Hz. The assignment of the proton signals is based on the chemical shift correlation experiments (COSY) while the carbon nuclei were assigned from the heteronuclear correlation experiments via one-bond (HSQC) coupling constants and long-range (HMBC)

coupling constants. HR-ESI-MS were measured in a Bruker microTOF-Q II mass spectrometer.

2.2. Enzyme production

ROL was produced by the Bioprocess Engineering and Applied Biocatalysis group of Universitat Autònoma de Barcelona (UAB). It has been obtained by a fed-batch cultivation mixed substrates fed-batch cultivation of a recombinant *P. pastoris* strain using a methanol non-limiting fed-batch strategy maintaining a set-point of methanol of 3 g L^{-1} by means of a predicitive-PI control strategy [44c]. After centrifuged and microfiltered the culture broth, the supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system equipped with an Omega membrane of 10 kDa cut-off, and subsequently dialyzed against 10mM Tris-HCl buffer pH 7.5 and thereafter lyophilized [47].

2.3. Enzymatic activity assay

The activities of the soluble and immobilized ROL preparations were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm produced by the release of p-nitrophenol (pNP) ($\in$= 5,150 M^{-1} cm^{-1}) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. Enzymatic activity is given as micromole of hydrolyzed pNPB per minute per milligram of enzyme (IU) under the conditions described above.

2.4 Immobilization of ROL on Octadecyl-Sepabeads® or LewatitVPOC1600.

One gram of dry octadecyl-Sepabeads (or Lewatit1600) was added to 100 mL of a solution water/acetone (50/50, v/v) for 2 h. Then, the solution was filtrated by vacuum and the support was added to 15 mL of protein solution (containing 12 mg of lipase in 25 mM sodium phosphate buffer) at pH 7. The mixture was then gently stirred at 25ºC and 250 rpm over night on Coulter stirrer. After that, the solution was filtered on sintered glass filter and the supported lipase was washed several times with abundant distilled water. The immobilization yield was more than 99% determined by the enzymatic activity assay described above and Bradford's

assay (ref Bradford). The prepared catalysts (containing 12 mg lipase per gram of support) were called Octadecyl-ROL and Lewatit-ROL respectively.

Effect of E/S and A/S ratios on conversion

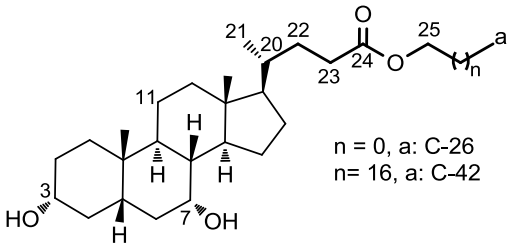
The effect of enzyme/substrate (E/S) and alcohol/substrate (A/S) ratio on the conversion of the chenodeoxycholic esters was studied by means of a central composite design (CCD) with Matlab using ROL1 (ROL immobilized on Octadecyl Sepabeads). The E/S and A/S ratios were selected according with preliminary experiments and were in the range 5-25. The values of both variables were codified from -1.41 to 1.41. The empirical response surfaces were built from the values of conversion after 48 h of conversion.

All the experiments were made under the same conditions of immobilized biocatalyst (dry) temperature and shaking were fixed at 55°C and 200 rpm, respectively. DIPE was used as solvent, and chenodeoxycholic acid was esterified using stearyl alcohol.

2.5. Enzymatic esterification

2.5.1. General procedure

Rhizopus oryzae lipase immobilized on octadecyl Sepabeads (2 g) was added to a solution of chenodeoxycholic acid (100 mg, 0.25 mmol) in diisopropylehter (10 mL) and ethanol (0.25 mL, 5 mmol) or stearyl alcohol (1.35 g, 5 mmol). The suspension was shaken (200 rpm) at 55°C and the progress of the reaction monitored by TLC/GC. When the acid was converted into the alkyl ester, the enzyme was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography employing mixtures hexane: ethyl acetate as eluent (9:1-7:3). Reuse experiments: the filtered and washed enzyme was used in the next enzymatic esterification under the same reaction conditions.



2.5.2. Ethyl 3α,7α-Dihydroxy-5β-cholan-24-oate (2).

Colorless oil. Yield: 366 mg (87 %).FT-IR (film) ν_{max} = 3414, 1754, cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) δ: 3.84 (dd, *J*= 3.7, 2.6 Hz, 1H, H-3), 3.46 (tt, *J*= 11.1, 4.3 Hz, 1H, H-7), 0.65 (s, 3H, H-18), 0.90 (s, 3H, H-19), 0.92 (d, *J*=6.6 Hz, 3H, H-21), 4.11 (q, *J*=7.1 Hz, 2H, H-26), 1.40 (t, *J*= 7.2 Hz, 3H, H-26). ^{13}C NMR ($CDCl_3$, 200 MHz): δ : 72.2 (C-3), 68.7 (C-7), 11.9 (C-18), 18.4 (C-19), 17.5 (C-21), 21.9 (C-23), 174.5 (C-24), 60.3 (C-25), 14.4 (C-26). ESI-HRMS *m/z*: calcd. for $C_{26}H_{44}O_4$: $[M+H]^+$ 421.3312, found 621.3317; $[M+Na]^+$ calcd: 443.3132, found 443.3135 [39b]. Analysis for $C_{42}H_{76}O_4$: calcd. C, 74.24; H, 10.54. found: C, 74.22; H, 10.55.

2.5.3. Octadecyl 3α,7α-Dihydroxy-5β-cholan-24-oate (3).

White solid. Yield: 543 mg (84 %). Mp: 61-62 °C. FT-IR (film) ν_{max} = 3420, 1761, cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) δ: 3.85 (dd, *J*= 3.8, 2.7 Hz, 1H, H-3), 3.46 (tt, *J*= 11.0, 4.3 Hz, 1H, H-7), 0.65 (s, 3H, H-18), 0.90 (s, 3H, H-19), 0.92 (d, *J*=6.5 Hz, 3H, H-21), 4.05 (t, *J*=6.7 Hz, 2H, H-25), 1.62 (m, 2H, H-26), 1.28-1.38, (broad signals, 30H, H-27-41), 0.88 (t, *J*=6.85 Hz, 3H, H-42). ^{13}C NMR ($CDCl_3$, 200 MHz): δ: 72.0 (C-3), 68.6 (C-7), 11.8 (C-18), 18.3 (C-19), 17.6 (C-21), 22.8 (C-23), 174.4 (C-24), 64.5 (C-25), 28.2 (C-26), 14.1(C-42). ESI-HRMS *m/z*: calcd. for $C_{42}H_{76}O_4$: $[M+H]^+$ 645.5816, found 645.5820; $[M+Na]^+$ cald. 667.5634 m/z; found 667.5638 m/z. Analysis for $C_{42}H_{76}O_4$: calcd. C, 78.08; H, 12.01. found: C, 78.12; H, 12.05.

3. Results

1. Enzymatic esterification

The enzyme-catalyzed esterification of chenodeoxycholic acid (**1**) allowed us to obtain two ester derivatives: ethyl chenodeoxycholate (**2**) and stearyl chenodeoxycholate (**3**) (Scheme 1).

Product **2** was fully identified by ESI-HRMS and ^1H NMR spectroscopy, which was in accordance with reported data [24].

The esterification with ethanol was confirmed by observing the typical signals in the ^1H NMR signals of methyl triplet resonance at 1.40 ppm and a quartet at 4.11 ppm corresponding to the

methylene group in the ethyl chenodeoxycholate. There was no variation on methyl-18 and methyl-19 chemical shift, at 0.65 ppm and 0.90 respectively in comparison with the same signals in proton NMR spectrum data of chenodeoxycholic acid. In carbon-13 NMR spectrum, the signals at δ: 60.3 and 14.4, corresponding to carbons in methylene and methyl groups respectively in the ethyl ester were observed as expected. Again, no variation was observed on the C-18, C-19 and C-21 chemical shift in comparison with data for chenodeoxycholic acid. Regarding the signal corresponding to the carbonyl group, it was observed a downfield shift from 178.9 ppm in **1** to 174.5 ppm in **2**.

Stearyl chenodeoxycholate **3** is a new compound. Its identity was completely determined by spectroscopic methods. The spectroscopic analysis of **3** was consistent with the formation of the stearyl ester of chenodeoxycholic acid. The molecular formula was determined as $C_{42}H_{76}O_4$, on the basis of the elemental analysis and ESI-HRMS spectrum observing the peaks: $[M+H]^{+:}$ 645.5820 and $[M+Na]^+$: 667.5638. The molecular ion was 645 larger from that of chenodeoxycholic acid, which indicated the formation of the stearyl ester.

The identity of **3** was also established by observing in the ^{1}H NMR spectrum the triplet at δ 0.88 corresponding to methyl group in the end of the stearyl group, multiplets at 1.28-1.38, ppm corresponding to the methylene groups in the stearyl chain and a triplet at 4.05 assigned to the methylene group bonded to the carboxylic oxygen of the ester. In the ^{13}C NMR spectrum we observed the new signals of the methylene at C-25 at 64.5 ppm and the methyl C-42 at 14.1 ppm from the new stearyl group. The chemical shifts assignments, made on the basis of the information obtained from ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and COSY (Supplementary data), allowed identifying **3** as octadecyl 3α,7α-dihydroxy-5β-cholan-24-oate.

In order to optimize the reaction conditions we have performed several experiments involving lipase screening and variation of the reaction parameters such as temperature, enzyme: substrate ratio (E/S), alcohol: substrate ratio (A/S) and solvent.

1.a. Enzyme screening and solvent effect

To begin, the behavior of CAL B, free ROL, and two immobilized preparations named: ROL 1 (immobilized on Octadecyl Sepabeads®) and ROL 2 (immobilized on Lewatit® 1600) was evaluated.

Table 1. Optimization of reaction parameters for ROL-catalyzed esterification of CDCA (**1**). [a]

Entry	Enzyme	Solvent	Temperature (°C)	E/S	A/S	Conversion (%)
Solvent and Lipase						
1	ROL 1	Hexane	55	30	30	75.6
2	**ROL 1**	**DIPE**	55	30	30	89.2
3	ROL 1	Acetonitrile	55	30	30	70.4
4	ROL 2	Hexane	55	30	30	51.1
5	ROL 2	DIPE	55	30	30	60.2
6	ROL 2	Acetonitrile	55	30	30	45.8
7	CAL B	Hexane	55	30	30	69.4
8	CAL B	DIPE	55	30	30	52.1
9	CAL B	Acetonitrile	55	30	30	58.3
E/S						
10	ROL 1	DIPE	55	2	20	30.2
11	ROL 1	DIPE	55	5	20	35.6
12	ROL 1	DIPE	55	10	20	71.0
13	ROL 1	DIPE	55	**20**	20	89.6
14	ROL 1	DIPE	55	30	20	89.9
A/S						
15	ROL 1	DIPE	55	20	5	28.8
16	ROL 1	DIPE	55	20	10	58.4
17	ROL 1	DIPE	55	20	**20**	88.9
18	ROL 1	DIPE	55	20	30	90.3
Temperature						
19	ROL 1	DIPE	30	20	20	65.3
20	ROL 1	DIPE	**55**	20	20	90.1

[a] time: 48h. Alcohol: stearyl aclohol.

Previous work on the acetylation reaction catalyzed by ROL [46e] showed that enzyme performance was improved when it was dried before use. After drying, the water percentage in free ROL and the two immobilized ROL was variable being 57% in free ROL, 54% in ROL 1and 43% in ROL 2. The four lipase preparations, CAL B, free ROL, ROL 1 and ROL 2, were evaluated in the esterification of **1** with stearyl alcohol. The solvents used for this screening were acetonitrile, hexane and diisopropyl ether (DIPE). Reactions were carried out at 55°C using E/S ratio 30 and stearyl alcohol/chenodeoxycholic acid ratio 30. TLC and GC monitoring allowed the identification of the lipase able to promote the esterification of **1.** In the absence of biocatalyst no product was obtained.

Among the tested enzymes, ROL 1 gave the most satisfactory results followed by CAL B (Table 1, entries 1-3 and 7-9 respectively). ROL 2 showed moderate to poor activity (Table 1, entries 10-12) and with free ROL, chenodeoxycholic acid and stearyl alcohol did not react at all.

If we compare the results of the enzyme using DIPE as solvent, even ROL 2 showed higher activity than CAL B, being both immobilized on the same support. Therefore, the best results obtained with ROL 1 in the esterification reaction could be attributed both to the lipase source and the support of immobilization. Moreover, it is interesting to mention that the amount of lipase powder in the immobilized enzyme is lower in ROL 1 or ROL2 (12 mg/ g of support) than in CAL B (~30 mg/ g of support)[43]. Indeed, the specific activity of ROL 1 was six fold higher than that of CAL B in the esterification of CDCA with stearyl alcohol, 0.62 and 0.10 µmmol.mg^{-1}lipase.h^{-1}, respectively. On the other hand the specific activity of ROL 2 was also slightly higher than CAL B (Table 2).

Table 2. Specific activity of ROL 1, ROL 2 and CAL B

Lipase	Conversion (%)	Time (h)	Specific activity (µmol/mg lipase.h)
ROL 1	22.3	5	0.62
ROL 2	20.5	11	0.17
CAL B	19.8	16	0.10

These results show how the immobilization of a lipase on different supports may alter its catalytic features. Octadecyl-Sepabeads (ROL 1) is a hydrophobic epoxyacrylic matrix with the surface functionalized with octadecyl groups in contrast to Lewatit (ROL 2 and CAL B)

which is a basic divinylbenzene resin. Although the immobilization of ROL was performed following the same procedure in both cases, at low ionic strength after a simple hydrophobic adsorption of the lipase, the esterification results suggest that the immobilization in the case of using Lewatit may involve some other kind of enzyme-support interactions, perhaps by ionic exchange, that could be enough to produce changes in the enzyme features.

In previous work, we have also observed large differences in activity and spectacular changes in enantiopreference when using these two types of immobilization methods applied to *Candida antarctica* lipase catalyzing reactions in aqueous media [43]. CAL B seems to exhibit good performance in more hydrophobic solvents such as hexane while ROL 1 is more active in a solvent with intermediate polarity as DIPE [35,36,39].

Considering that free ROL was completely inactive, the immobilization on Sepabeads made ROL 1 a new and excellent biocatalyst for esterification reactions of chenodeoxycholic acid and potentially overall bile acids.

Once the optimal catalyst was selected, we turned our attention to the solvent effect in the ROL 1 catalyzed reaction. Therefore, hexane, diisopropyl ether and acetonitrile were tested. It is well-known that hydrophobic water-immiscible solvents such as hexane are a good medium for lipase-catalyzed reactions. The organic medium shows interesting advantages, such as the enhancement of solubility of reactants, activity and stability of the enzyme, the shift of the equilibrium towards product formation and easier separation of the enzyme from the reaction medium at the end of the reaction [30]. On the other hand, water-miscible solvents shows a high tendency of stripping off tightly bound water from enzyme and ability to partition deeper into the enzyme active site which in turn causes loss of both structure and activity of enzymes [48]. Due to the presence of the carboxyl and two hydroxyl groups, chenodeoxycholic acid is a polar molecule, insoluble in non polar solvents (hexane) and soluble in polar solvents (acetonitrile). As acetonitrile proved to be useful as solvent in previous work using a variety of substrates, we decided to test it [49]. However, enzymes performance was poor in this solvent as can be observed in Table 1 (entries 3, 6 and 9). Only ROL 1 afforded the product **3** in good conversion (70.4%) using acetonitrile as solvent. This result was improved performing the esterification in DIPE, therefore this was the solvent of choice.

1.b. Influence of temperature

With the aim of investigating the influence of temperature on the enzymatic esterification we performed it at 30 °C and 55 °C. The other reaction parameters were settled to their optimal values (ROL 1, DIPE, E/S and A/S: 20). The results in Table 1 (entries 19 and 20) show a better performance with the increase in temperature. Therefore we selected 55°C as the reaction temperature. It is interesting to observe that the stability of the enzyme was kept unaltered with the increase of temperature reaction.

1.c. Effect of enzyme: substrate ratio

Then the reaction was analyzed studying the influence of the enzyme: substrate ratio in the enzymatic esterification. This was evaluated at 48 h, using A/S: 20, DIPE as solvent at 55°C and variable amounts of ROL 1. From the obtained results, E/S:20 (Table 1, entry 13) resulted the ratio of choice.

1.d. Effect of alcohol and alcohol agent : substrate ratio

In order to test the influence of the alkyl chain length of the alcohol on enzymatic esterification of chenodeoxycholic acid with the immobilized recombinant lipase ROL 1, we prepared ethyl (**2**) and stearyl (**3**) chenodeoxycholic esters. The results were both very good and much the same, the ethyl chenodeoxycholate (**2**) was obtained in 87% yield and the stearyl chenodeoxycholate (**3**) in 84 % yield. The alkyl chain length of the alcohol had no significance on yield in the esterification catalyzed by ROL 1.

The influence of alcohol: substrate ratio on reaction yield was evaluated in the esterification of chenodeoxycholic acid **1** with stearyl alcohol in DIPE using ROL 1. As expected, it was observed that a molar excess of alcohol was advantageous for the reaction (Table 1, entries 15-18) with A/S 20 (entry 17) giving very good results (88.9 % of conversion). A slightly better conversion was observed using a higher excess of alcohol (A/S:30 afforded 90.3 %). The optimum reaction time was also studied and conversion was not improved after 48 h of reaction.

E/S and A/S ratios are key parameters in order to establish an optimal relationship not only in conversion but also in economics terms. Thus, a response surface methodology and a central

composite experimental design were performed. E/S and A/S selected range is described in Figure 2, according with the described previous individual experiments.

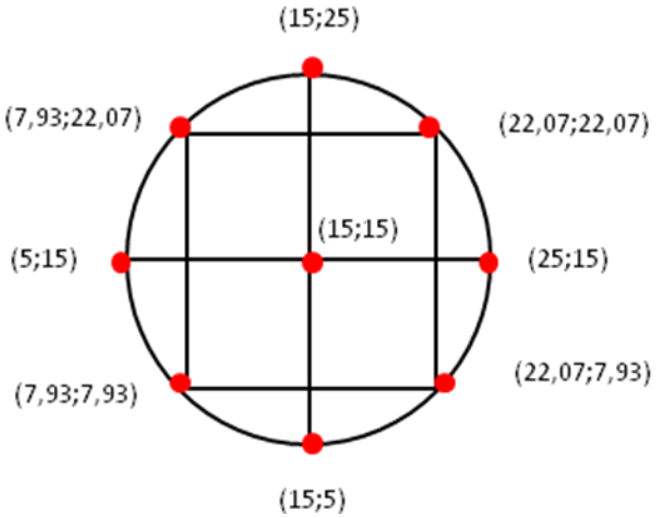


Figure 2. Diagram of the central composite rotatable design, using the nomenclature (E/S); (A/S).

The constant operational conditions were fixed following the results obtained in the esterification reaction. Therefore, the biocatalyst was previously dried, temperature and shaking were fixed at 55 °C and 200 rpm, respectively, stearyl alcohol as esterifying agent and DIPE as solvent. The conversions of each reaction are presented in Table 3.

Table 3. Conversion reached in each reaction at different (E/S) and (A/S) ratio.

Experiment number	Codified factor (E/S)	Codified factor (A/S)	Non codified factor (E/S)	Non codified factor (A/S)	Conversion (%)
1	-1	-1	7.93	7.93	15.2
2	-1	1	7.93	22.07	27.4
3	1	-1	22.07	7.93	35.3
4	1	1	22.07	22.07	88.5
5	-1.4142	0	5	15	12.6
6	1.4142	0	25	15	85.3
7	0	-1.4142	15	5	21.9
8	0	1.4142	15	25	83.2
9	0	0	15	15	84.1
10	0	0	15	15	83.9
11	0	0	15	15	85.3
12	0	0	15	15	87.3
13	0	0	15	15	84.5
14	0	0	15	15	85.0
15	0	0	15	15	81.0
16	0	0	15	15	86.5

The results obtained were fitted to the mathematical modelling expressed in equation 1, and the corresponding response surface is plotted in Figure 3.

$$X = \beta_1 + \beta_2 \cdot ES + \beta_3 \cdot AS + \beta_4 \cdot ES \cdot AS + \beta_5 \cdot ES^2 + \beta_6 \cdot AS^2 \text{ (equation 1)}$$

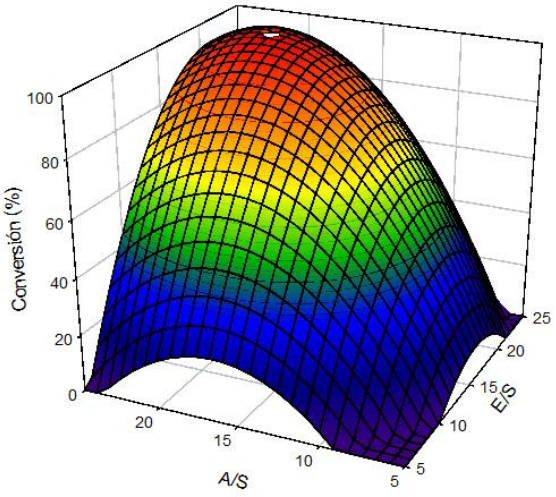


Figure 3. Response surface of the experimental design, corresponding to the conversion of stearyl deoxycholate (**3**) under different ratios of E7S and A/S. Reactions were carried out at 55 °C and 200 rpm, using dry ROL 1, DIPE as solvent and stearyl alcohol. Reaction time was 48 h.

The values of each coefficient and the statistical parameter p values, are shown in Table 4.

Table 4. Parameter and statistical p values of the equation 1.

Coefficient	β_1	β_2	β_3	β_4	β_5	β_6
Value	84.700	23.002	19.012	10.250	-20.163	-18.363
p value	$1.063 \cdot 10^{-12}$	$3.620 \cdot 10^{-7}$	$2.111 \cdot 10^{-6}$	0.004	$1.232 \cdot 10^{-6}$	$2.895 \cdot 10^{-6}$

All the terms of the equation 1 has significant effect on conversion ($P < 0.05$). The term ES·AS has the lowest effect on the estimation. As it has been observed in previous individual experiments about effect of E/S and A/S ratios, the optimum value of (E/S) and (A/S) to obtain practically a total conversion were 20. However from an economical point of view the optimum value can be reduced up to 15 for both ratios.

Thus, optimal conditions from a conversion and economic point of view for the esterification of chenodeoxycholic acid using ROL 1 as biocatalyst are: E/S: 15, solvent: DIPE, temperature: 55ºC and stearyl alcohol/CDCA: 15.

Finally, we studied the possibility of recycling ROL 1. Due to ROL 1 is immobilized, it is insoluble in DIPE. Therefore, it can be easily removed by filtration at the end of the process

and can be reused. For the esterification of CDCA with stearyl alcohol ROL 1 kept almost 90% of its original activity after three reaction cycles (Figure 4).

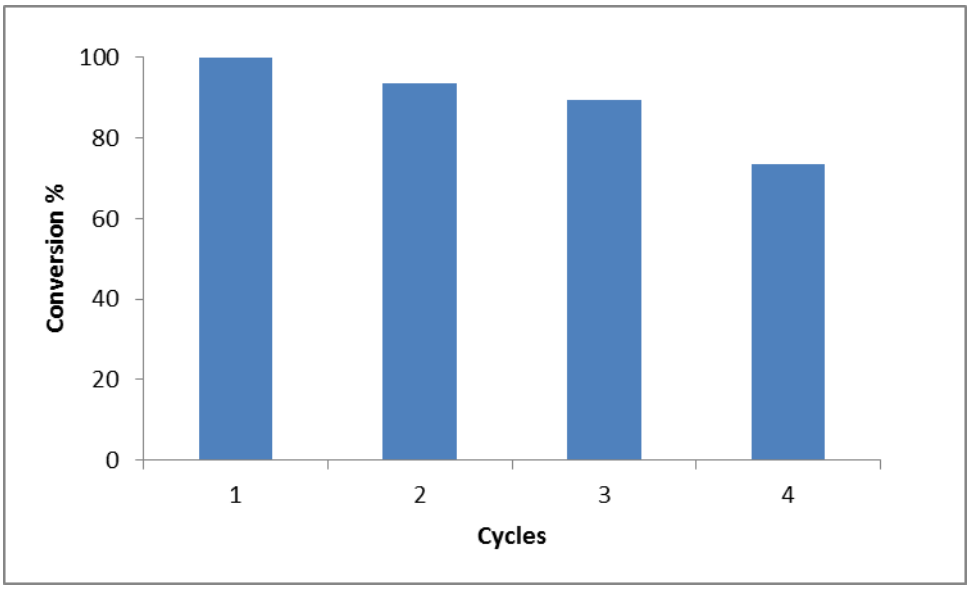


Figure 4. ROL 1 reuse in the esterification of chenodeoxycholic acid under standard conditions.

4. Discussion

In this report we have studied the performance of CAL B and two immobilized heterologous *Rhizopus oryzae* lipases as catalysts in the esterification of chenodeoxycholic acid. For the first time two bile acid esters were prepared in very good yield following the biocatalytic approach. One of them, the stearyl chenodeoxycholate is a new product. ROL immobilized on Octadecyl Sepabeads (ROL 1) and using DIPE as solvent, was the best biocatalyst in the preparation of the steroid derivatives, showing an activity six times higher than CAL B. Moreover, ROL 1 performance was not affected by the chain length in the alcohol, affording both esters in similar yield and it kept almost 90% of its original activity after three reaction cycles.

By means of a response surface methodology and a central composite rotatable design, the optimal values of enzyme:substrate ratio and alcohol:substrate ratio were determined in order to maximize conversion and minimize economics of the bioprocess. The optimal values of

(E/S) and (A/S) were 15 for both, lower than the values obtained from individual experiments, saving both enzyme and alcohol.

The enzymatic approach provided a simple and mild alternative method for the synthesis of chenodeoxycholic esters and potentially other bile acid derivatives. It proved to be a convenient way to prepare bile acid derivatives in high purity and free of toxic reagents, which is a great advantage in the case of products designed for human consumption such as pharmaceuticals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at .....

References

[1] Ruggles KV, Turkish A, Sturley SL. Making, baking and breaking: The synthesis, storage and hydrolysis of neutral lipids. Ann Rev Nutr 2013;33:413-451.

[2] Negovsky VA. Physiological and molecular biochemical mechanisms of bile formation. World J Gastroenterol 2013;19:7341-7360.

[3] a) Hylemon, PB, Zhou H, Pandak WM, Ren S, Gil G, Dent P. Biles acids as regulatory molecules. J Lipid Res 2009;50:1509-1520. b) Zhou H, Hylemon PB. Bile acids as nutrient signaling hormones. Steroids 2014;86:62-68.

[4] Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB. A regulatory casacade of the nuclear receptors FXR, SHP-1 and LRH-1 represses bile acid biosyntehsis. Mol Cell 2000; 6:517-526.

[5] Zhou H, Hylemon P. Bile acids are nutrient signaling hormones. Steroids 2014;86:62-68.

[6] Sharma P, Chawla HPS, Panchagnula R. The role of sorption promoters in increasing the bioavailability of drugs in oral preparations. Drugs Fut 1999;24:1221-1240.

[7] Michael S, Thole M, Dillmann R, Fahr A, Drewe J, Fricker G. Improvement of intestinal peptide absorption by a synthetic bile aicd derivativel cholylsarcosine. Eur J Pharm Sci 2000;10:133-140.

[8] Mikov M, Fawcett JP, Kuhajda K, Kevresan S. Pharmacology of bile acis and their derivatives: absorption promoters and therapeutic agents. Eur J Drug Metab Pharmacokinet 2006;31:237-251.

[9] Mrózek L, Dovořáková L, Mandelová Z, Rárová L, Řezáčová A, Plaček L, Opatřilová R, Dohnal J, Paleta O, Král, Drašar, Jampílek J. Investigation of new acyloxy derivatives of cholic acid and their esters as drug absorption modifiers. Steroids 2011; 76:1082-1097.

[10] Jampilek J, Brychtova K, Azone analogs: classification, design and transdermal penetration principles. Med Res Rev 2012;32:907.

[11] Bortolini O, Fantin G, Fogagnolo M. Bile acids in asymmetric synthesis and chiral discrimination. Chirality 2010;22:486-494.

[12] Monte MJ, Marín JJ, Vazquez-Tato J. Bile acids: chemistry, physiology and pathophysiology. J Gastroenterol 2009;15:804-816.

[13] Campanelli AR, Candeloro de Sanctis S, Giglio E, Pavel NV, Quagliata C. From crystal to micelle: a new approach to the micellar structure. J Inclusion Phenom Macrocyclic Chem 1989;7:391.400.

[14] Alvarez M, Jover A, Carrazana J, Meijide F, Soto VH, Vázquez Tato J. Crystal structure of chenodeoxycholic acid, ursodeoxycholic acid and their two 3β,7α- and 3β,7β-dihydroxy epimers. Steroids 2007;72:535-544.

[15] Small DM. Size and structure of bile acids micelles. Influence of structure, concentraton, counterion concentration, pH and temperature. Adv Chem Ser 1968;84:31-52.

[16] Chikada M, Sada K, Miyata M. Intercalation and polymerization in chenodeoxycholic acid channels with retention of a crystalline state. Polym J 1999;31:1061-1064.

[17] Iser JH, Sali A. Chenodeoxycholic acid: A review of its pharmacological properties and therapeutic use. Drugs 1981;21:90-119.

[18] Meyer-Gerspach AC, Steinert RE, Keller S, Malarski A, Schulte FH, Beglinger C. Effects of chenodeoxycholic acid on the secretion of gut pepetides and fibroblast growth factor in healthy humans. J Clin Endrocrinol Metabol 2013;98:3351-3358.
[19] Liang D, Zhou Q, Zhang J, Gong W, Xu Ch, Li B, Wang Y, Li J. A novel chenodeoxycholic acid-verticinone ester induces apoptosis and cell cycle arrest in HepG2 cells. Steroids 2012;77:1381-1390.
[20] Zhang J, Wang H, Pi H, Ruan H, Zhang P, Wu J. Structural analysis and antitussive evaluation of five novel esters of verticinone and bile acids. Steroids 2009;74:424-434.
[21] Shi Z, Zha Z, Liu M, Wang X. Solvent-free synthesis of novel unsymmetric chenodeoxycholic acid bis thiocarbazone derivatives promoted by microwave irradiation and evaluation of their antibacterial activity. CR Chim 2013;16:977-984.
[22] Sepe V, Ummarino R, D'Auria MV, Chini MG, Bifulco G, Renga B, D'Amore C, Debitus C, Fiorucci S, Zampella A. Conicasterol E, a small heterodimer partner sparing farnesol X receptor modulator endowed with a pregnan X receptor agonistic activity , from the marine sponge *Theonella swinhoei*. J Med Chem 2012;55:84-93.
[23] Sharma R, Majer F, Peta VL. Wang J, Keaveney R, Kelleyer D, Long A, Gilmer JF. Bile acid toxicity structure-activity relationships: Correlation between cell viability and lipophilicity in a panel of new and known bile acids using an oesophageal cell line (HET-1A). Biorg Med Chem 2010;18:6886-6895.
[24] Uekawa T, Ishigami K, Kitahara T.Short step synthesis of chenodiol from stigmasterol. Biosc Biotech Biochem. 2004;68:1332-1337.
[25] Bai X, Barnes Ch, Dias JR. Synthesis and comparative spectroscopic analysis of two chenodeoxycholic acid (CDCA) derivatives with closely related 7alfa-ester moieties. Tetrahedron Lett 2009;50:503-505.
[26] Dayal B, Keshava R, Salen G. Microwave-induced organic reactions of bile acids: esterification, deformylation and deacetylation using mild reagents. Steroids 1995;60:453-457.
[27] Faber K. Biotransformations in Organic Chemistry, 6th ed. Heidelberg: Springer Verlag; 2011.
[28] Whitthall J, Sutton PW. Practical Methods for Biocatalysis and Biotransformations 2, New York: John Wiley & Sons Ltd.; 2012.
[29] Tao J, Lin G-Q, Liese A. Biocatalysis for the Pharmaceutical Industry: Discovery, Development and Manufacturing, New York: John Wiley & Sons Ltd.; 2009.
[30] Carrea G, Riva S, editors. Organic Synthesis with Enzymes in Non-aqueous Media, Weinheim: Wiley–VCH; 2008.
[31] Baldessari A. Lipases as catalysts in synthesis of fine chemicals. In Georgina Sandoval: editor. Lipases and Phospholipases, Methods and Protocols, New York: Humana Press; 2012, p. 445-456.
[32] a) Rustoy EM, Y. Sato Y, Nonami H, Erra-Balsells R, Baldessari A. Lipase-catalyzed synthesis and characterization of copolymers from ethyl acrylate as the only monomer starting material. Polymer 2007; 48:1517-25. b) Monsalve LN, Fatema KM, Nonami H, Erra-Balsells R, Baldessari A. Lipase-catalyzed synthesis and characterization of a novel linear polyamidoamine oligomer. Polymer 2010;51:2998-3005 c) García Liñares G, Baldessari A. Lipases as efficient catalysts in the synthesis of monomers and polymers with biomedical applications. Curr Org Chem 2013;17:719-743. d)Monsalve LN, Petroselli G, Vázquez A, Erra-Balsells R, Baldessari A. Chemoenzymatic synthesis

of novel N-(2-hydroxyethyl)-beta peptoid oligomer derivatives and application to porous polycaprolactone films. Polymer Int 2014;63:1523-1530.
[33] Gotor V, Alfonso I, García-Urdiales E. Asymmetric Organic Synthesis with Enzymes, Weinheim: Wiley–VCH; 2007.
[34] Hall M, Kroutil W, Faber K. The Evolving Role of Biocatalysis in Asymmetric Synthesis II: More Methods and Applications, New York: Wiley-VCH; 2013.
[35] a) Monsalve, LN, Roselli S, Bruno M, Baldessari A. Lipase-catalyzed preparation of acyl derivatives of the germacranolide cnicin. J Mol Catal B: Enzym 2009;57:40-47.
[36] a) Monsalve LN, Machado Rada MY, Ghini AA, Baldessari A. An efficient enzymatic preparation of 20-pregnane succinates: Chemoenzymatic synthesis of 20β-hemisuccinyloxy-5αH-pregnan-3-one. Tetrahedron 2008; 64:1721-1730. b) Quintana GP, Baldessari A. Lipase-catalyzed regioselective preparation of fatty acid esters of hydrocortisone. Steroids 2009;74:1007-1014 c) Quintana PG, Guillén M, Marciello M, Palomo JM, Valero F, Baldessari A. Eur J Org Chem 2012: 4306-4312. d) Baldessari A, Iglesias, LE. Lipases in Green Chemistry: acylation and alcohólisis of steroids and nucleosides. In Georgina Sandoval: editor. Lipases and Phospholipases, Methods and Protocols, New York: Humana Press; 2012, p. 445-456.
[37] a) Mangone CP, Pereyra EN, Argimón S, Moreno S, Baldessari A. Chemo- and stereoselective reduction of β-ketoesters by spores and various morphological forms of *Mucor rouxii*. Enzyme Microb Tech 2002,;30:596-601. b) Rustoy EM, Pereyta EN, Moreno S, Baldessari A. Combined strategy using pure enzymes and whole cells as biocatalysts in the preparation of 2-hydroxyesters and gamma-butyrolactones from 2-oxoglutaric acid. Tetrahedron Asymm 2004;15:3763-3768. c) Monsalve LN, Cerrutti P, Galvagno MA, Baldessari A. An efficient biotransformation of dialkyl esters of 2-oxoglutaric acid by *Rhodotorula minuta* whole cells. Biocatal Biotransf 2008;26:204-209. d) Monsalve LN, Cerrutti P, Galvagno MA, Baldessari A. *Rhodotorula minuta*-mediated bioreduction of 1,2-diketones. Biocatal Biotransf 2010;28:137-143. e) Quintana PG, Romero SM, Vaamonde G, Baldessari A. New metabolites of drospirenone obtained in Mucorales fungi culture. J Mol Catal B: Enzym 2013;97:110-117.
[38] a)Afzal M, Oommen S, Al-Awadi S. Transformation of chenodeoxycholic acid by thermophilic *Geobacillus stearothermophilus*. Biotechnol Appl Biochem 2011;58:250-255. b) Giovannini PP, Grandini A, Perrone D, Pedrini P, Fantin G, Fogagnolo M. 7α- and 12α-Hydroxysteroid dehydrogenases from *Acinetobacter calcoaceticus lwoffii*: a new integrated chemo-enzymatic route to ursodeoxycholic acid. Steroids 2008;73:1385-1390. c) Medici A, Pedrini P, Bianchini E, Fantin G, Guerrini A, Natalini B, Pellicciari R. 7α-OH epimerization of bile acids via oxido-reduction with *Xanthomonas maltophilia*. Steroids 2002;67:51-56.
[39] a) Riva S, Bovara R, Ottolina G, Secundo F, Carrea G. Regioselective acylation of bile acid derivatives with *Candida cylindracea* lipase in anhydrous benzene. J Org Chem 1989;54:3161-3164. b) Sugai T, Takizawa M, Bakke M, Ohtsuka Y, Ohta H. Efficient lipase-catalyzed preparation of long-chain fatty acid esters of bile acids: Biological activity and synthetic application of the products. Biosc Biotech Biochem 1996;60:2059-2063.
[40] a) Bhandari K, Chaurasia SP, Dalai AK, Gupta A, Singh K. Kinetic study on enzymatic esterification of tuna fish oil fatty acids with butanol. J Mol Catal B: Enzym 2013; 94:104-110. b) Oliveira P, Jares F, Ikegaki M. Enzymatic resolution of (*R*,*S*)-ibuprofen

and (*R*,*S*)-ketoprogen by microbial lipases from native and commercial sources. Braz J Microbiol 2006;37:329-337. c) Méndes J, Oromi M, Cervero M, Balell M, Torres M, Canela R. Combining regio- and enantioselectivity of lipases for the preparation of (*R*)-4-chloro-2-butanol. Chirality 2007;19: 44-50.

[41] Tejo BA, Salleh AB, Pleiss J. Structure and dynamics of *Candida rugosa* lipase: The role of organic solvent. J Mol Model 2004;10:358-366.

[42] a) Palomo JM, Muñoz G, Fernández-Lorente G, Mateo C, Fernández-Lafuente R, -Guisán JM. Interfacial adsorption of lipases on very hydrophobic support (Octadecyl-Sepabeads): Immobilization, hyperactivation and stabilization of the open form of lipases. J Mol Cat B: Enzym 2002;19-20:279-286. b) Palomo JM, Filice M, Romero O, Guisan JM. Improving lipase activity by immobilization and post-immobilization strategies. Method Mol Biol 2013;1051:255-273. c) Brabcova J, Demianova Z, Vondrasek J, Jagr M, Zarevucka M, Palomo J.M. Highly selective purification of three lipases from *Geotrichum candidum* 4013 and their characterization and biotechnological applications. J Mol Cat B: Enzym 2013;98:62-72. d) Marciello M, Filice M, Palomo JM. Different strategies to enhance activities of lipase catalysts. Catal Sci Technol 2012; 2:1531-1543).

[43] Cabrera Z, Fernández-Lorente G, Fernandez-Lafuente R, Palomo JM, Guisan JM. Novozym 435 displays very different selectivity compared to *Candida antarctica* B lipase adsorbed on standard hydrophobic supports. J.Mol Cat B: Enzym 2009;57:171-176).

[44] a) Cos O, Resina D, Ferrer P, Montesinos JL, Valero F. Heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fed-batch cultures. Biochem Engi J 2005; 26:. 86-94. b) Arnau C, Ramón R, Casas C, Valero F. Optimization of the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. Enzyme Microb Technol 2010; 46: 494-500. c) Barrigón JM, Montesinos JL, Valero F. Searching the best operational strategies for *Rhizopus oryzae* lipase production in *Pichia pastoris* Mut^+ phenotype: Methanol limited or methanol non-limited fed-batch cultures? Biochem Eng J 2013; 75: 47-54.

[45] Guillén M, Benaiges MD, Valero F. Comparison of the biochemical properties of a recombinant lipase extract from *Rhizopus oryzae* expressed in *Pichia pastoris*. Biochem Eng J 2011; 54:117-123.

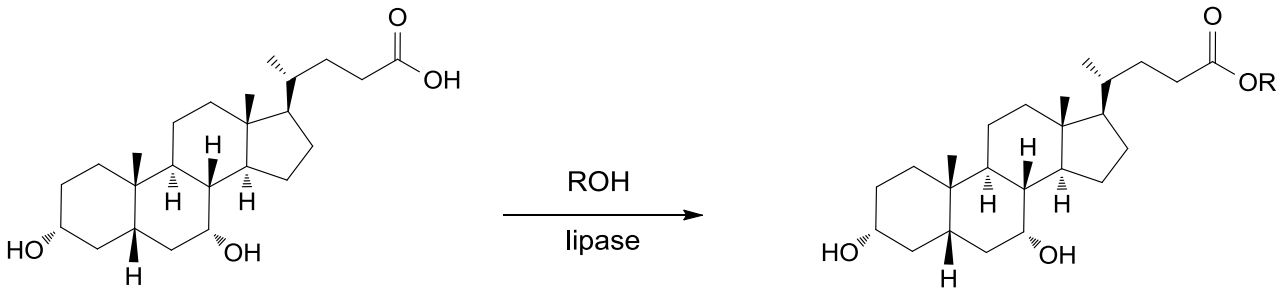
[46] a) Nunes PA, Pires-Cabral P, Guillén M, Valero F, Ferreira-Dias S. Optimize production of LM triacylglycerols catalyzed by immobilized heterologous. *Rhizopus oryzae* lipase. JAOCS 2012;89:1287-1295. b) Simoes T, Valero F, Tecelao C, Ferreira-Dias S. Production of human milk fat substitutes catalyzed by a heterologous *Rhizopus oryzae* lipase and commercial lipases. JAOCS 2014;91:411-419. c) Guillén M, Benaiges MJ, Valero F. Biosynthesis of ethyl butyrate by immobilized recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris*. Biochem Eng J 2012;65:1-9. d) Canet A, Benaiges MD, Valero F. Biodiesel synthesis in a solvent-free system by recombinant *R. oryzae* lipase. Study of the catalytic reaction progress JAOCS 2014; (in press). e) Quintana PG, Guillén M, Marciello M, Palomo JM, Valero F, Baldessari A. Eur J Org Chem 2012: 4306-4312.

[47] Ferrer P, Alarcón M, Ramón R, Benaiges MD, Valero F. Recombinant *Candida rugosa* LIP2 expression in *Pichia pastoris* under the control of the AOX1 promoter. Biochem Eng J 2009;43:271-277.

[48] Yang L, Dordick JS, Garde S. Hydration of enzyme in nonaqueous media is consistent with solvent dependence of its activity. Biophys J 2004;87:812-821

[49] a) Bruttomesso AC, Baldessari A. Lipase-catalyzed deacetylation of androstane and pregnane derivatives: influence of ring D substitution. J Mol Catal B: Enzym 2004;29:149-153. b) da Silva MR, de Mattos MC, Ferreira de Oliveira MC, Gomes de Lemos TL, Pontes Silva NM, de Gonzalo G, Lavandera I, Gotor-Fernandez V, Gotor V. Asymmetric chemoenzymatic synthesis of N-acetyl-α-amino esters based on lipase-catalyzed kinetic resolutions through interesterification reactions. Tetrahedron 2014;70:2264-2271. c) De Diego T, Manjón A , Iborra JL. Selective synthesis of panthenyl esters by a kinetically controlled enzymatic process. Biocatal Biotransf 2013;31:175-180.

Figure

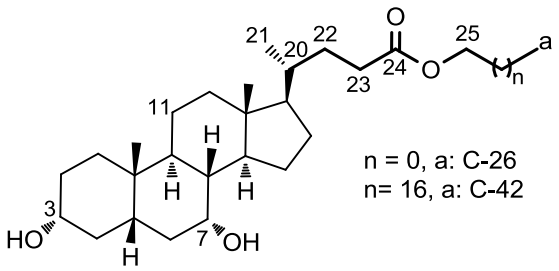


1

2 - 3

R: $CH_3 (CH_2)_n$-, **2**: n = 1; **3**: n = 17

Scheme 1.



Formula 1

Scheme and Figure captions

Scheme 1. Enzyme-catalyzed esterification of chenodeoxycholic acid.

Figure 1. Oriented immobilized lipases on hydrophobic supports.

Figure 2. Diagram of the central composite rotatable design, using the nomenclature (E/S); (A/S).

Figure 3. Response surface of the experimental design, corresponding to the conversion of stearyl deoxycholate (**3**) under different ratios of E7S and A/S. Reactions were carried out at 55 °C and 200 rpm, using dry ROL 1, DIPE as solvent and stearyl alcohol. Reaction time was 48 h.

Figure 4. ROL 1 reuse in the esterification of chenodeoxycholic acid under standard conditions.

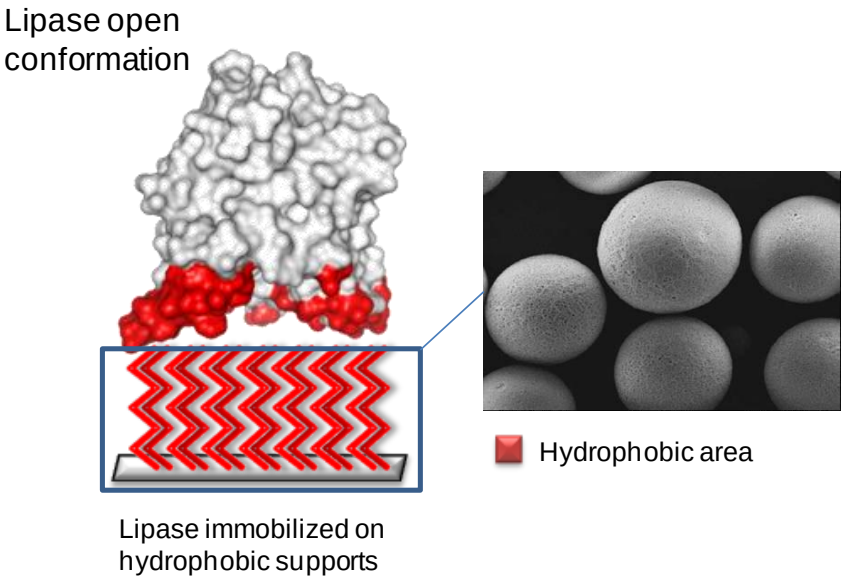

Lipase open conformation
Hydrophobic area
Lipase immobilized on hydrophobic supports

Figure 1.

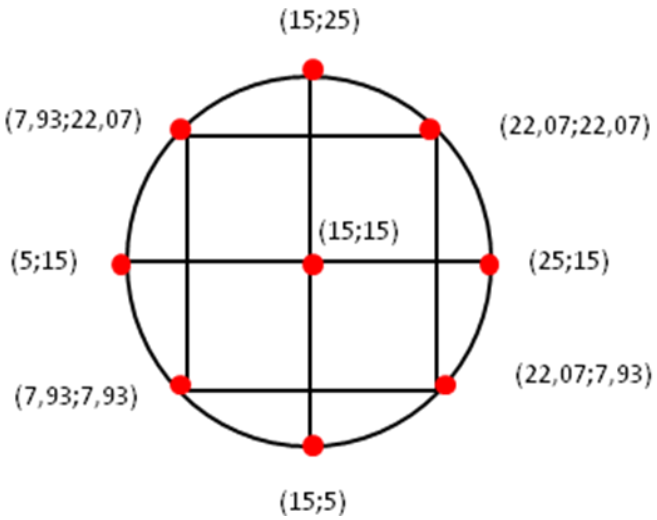


Figure 2.

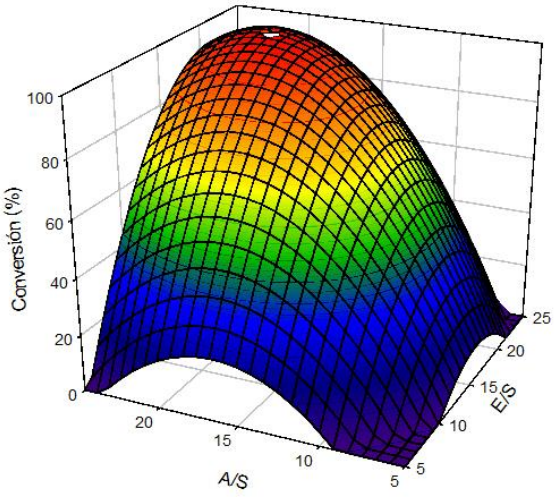


Figure 3.

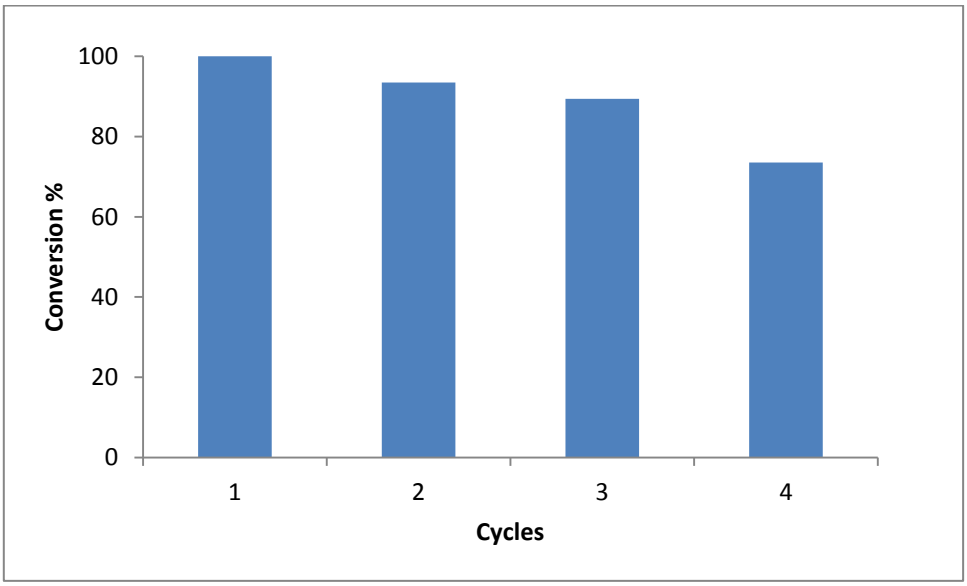


Figure 4.

Table

Table 1. Optimization of reaction parameters for ROL-catalyzed esterification of CDCA (**1**). [a]

Entry	Enzyme	Solvent	Temperature (°C)	E/S	A/S	Conversion (%)
Solvent and Lipase						
1	ROL 1	Hexane	55	30	30	75.6
2	**ROL 1**	**DIPE**	55	30	30	89.2
3	ROL 1	Acetonitrile	55	30	30	70.4
4	ROL 2	Hexane	55	30	30	51.1
5	ROL 2	DIPE	55	30	30	60.2
6	ROL 2	Acetonitrile	55	30	30	45.8
7	CAL B	Hexane	55	30	30	69.4
8	CAL B	DIPE	55	30	30	52.1
9	CAL B	Acetonitrile	55	30	30	58.3
E/S						
10	ROL 1	DIPE	55	2	20	30.2
11	ROL 1	DIPE	55	5	20	35.6
12	ROL 1	DIPE	55	10	20	71.0
13	ROL 1	DIPE	55	**20**	20	89.6
14	ROL 1	DIPE	55	30	20	89.9
A/S						
15	ROL 1	DIPE	55	20	5	28.8
16	ROL 1	DIPE	55	20	10	58.4
17	ROL 1	DIPE	55	20	**20**	88.9
18	ROL 1	DIPE	55	20	30	90.3
Temperature						
19	ROL 1	DIPE	30	20	20	65.3
20	ROL 1	DIPE	**55**	20	20	90.1

[a] time: 48h. Alcohol: stearyl aclohol.

.

Table 2. Specific activity of ROL 1, ROL 2 and CAL B

Lipase	Conversion (%)	Time (h)	Specific activity (µmol/mg lipase.h)
ROL 1	22.3	5	0.62
ROL 2	20.5	11	0.17
CAL B	19.8	16	0.10

Table 3. Conversion reached in each reaction at different (E/S) and (A/S) ratio.

Experiment number	Codified factor (E/S)	Codified factor (A/S)	Non codified factor (E/S)	Non codified factor (A/S)	Conversion (%)
1	-1	-1	7.93	7.93	15.2
2	-1	1	7.93	22.07	27.4
3	1	-1	22.07	7.93	35.3
4	1	1	22.07	22.07	88.5
5	-1.4142	0	5	15	12.6
6	1.4142	0	25	15	85.3
7	0	-1.4142	15	5	21.9
8	0	1.4142	15	25	83.2
9	0	0	15	15	84.1
10	0	0	15	15	83.9
11	0	0	15	15	85.3
12	0	0	15	15	87.3
13	0	0	15	15	84.5
14	0	0	15	15	85.0
15	0	0	15	15	81.0
16	0	0	15	15	86.5

Supplementary Material

Click here to download Supplementary Material: Baldessari Suppl. Data.doc