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New cholic acid derivatives: Biocatalytic synthesis and molecular docking study

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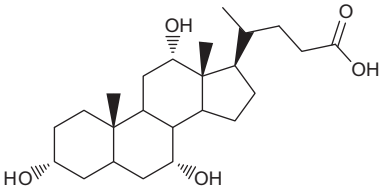
ABSTRACT

A series of cholic acid derivatives was synthesized by enzyme catalysis. Eleven acetyl and ester derivatives of cholic acid, eight of them new compounds, were obtained through regioselective lipase-catalyzed reactions in very good to excellent yield. The influence of various reaction parameters in the enzymatic esterification, acetylation and alcoholysis reactions, such as enzyme source, alcohol or acylating agent: substrate ratio, enzyme: substrate ratio, solvent and temperature, was studied. Moreover, in order to shed light to cholic acid behavior in the enzymatic reactions, molecular docking of the lipase with cholic acid and some derivatives was carried out.

1. Introduction

Enzymes are highly active and selective biocatalysts that turned out to be highly suitable for the synthesis of many complex organic compounds [1,2]. The application of enzymes for synthesizing specific compounds and the development of chemoenzymatic multi-step processes are key research topics nowadays. Among the enzymes exploited for synthetic purposes, lipases are very attractive due to their special properties: stability, recycling, broad substrate tolerance and non-aqueous media activity. Due to their stereo-. chemo- and regioselective nature, lipases have been employed for the preparation of compounds, which are not easily obtainable by chemical methods. Steroids have been suitable substrates for enzymatic transformations. In particular, the regioselective synthesis of steroid derivatives containing multiple hydroxyl groups has been undertaken through lipase-catalyzed acylation or deacylation procedures [3]. These mild and selective interconversions of functional groups are more accessible than the standard chemical approaches, because they avoid the tedious protection and deprotection steps [4].

Bile acids, widely distributed in nature as oxygenated metabolites of steroids, have been the subject of numerous pharmacological studies [5,6]. Their amphiphilic character generally increases cell membrane permeability [3]. For this reason their analogues are widely used in drug formulations as excipients (intestinal absorption enhancers, promoters, etc.) that can influence gastrointestinal solubility, and absorption of drugs [7].



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Cholic acid (CA, **1**) is one of the most important human bile acids and has attracted significant attention primarily due to its wide availability and relatively low cost. Synthetic analogues and derivatives of cholic acid have been widely explored in different scientific areas such as combinatorial and supramolecular chemistry [8], syntheses of various receptors [9], as antimalarials and antiproliferatives, etc. [10].

As other bile acids, CA exhibits two different faces: a hydrophobic face (β) containing angular methyl groups and a hydrophilic face (α) with hydroxyl groups. This structure is responsible for their amphipathic nature and, consequently, in water self-associates to form multimers above a critical concentration, where hydrogen bonding plays a key role. Because of its amphipathic properties and ability to form micelles, cholic acid has been used

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as a building block in supramolecular chemistry to transport ion and polar molecules across the membrane [11].

Unlike other bile acids, CA is the only one that has three hydroxyl groups at the C3, C7 and C12 positions facing the concave side of the molecule. This feature gives higher water solubility and lower detergent power than the other bile acids [12]. Sometimes, the potential use of bile acid is difficult because of their low solubility in both hydrophilic and hydrophobic media. In order to find an increase in lipophilicity and enhance permeability features, we have recently reported the synthesis of derivatives of chenodeoxycholic (CDA), deoxycholic (DA) and lithocholic (LA) acids [13].

In the present work we describe an enzymatic strategy for the synthesis of a series of new derivatives of cholic acid, obtained by lipase-catalyzed esterification, acetylation and alcoholysis reactions. Due to its singular structure containing three hydroxyl groups, in the enzymatic reactions CA showed a different behavior from that observed for the three bile acids previously studied, containing one (LA) or two hydroxyl groups (CDA and DA). In fact, we set about examining the particular reaction conditions to obtain CA derivatives. This paper reports the results of our investigation in detail. Moreover, with the aim to rationalize the selective behavior of lipases with CA as substrate, molecular modeling studies were applied.

2. Experimental

2.1. General

Chemicals and solvents were purchased from Merck Argentina and Sigma–Aldrich de Argentina and used without further purification. Lipase from *Candida rugosa* (CRL) (905 U/mg solid) and type II crude form porcine pancreas lipase ((PPL) (190 U/mg protein) were purchased from Sigma Chemical Co.; *Candida antarctica* lipase B (CAL B): Novozym 435 (7400 PLU/g) and Lipozyme RM 1 M (LIP) (7800 U/g) were generous gifts of Novozymes Spain and *Carica papaya* lipase (CPL) is the remaining solid fraction of papaya latex, after wash off of proteases using distilled water was a generous gift of Dr. Georgina Sandoval (CIATEJ). All enzymes were used "straight from the bottle". Enzymatic reactions were carried out on Innova 4000 digital incubator shaker, New Brunswick Scientific Co. at the corresponding temperature and 200 rpm. E/S: enzyme amount in mg/substrate amount in mg. 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 analytical reverse-phase HPLC employing a Phenomenex Phenogel column 5 μM 10E5A, 300 × 7.8 mm and eluting with MeOH:H_2O 80:20 at 1.00 mL/min. Melting points were measured in a Fisher Johns apparatus and are uncorrected. Optical rotation values were measured in a $CHCl_3$ solution with a Perkin Elmer-343 automatic digital polarimeter at 25 °C. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature in $CDCl_3$ as solvent using a Bruker AM-500 NMR instrument operating at 500.14 MHz and 125.76 MHz for ^{1}H and ^{13}C respectively. The ^{1}H NMR spectra are referenced with respect to the residual $CHCl_3$ proton of the solvent $CDCl_3$ at δ = 7.26 ppm. Coupling constants are reported in Hertz (Hz). ^{13}C NMR spectra were fully decoupled and are referenced to the middle peak of the solvent $CDCl_3$ at δ = 77.0 ppm. Splitting patterns are designated as: s, singlet; d, doublet; t, triplet; q, quadruplet; qn, quintet; dd, double doublet, etc. IR spectra were recorded with a Nicolet Magna 550 spectrometer. High Resolution Mass Spectrometry was recorded with Thermo Scientific EM/DSQ II – DIP. The results were within ± 0.02% of the theoretical values.

2.2. Enzymatic esterification. General procedure

Lipozyme (1 g) was added to a solution of cholic acid (100 mg) in DIPE (10 mL) and the corresponding alcohol (20 eq). The suspension was shaken (200 rpm) at 40 °C and the progress of the reaction monitored by TLC/HPLC. Once the reaction was finished, the enzyme was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography on silicagel employing mixtures hexane:ethyl acetate as eluent (6:4–2:8). Reuse experiments: the filtered and washed enzyme was used in the next enzymatic esterification under the same reaction conditions.

2.3. Enzymatic acetylation. General procedure

CAL B (1 g) was added to a solution of ethyl cholate (100 mg) in hexane (10 mL) and ethyl acetate (0.25 mL). The suspension was shaken (200 rpm) at 40 °C and the progress of the reaction monitored by TLC/HPLC. Once the reaction was finished, the enzyme was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography on silicagel employing mixtures hexane:ethyl acetate as eluent (7:3–1:1). Reuse experiments: the filtered and washed enzyme was used in the next enzymatic acetylation under the same reaction conditions.

2.4. Enzymatic alcoholysis. General procedure

To a solution of the peracetylated cholic acid (100 mg) in 10 ml of the indicated solvent containing 5 mol equiv of ethanol, CAL B (1 g) was added. The suspension was shaken (200 rpm) at 40 °C and the progress of the reaction was monitored by TLC. After indicated time, the enzyme was filtered off, the solvent was evaporated, and the crude residue purified by column chromatography on silica gel employing mixtures hexane:ethyl acetate as eluent (8:2–1:1).

2.5. Chemical peracetylation

In a typical procedure, a mixture of cholic acid (400 mg, 1 mmol), acetic anhydride (2 mL, 20 mmol), pyridine (2 mL) and DMAP (10 mg) was stirred at room temperature for 4 h. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was partitioned between saturated solution ammonium chloride (20 mL) and methylene chloride (20 mL). The aqueous phase was extracted with methylene chloride (3 × 10 mL). The combined organic layers were washed with saturated solution of sodium chloride (5 × 20 mL), dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography (silica gel) employing mixtures of hexane/EtOAc as eluent (8:2–1:1).

2.5.1. Ethyl 3α,7α,12α-trihydroxy-5β-cholanate (2a)

Yield 85%; white solid, mp 160–161 °C; $[\alpha]_D^{25}$ + 22.2° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ4.12 (2H, q, *J* = 6.7 Hz, –OC*H*$_2$-CH_3), 3.96 (1H, t, *J* = 2.9 Hz, H-12), 3.83 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 3.43 (1H, tt, *J* = 11.2, 4.1 Hz, H-3), 2.35 (1H, m, H-23b), 2.22 (1H, m, H-23a), 1.24 (3H, t, *J* = 6.8 Hz, –OCH$_2$C*H*$_3$), 0.98 (3H, d, *J* = 6.4 Hz, H-21), 0.88 (3H, s, H-19), 0.67 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 73.1 (C-12), 71.9 (C-7), 68.4 (C-3), 60.2 (–O*C*H$_2$CH$_3$), 30.9 (C-23), 22.4 (C-19), 17.3 (C-21), 14.2 (–OCH$_2$C*H*$_3$), 12.5 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{26}H_{44}NaO_5$ 459.3086 Found: $C_{26}H_{44}NaO_5$ 459.3090.

2.5.2. Propyl 3α,7α,12α-trihydroxy-5β-cholanate (2b)

Yield 70%; white solid, mp 120–121 °C; $[\alpha]_D^{25}$ + 25.3° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.02 (2H, t, *J* = 6.7 Hz, –O*CH*$_2$-CH_2CH_3), 3.97 (1H, t, *J* = 3.0 Hz, H-12), 3.84 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 3.45 (1H, tt, *J* = 10.8, 4.0 Hz, H-3), 2.35 (1H, m, H-23b), 2.23 (1H, m, H-23a), 1.64 (2H, m, –$OCH_2CH_2CH_3$), 0.98 (3H, d, *J* = 6.2 Hz, H-21), 0.95 (3H, t, *J* = 7.0 Hz, –$OCH_2CH_2CH_3$), 0.88 (3H, s, H-19), 0.68 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 73.0 (C-12), 71.9 (C-7), 68.4 (C-3), 65.9 (–O*CH*$_2$$CH_2CH_3$), 30.9 (C-23), 22.5 (C-19), 17.3 (C-21), 12.5 (C-18), 10.4 (–OCH_2CH_2-*CH*$_3$). HRMS: $[M+Na]^+$ Calcd. $C_{27}H_{46}NaO_5$ 473.3243 Found: $C_{27}H_{46}$-NaO_5 473.3239.

2.5.3. Butyl 3α,7α,12α-trihydroxy-5β-cholanate (2c)

Yield 66%; white solid, mp 163–164 °C; $[\alpha]_D^{25}$ + 23.8° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.06 (2H, t, *J* = 6.7 Hz, –O*CH*$_2$-$CH_2CH_2CH_3$), 3.98 (1H, t, *J* = 2.9 Hz, H-12), 3.85 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 3.45 (1H, tt, *J* = 10.8, 4.0 Hz, H-3), 2.35 (1H, m, H-23b), 2.23 (1H, m, H-23a), 1.61 (2H, m, –$OCH_2CH_2CH_2CH_3$), 0.98 (3H, d, *J* = 6.2 Hz, H-21), 0.92 (3H, t, *J* = 7.0 Hz, –$OCH_2CH_2CH_2CH_3$), 0.89 (3H, s, H-19), 0.69 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 72.9 (C-12), 72.0 (C-7), 68.4 (C-3), 64.2 (–O*CH*$_2$$CH_2$-$CH_2CH_3$), 30.9 (C-23), 22.5 (C-19), 17.3 (C-21), 13.7 (–OCH_2CH_2-CH_2CH_3), 12.5 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{28}H_{48}NaO_5$ 487.3399 Found: $C_{28}H_{48}NaO_5$ 487.3392.

2.5.4. Dodecyl 3α,7α,12α-trihydroxy-5β-cholanate (2d)

Yield 55%; white solid, mp 71–72 °C; $[\alpha]_D^{25}$ + 26.2° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.05 (2H, t, *J* = 6.7 Hz, –O*CH*$_2$$(CH_2)_{10}$-$CH_3$), 3.98 (1H, t, *J* = 2.8 Hz, H-12), 3.85 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 3.45 (1H, tt, *J* = 10.9, 4.2 Hz, H-3), 2.35 (1H, m, H-23b), 2.23 (1H, m, H-23a), 1.59 (2H, m, –$OCH_2CH_2(CH_2)_9CH_3$), 0.98 (3H, d, *J* = 6.2 Hz, H-21), 0.89 (3H, s, H-19), 0.88 (3H, t, *J* = 7.0 Hz, –OCH_2 $(CH_2)_{10}CH_3$), 0.68 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 73.0 (C-12), 71.9 (C-7), 68.4 (C-3), 64.5 (–O*CH*$_2$$(CH_2)_{10}CH_3$), 30.9 (C-23), 22.5 (C-19), 17.3 (C-21), 14.1 (–$OCH_2(CH_2)_{10}CH_3$), 12.5 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{36}H_{64}NaO_5$ 599.4651 Found: $C_{36}H_{64}NaO_5$ 599.4647.

2.5.5. Tetradecyl 3α,7α,12α-trihydroxy-5β-cholanate (2e)

Yield 54%; white solid, mp 65–66 °C; $[\alpha]_D^{25}$ + 30.1° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.05 (2H, t, *J* = 6.7 Hz, –O*CH*$_2$$(CH_2)_{12}$ CH_3), 3.98 (1H, t, *J* = 2.9 Hz, H-12), 3.85 (1H, dd, *J* = 2.4, 3.0 Hz, H-7), 3.45 (1H, tt, *J* = 11.0, 4.0 Hz, H-3), 2.35 (1H, m, H-23b), 2.22 (1H, m, H-23a), 1.59 (2H, m, –$OCH_2CH_2(CH_2)_{11}CH_3$), 0.98 (3H, d, *J* = 6.1 Hz, H-21), 0.89 (3H, s, H-19), 0.87 (3H, t, *J* = 7.0 Hz, –OCH_2 $(CH_2)_{12}CH_3$), 0.68 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 73.0 (C-12), 71.9 (C-7), 68.4 (C-3), 64.5 (–O*CH*$_2$$(CH_2)_{12}CH_3$), 30.9 (C-23), 22.5 (C-19), 17.3 (C-21), 14.1 (–$OCH_2(CH_2)_{12}CH_3$), 12.5 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{38}H_{68}NaO_5$ 627.4964 Found: $C_{38}H_{68}NaO_5$ 627.4968.

2.5.6. Hexadecyl 3α,7α,12α-trihydroxy-5β-cholanate (2f)

Yield 58%; white solid, mp 74–75 °C; $[\alpha]_D^{25}$ + 29.3° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.05 (2H, t, *J* = 6.7 Hz, –O*CH*$_2$$(CH_2)_{14}$ CH_3), 3.98 (1H, t, *J* = 2.9 Hz, H-12), 3.84 (1H, dd, *J* = 2.4, 3.0 Hz, H-7), 3.45 (1H, tt, *J* = 10.9, 4.0 Hz, H-3), 2.36 (1H, m, H-23b), 2.22 (1H, m, H-23a), 1.60 (2H, m, –$OCH_2CH_2(CH_2)_{13}CH_3$), 0.98 (3H, d, *J* = 6.1 Hz, H-21), 0.89 (3H, s, H-19), 0.88 (3H, t, *J* = 7.0 Hz, –OCH_2 $(CH_2)_{14}CH_3$), 0.68 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 73.0 (C-12), 71.9 (C-7), 68.4 (C-3), 64.5 (–O*CH*$_2$$(CH_2)_{14}CH_3$), 30.9 (C-23), 22.5 (C-19), 17.3 (C-21), 14.1 (–$OCH_2(CH_2)_{14}CH_3$), 12.5 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{40}H_{72}NaO_5$ 655.5278 Found: $C_{40}H_{72}NaO_5$ 655.5280.

2.5.7. Octadecyl 3α,7α,12α-trihydroxy-5β-cholanate (2g)

Yield 51%; white solid, mp 81–82 °C; $[\alpha]_D^{25}$ + 30.6° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.05 (2H, t, *J* = 6.8 Hz, –O*CH*$_2$$(CH_2)_{16}$ CH_3), 3.98 (1H, t, *J* = 3.0 Hz, H-12), 3.84 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 3.45 (1H, tt, *J* = 11.0, 4.1 Hz, H-3), 2.35 (1H, m, H-23b), 2.23 (1H, m, H-23a), 1.60 (2H, m, –$OCH_2CH_2(CH_2)_{15}CH_3$), 0.98 (3H, d, *J* = 6.0 Hz, H-21), 0.89 (3H, s, H-19), 0.88 (3H, t, *J* = 7.0 Hz, –OCH_2 $(CH_2)_{16}CH_3$), 0.68 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 73.0 (C-12), 71.9 (C-7), 68.4 (C-3), 64.5 (–O*CH*$_2$$(CH_2)_{16}CH_3$), 30.9 (C-23), 22.5 (C-19), 17.3 (C-21), 14.1 (–$OCH_2(CH_2)_{16}CH_3$), 12.5 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{42}H_{76}NaO_5$ 683.5591 Found: $C_{42}H_{76}NaO_5$ 683.5590.

2.5.8. Ethyl 3α-acetoxy-7α,12α-dihydroxy-5β-cholanate (3a)

Yield 87%; white solid, mp 95–96 °C; $[\alpha]_D^{25}$ + 24.5° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.58 (1H, tt, *J* = 11.2, 4.2 Hz, H-3), 4.12 (2H, q, *J* = 6.7 Hz, –O*CH*$_2$$CH_3$), 3.99 (1H, t, *J* = 2.9 Hz, H-12), 3.85 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 2.35 (1H, m, H-23b), 2.22 (1H, m, H-23a), 1.25 (3H, t, *J* = 6.7 Hz, –OCH_2CH_3), 0.98 (3H, d, *J* = 6.5 Hz, H-21), 0.90 (3H, s, H-19), 0.70 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.2 (C-24), 170.8 (*C*OCH_3), 74.3 (C-12), 72.7 (C-7), 68.2 (C-3), 60.2 (–O*CH*$_2$$CH_3$), 30.9 (C-23), 22.5 (C-19), 21.5 (*CH*$_3$-CO–), 17.3 (C-21), 14.2 (–OCH_2CH_3), 12.6 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{28}H_{46}NaO_6$ 501.3192 Found: $C_{28}H_{46}NaO_6$ 501.3195.

2.5.9. 3α,7α,12α-triacetoxy-5β-cholanic acid (4a)

Yield 90%; white solid, mp 69.5–70 °C; $[\alpha]_D^{25}$ + 26.9° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.08 (1H, t, *J* = 3.0 Hz, H-12), 4.90 (1H, dd, *J* = 3.0, 2.5 Hz, H-7), 4.57 (1H, tt, *J* = 11.2, 4.3 Hz, H-3), 2.32 (1H, m, H-23b), 2.22 (1H, m, H-23a), 2.14 (3H, s, CO*CH*$_3$), 2.09 (3H, s, CO*CH*$_3$), 2.05 (3H, s, CO*CH*$_3$), 0.91 (3H, s, H-19), 0.81 (3H, d, *J* = 6.5 Hz, H-21), 0.73 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 176.6 (C-24), 170.5 (2 × *C*OCH_3), 170.4 (*C*OCH_3), 75.4 (C-12), 74.1 (C-7), 70.7 (C-3), 30.8 (C-23), 22.6 (C-19), 21.6 (*CH*$_3$CO–), 21.5 (*CH*$_3$CO–), 21.4 (*CH*$_3$CO–), 17.5(C-21), 12.2 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{30}H_{46}NaO_8$ 557.3090 Found: $C_{30}H_{46}NaO_8$ 557.3086.

2.5.10. Ethyl 3α,7α,12α-triacetoxy-5β-cholanate (4b)

Yield 92%; white solid, mp 69.5–70 °C; $[\alpha]_D^{25}$ + 28.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.08 (1H, t, *J* = 3.0 Hz, H-12), 4.90 (1H, dd, *J* = 3.0, 2.5 Hz, H-7), 4.57 (1H, tt, *J* = 11.2, 4.3 Hz, H-3), 4.11 (2H, q, *J* = 6.7 Hz, –O*CH*$_2$$CH_3$), 2.32(1H, m, H-23b), 2.20 (1H, m, H-23a), 2.13 (3H, s, CO*CH*$_3$), 2.08 (3H, s, CO*CH*$_3$), 2.04 (3H, s, CO*CH*$_3$), 1.24 (3H, t, *J* = 6.7 Hz, –OCH_2CH_3), 0.91 (3H, s, H-19), 0.81 (3H, d, *J* = 6.5 Hz, H-21), 0.73 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.1 (C-24), 170.6 (*C*OCH_3), 170.5 (*C*OCH_3), 170.4 (*C*OCH_3), 75.4 (C-12), 74.1 (C-7), 70.7 (C-3), 60.2 (–O*CH*$_2$$CH_3$), 30.8 (C-23), 22.6 (C-19), 21.6 (*CH*$_3$CO–), 21.5 (*CH*$_3$CO–), 21.4 (*CH*$_3$-CO–), 17.5(C-21), 12.2 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{32}H_{50}NaO_8$ 585.3403 Found: $C_{32}H_{50}NaO_8$ 585.3408.

2.5.11. Ethyl 7α,12α-diacetoxy-3α-hydroxy-5β-cholanate (5b)

Yield 70%; white solid, mp 75–76 °C; $[\alpha]_D^{25}$ + 28.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.08 (1H, t, *J* = 3.0 Hz, H-12), 4.90 (1H, dd, *J* = 3.0, 2.5 Hz, H-7), 4.11 (2H, q, *J* = 6.7 Hz, –O*CH*$_2$$CH_3$), 3.64 (1H, tt, *J* = 11.0, 4.1 Hz, H-3), 2.32 (1H, m, H-23b), 2.20 (1H, m, H-23a), 2.13 (3H, s, CO*CH*$_3$), 2.08 (3H, s, CO*CH*$_3$), 1.25 (3H, t, *J* = 6.7 Hz, –OCH_2CH_3), 0.90 (3H, s, H-19), 0.81 (3H, d, *J* = 6.5 Hz, H-21), 0.72 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.1 (C-24), 170.7 (*C*OCH_3), 170.6 (*C*OCH_3), 75.4 (C-12), 71.7 (C-7), 70.8 (C-3), 60.3 (–O*CH*$_2$$CH_3$), 30.8 (C-23), 22.6 (C-19), 21.6 (*CH*$_3$CO–), 21.5 (*CH*$_3$-CO–), 17.5(C-21), 12.2 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{30}H_{48}NaO_7$ 543.3298 Found: $C_{30}H_{48}NaO_7$ 543.3301.

2.6. Modecular modeling

CAL B structure was downloaded from RCSB Protein DataBank (http://www.rcsb.org/pdb/), PDB code for CAL B is 1TCA. All substrates were minimized using semiempirical AM1 method with the algorithm Polak-Ribiere in Hyperchem. The docking calculations were carried out with Autodock 4.2 program [14]. The Autodock 4.2 method was applied considering a pair of rotatable bonds for cholic acid and all the protein were defined it was considered stiff. For the location and extent of the 3D area, the search space is defined by specifying a center, the number of points in each dimension, and points between spaces to focus the search space in the active site of the enzyme, it was taken as grid box center the coordinates *x*, *y*, *z* of a water molecule close to Ser105.

3. Results and discussion

In this section we are going to describe the results obtained by applying lipases as catalysts in three reactions: esterification, acetylation and alcoholysis.

3.1. Enzymatic esterification

According to literature, only methyl and ethyl esters of cholic acid have been described so far. CA methyl ester was prepared using methanol and acids such as hydrochloric acid, methanesulfonic or *p*-toluensulfonic acid [15–17]. Ethyl ester was obtained by treatment with absolute ethanol and fuming sulfuric acid (20–30% SO_3) [18].

With the aim to get a mild procedure applied also to longer chain alcohols, we carried out the esterification of CA with different alcohols and lipases as catalysts (Scheme 1).

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

Initially we examined the catalytic activity of five lipases, four of them commercially available, in the esterification reaction of **1** with ethanol: *C. rugosa* lipase (CRL), *C. antarctica* lipase B (CAL B), Lipozyme, lipase from the fungus *Rhizomucor miehei* (LIP), porcine pancreas lipase (PPL) and *C. papaya* lipase (CPL). Some satisfactory results are shown in Table 1. Among the enzymes tested, only LIP and CAL B were active enough toward the esterification of CA. In terms of conversion the former was the best while with CRL, PPL and CPL and in the absence of biocatalyst no product was obtained.

Although several studies have already been reported on the effect of organic solvents on lipase-catalyzed reactions [19], it is still difficult to predict the solvent effect. Therefore the practical way is to select an appropriate solvent through screening experiments. We tested hexane, diisopropyl ether (DIPE) and acetonitrile as low, medium and high polar solvents respectively. In addition, the reaction without co-solvent using ethanol both as nucleophile and solvent was also performed.

Table 1
Optimization of reaction parameters for lipase-catalyzed esterification of CA with ethanol.

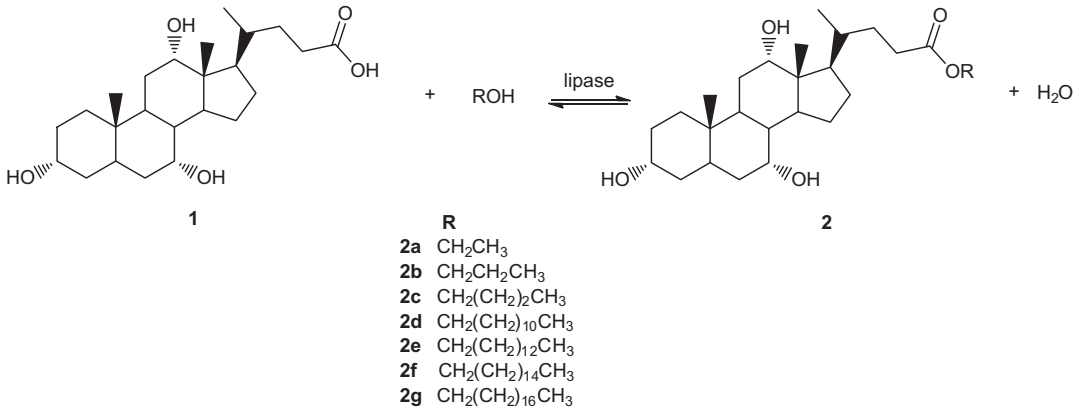
Entry	Lipase	Solvent	*T* (°C)	E/S[a]	Nu/S	*t* (h)	Conversion
Lipase and solvent							
1	CAL B	Ethanol	40	10	20	96	18
2	CAL B	Hexane	40	10	20	96	43
3	CAL B	DIPE	40	10	20	96	40
4	CAL B	Acetonitrile	40	10	20	>96	–
5	LIP	Ethanol	40	10	20	72	60
6	LIP	Hexane	40	10	20	72	50
7	LIP	DIPE	**40**	**10**	**20**	**48**	**85**
8	LIP	Acetonitrile	40	10	20	>96	10
E/S							
9	LIP	DIPE	40	50	20	48	87
10	LIP	DIPE	40	20	20	48	84
11	LIP	DIPE	40	5	20	48	50
12	LIP	DIPE	40	1	20	48	22
Nu/S							
13	LIP	DIPE	40	10	500	48	88
14	LIP	DIPE	40	10	100	48	85
15	LIP	DIPE	40	10	10	48	76
Temperature							
16	LIP	DIPE	30	10	20	96	-
17	LIP	DIPE	55	10	20	48	50

Optimal conditions are showed in bold values.
[a] E/S: enzyme amount in mg/substrate amount in mg.

Among the tested solvents, the lipase from *R. miehei* (LIP) showed the highest activity in DIPE, affording **2a** at 85% conversion in 48 h of reaction (Table 1, entry 7). This lipase was also active in hexane and without co-solvent, in neat ethanol, but to a lesser extent (Table 1, entries 5 and 6). CAL B showed lower activity: about 40% conversion in hexane and DIPE after 96 h. (Table 1, entries 2 and 3).

Regarding the influence of temperature on the enzymatic esterification, the reaction was tested at 30 °C, 40 °C and 55 °C using an enzyme:substrate ratio E/S: 10 and ethanol:substrate ratio: 20. The dependence of the reaction performance with temperature was interesting because no reaction was observed at 30 °C and yield was only 50% at 55 °C affording **2a** with a mixture of products. Therefore 40 °C was selected as the optimum temperature.

To further optimize the reaction efficiency, the influence of the enzyme:substrate ratio in the enzymatic esterification was



Scheme 1. Enzymatic synthesis of esters of CA (**1**).

Table 2
Lipase-catalyzed obtention of cholic acid esters.

Entry	Alcohol	Product	Yield (%)
1	Ethanol	**2a**	85
2	*n*-Propanol	**2b**	70
3	*n*-Butanol	**2c**	66
4	*n*-Dodecanol	**2d**	55
5	*n*-Tetradecanol	**2e**	54
6	*n*–Hexadecanol	**2f**	58
7	*n*-Octadecanol	**2g**	51

Reaction conditions: Enzyme: LIP; Solvent: DIPE; Temperature: 40 °C; E/S: 10; Nu/S: 20; t: 48 h.

evaluated at 48 h, using ethanol as nucleophile at Nu/S ratio of 20, DIPE as solvent at 40 °C and variable amounts of LIP (Table 1, entries 9–12). From the results, it can be observed that an E/S ratio of 50, 20 and 10 (Table 1, entries 9, 10 and 7) are very similar. A conversion slightly lower was observed at E/S ratio of 10 and this was the ratio of choice, considering the convenience of using the lowest amount of enzyme. Anyway, although E/S = 10 could seem very high for a catalyst, in general the amount of lipase in immobilized enzymes is very low. For example the ratio lipase:support is 30 mg of lipase/g support in the case of CAL B [20]. Moreover, due the immobilization, LIP can be reused. In this particular case it was observed that it retained 80% activity after three reaction cycles.

Finally, the influence of the Nu/S ratio on esterification yield was evaluated at 48 h using LIP (E/S = 10) in DIPE at 40 °C, reaction time 48 h and variable amounts of ethanol. From the results (Table 1, entries 13–16), it can be observed that there is not an important dependence of the reaction yield with the ratio Nu/S ratio. Then, Nu/S: 20 was the ratio of choice.

Considering the previously mentioned experiments, the following standard conditions for the enzymatic esterification of cholic acid with ethanol were chosen: LIP as biocatalyst, temperature: 40 °C, E/S: 10, Nu/S: 20 and DIPE as solvent (Table 1, entry 7).

After the establishment of the optimal conditions, the enzymatic strategy in the esterification of **1** with a variety of alcohols was explored (Table 2).

Alkyl esters containing linear alkyl chains from two to eighteen carbon atoms were synthesized in moderate to very good yield (50–80%). It was observed that yield decreased as the alkyl chain length increased. Every product was completely characterized by spectroscopic methods and six of them (**2b**–**2g**) were not previously described in literature. The advantage of the enzymatic procedure becomes more relevant in the case of longer-chain derivatives, where the use of excess of alcohol in the traditional synthetic approach is not convenient, in terms of product isolation and economy.

3.2. Enzymatic acetylation

At our best knowledge, the preparation of monoacetyl derivatives of cholic acid was not reported so far. Some acyloxy derivatives of cholic acid, such as butanoyl, decanoyl- and hexadecanoyloxy CA were prepared through protection and deprotection steps [21]. In a previous work, peracetylation of methyl ester CA was carried out in acetic anhydride, pyridine and DMAP at room temperature under microwave irradiation [16]. In the same report, it was also described the obtention of methyl 3α-hydroxy-7α,12α-diacetoxy-5β-cholanate by deacetylation using methanesulfonic acid in methanol. Moreover, a one-pot esterification of carboxyl group and 3α-acetylation of CA was carried out using the appropriate esters (ethyl-, propyl- and butyl acetate) in *p*-toluensulfonic acid and water at reflux. Under these conditions the reaction was not selective and the monoacetyl derivative was obtained in a mixture with ethyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholanate [22].

Recently, we have reported the synthesis of 3α-acetyl derivatives of chenodeoxycholic and deoxycholic acids using CAL B as biocatalyst, ethyl acetate as acylation agent in hexane at 55 °C. Under the same reaction conditions, with lithocholic acid as substrate, the lipase catalyzed both the acetylation and esterification reactions affording ethyl lithocholate 3-acetate as the only product in one step [13].

The presence of the three hydroxyl groups in CA makes it an interesting model for studying the selectivity of the enzymatic acetylation. Therefore, we applied the enzymatic procedure, studying a variety of biocatalysts and reaction conditions in the acetylation of CA. Unfortunately no reaction was observed in any case, even when using activated acylating agents such as vinyl or isopropenyl acetate. From these results and considering the work previously reported, it is obvious that it is not possible to acetylate CA in a regioselective way, chemically or enzymatically.

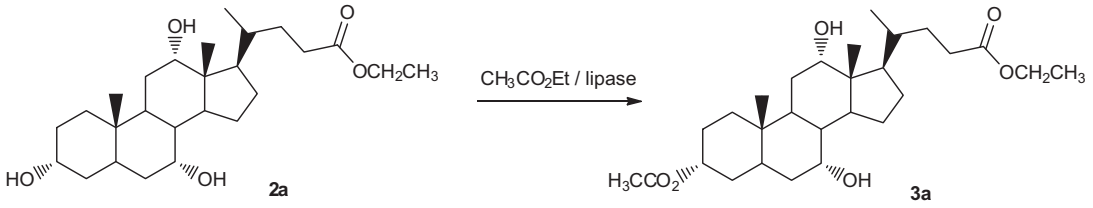
After this result, we decided to explore the reaction conditions for the lipase-catalyzed acetylation of CA ethyl ester (**2a**) (Scheme 2). As usual, we conducted a screening varying different parameters: lipases, solvents, temperature and E/S and acylating agent A/S ratios. The results can be observed in Table 3.

In the absence of biocatalyst no product was obtained. In this reaction CAL B was the only active enzyme giving the best results in hexane. The lipase was highly regioselective affording ethyl 3-acetyl cholate (**3a**) at 90% conversion in 96 h of reaction as the only acetylation product (Table 3, entry 2). CAL B was also active in DIPE but to a lesser extent (Table 3, entry 3) and working without co-solvent with ethyl acetate as acylating agent and solvent, **3a** conversion decreased to 70% (Table 3, entry 1).

Taking into account these studies, we have chosen as optimal conditions for the enzymatic acetylation of CA ethyl ester: CAL B as biocatalyst, ethyl acetate as acylating agent, hexane as solvent, temperature: 40 °C, E/S and A/S: 10.

Finally, in order to analyze the influence of the ester chain length on enzyme reactivity, we applied the optimal conditions to the acetylation of the lauryl ester of CA (**2d**). Under these conditions, no reaction was observed.

It is interesting to note that, despite the differences in structure and water solubility, in the enzymatic acetylation of bile acid ethyl esters, CAL B showed the same regioselective behavior for ethyl



Scheme 2. Enzymatic acetylation of ethyl cholate (**2a**).

Table 3
Optimization of reaction parameters for lipase-catalyzed acetylation of ethyl cholate (**2a**).

Entry	Lipase	Solvent	*T* (°C)	E/S[a]	A/S	*t* (h)	Conversion (%)
Solvent							
1	CAL B	Ethyl acetate	40	10	–	96	70
2	CAL B	Hexane	**40**	**10**	**10**	**96**	**90**
3	CAL B	DIPE	40	10	10	96	65
E/S							
4	CAL B	Hexane	40	5	10	96	76
5	CAL B	Hexane	40	2	10	96	54
6	CAL B	Hexane	40	1	10	96	35
A/S							
7	CAL B	Hexane	40	10	20	96	90
8	CAL B	Hexane	40	10	5	96	80
9	CAL B	Hexane	40	10	1	96	61
Temperature							
10	CAL B	Hexane	30	10	10	96	-
11	CAL B	Hexane	55	10	10	96	90

Lipases: LIP, PPL and CPL: inactive.
Optimal conditions are showed in bold values.
[a] E/S: enzyme amount in mg/substrate amount in mg.

esters of CA, chenodeoxycholic and deoxycholic acid, affording the monoacetyl derivative in the 3α position in every case [13].

3.3. Enzymatic alcoholysis

Since enzymatic acetylation was not a suitable way to get mono- or diacetyl derivatives of cholic acid, we tried to carry out the enzymatic alcoholysis of peracetylated derivatives both CA and its ethyl ester. Because CAL B was the only active enzyme in the acetylation reaction, it was the enzyme of choice (Scheme 3).

The substrates for the enzymatic alcoholysis **4a** and **4b**, were obtained in 90% and 92% yield respectively, by treatment of **1** and **2a** with acetic anhydride and pyridine at room temperature during 16 h.

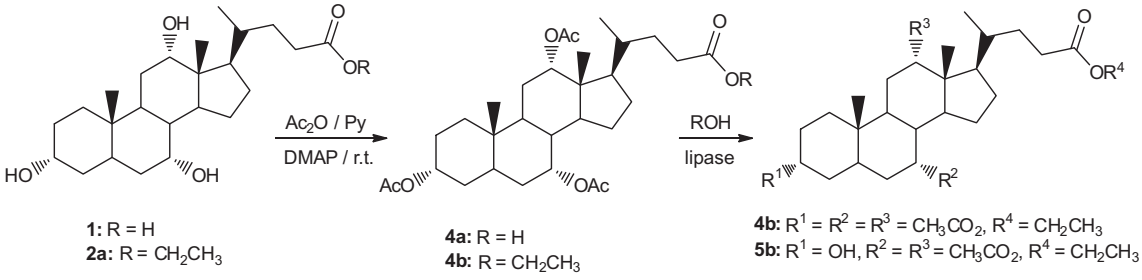
At this point, we attempted to carry out the enzymatic alcoholysis of **4a** and **4b** using ethanol as nucleophile and DIPE or hexane as solvents (Table 4). The best results were achieved when employing ethanol as nucleophile and hexane as solvent.

To our surprise under the above mentioned conditions, the alcoholysis of peracetylated cholic acid (**4a**) afforded 3,7,12-triacetyl ethyl ester (**4b**) showing that deacetylation did not take place in any of the three acyl positions. The lipase catalyzed the esterification of the carboxyl group with ethanol instead of performing the nucleophilic attack to acetate groups. On the other hand, with **4b** as substrate, alcoholysis occurred at 3-position while keeping unaltered the other two (7 and 12). Therefore, ethyl 7,12-diacetyl cholate (**5b**) was obtained, using CAL B as catalyst (E/S:10), ethanol as nucleophile (Nu/S:1.2) and hexane as solvent at 40 °C, after 72 h of reaction. It is remarkable that, at longer periods of time, some deacetylation product afforded from **4b** obtained from enzymatic alcoholysis of **4a**.

3.4. Molecular modeling

From the experimental results it is clear that only CAL B was able to perform the acetylation of CA ethyl ester within reasonable reaction time and at mild reaction conditions. It was also active in the esterification of CA with ethanol, although to a lesser extent than Lipozyme. According to the mechanism proposed for lipases, the acyl-enzyme intermediate is first formed by reaction with a serine residue in the active site [23]. Therefore, both steric and electronic properties of the substrate are likely to influence the outcome of CAL B-catalyzed reactions.

To shed light this influence in the case of CA, we applied molecular modeling. This approach helps to understand the structural relation between the enzymatic pocket and the characteristic steroidal framework. Then, molecular docking studies were performed on cholic acid with CAL B to evaluate the ability of the catalytic pocket to accommodate this substrate. The aim of this part of the work was to explain the lack of reactivity of CA in the enzymatic



Scheme 3. Chemical synthesis and enzymatic alcoholysis of peracetylated CA (**4a**) and its ethyl ester (**4b**).

Table 4
CAL B-catalyzed alcoholysis of 3α,7α,12α-triacetyl cholic acid (**4a**) and ethyl-3α,7α,12α-triacetyl cholate (**4b**).

Entry	Substrate	Solvent	Nu	Nu/S	*t* (h)	Conversion (%)	
						4b	**5b**
1	**4a**	Hexane	EtOH	1.2	72	95	
2	**4a**	Hexane	EtOH	5	48	85	
3	**4a**	DIPE	EtOH	1.2	72	10	
4	**4a**	DIPE	EtOH	5	72	30	
5	**4b**	Hexane	EtOH	1.2	72		50
6	**4b**	Hexane	EtOH	5	72		74
7	**4b**	DIPE	BuOH	5	72		10
8	**4b**	DIPE	OctOH	5	72		15

Experimental conditions: Enzyme: CAL B; Temperature: 40 °C; E/S: 10.

Table 5
Distance in representative conformers of bile acids.

Bile acid			Yield (%)
Esterification			
		COOH-Ser distance (Å)	
CA		2.48	43[a]
CDCA		1.83	99
DCA		1.94	89
LA		1.61	93
Acetylation			
	OH position	OH-Ser distance (Å)	
CA	3	10.07	
	7	11.02	–
	12	12.41	
Et-CA	3	2.07	87
La-CA	3	9.24	–
CDCA	3	1.94	90
CDCA	7	4.38	–
DCA	3	1.95	99
DCA	12	4.73	–
LA	3	1.95	75[b]

[a] Using CAL B, with Lipozyme under the optimal conditions the product was obtained in 80% yield.
[b] Ethyl ester LA-3-AcO, one-pot acetylation and esterification [13].

acetylation in comparison with the good performance showed when the ethyl ester was used as substrate.

The analysis of the results led to the selection of a possible conformation for CA, using the interaction with the amino acids of the catalytic site (Asp187–His224–Ser105) and the population of the clusters as selection criteria. Docking analysis showed a majority cluster, in which the substrate interacts with the active site (Ser105) of the enzyme through the carboxyl moiety showing a distance of 2.48 Å (Table 5, Fig. 1a). This optimal distance could explain that the enzymatic esterification reaction of CA is favored.

In addition, a less representative but considerable cluster, in which CA tries to enter the active site of the lipase by the end of the hydroxyl groups, was also observed. This entrance was unsuccessful as it can be assumed from Fig. 1b. It can be observed that the distance from Ser105 to the hydroxyl groups in C-3 (10.07 Å), C-7 (11.02 Å) and C-12 (12.41 Å) (Table 5). This confirms the lack of reactivity of CA towards the enzymatic acetylation.

In the case of CAL B-catalyzed acetylation of CA ethyl ester, it could be assumed that a decrease in the polarity of the substrate by esterification favors the income by the end of OH groups in CA. As it could be expected, OH on C-3 is the only one located

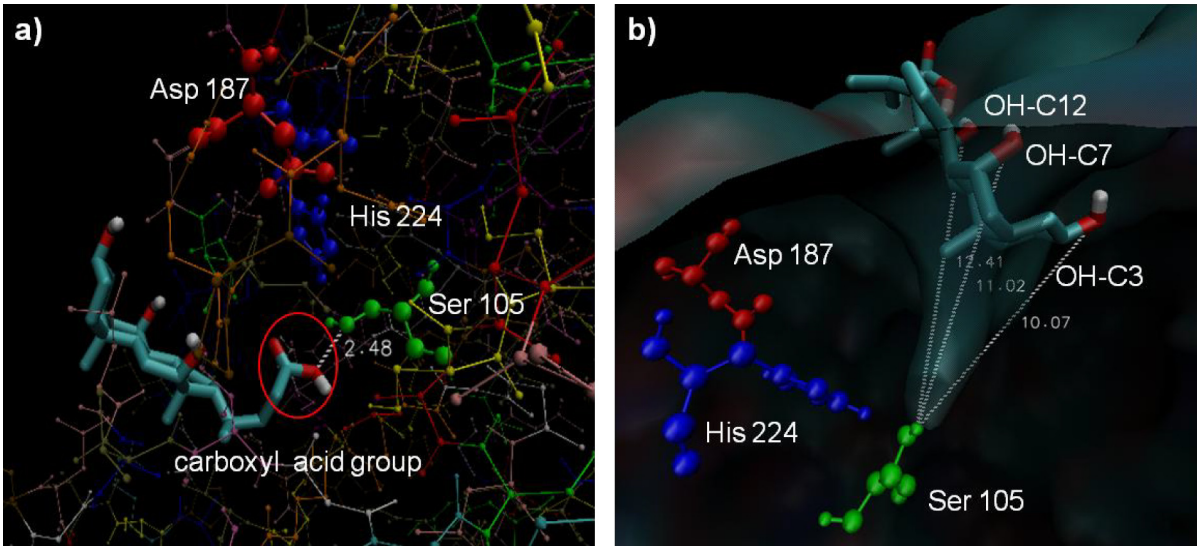


Fig. 1. Docking of CALB with cholic acid: interaction of substrate with Ser 105 through: (a) the carboxyl moiety; (b) hydroxyl gropus.

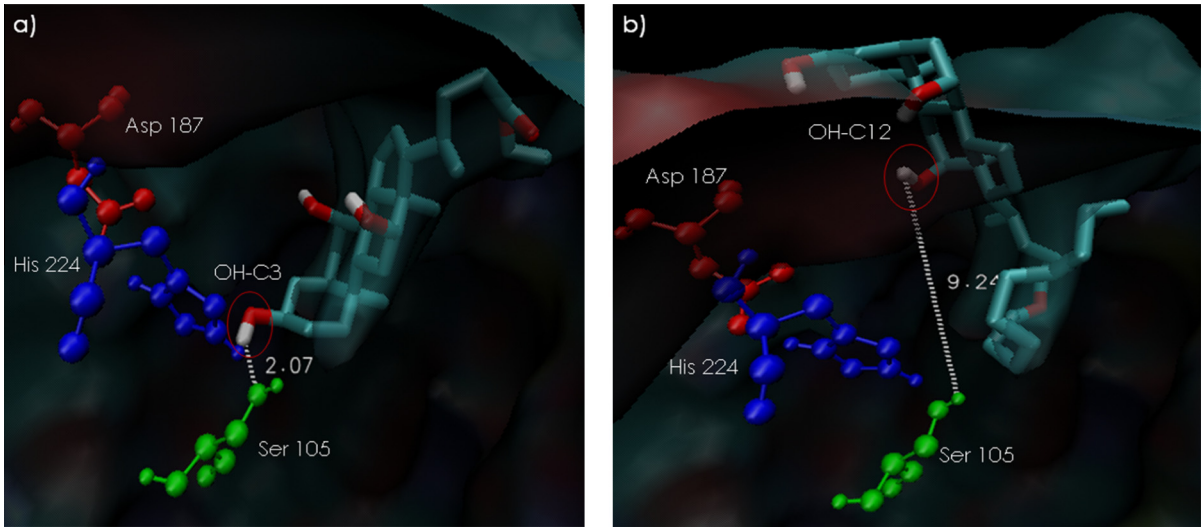
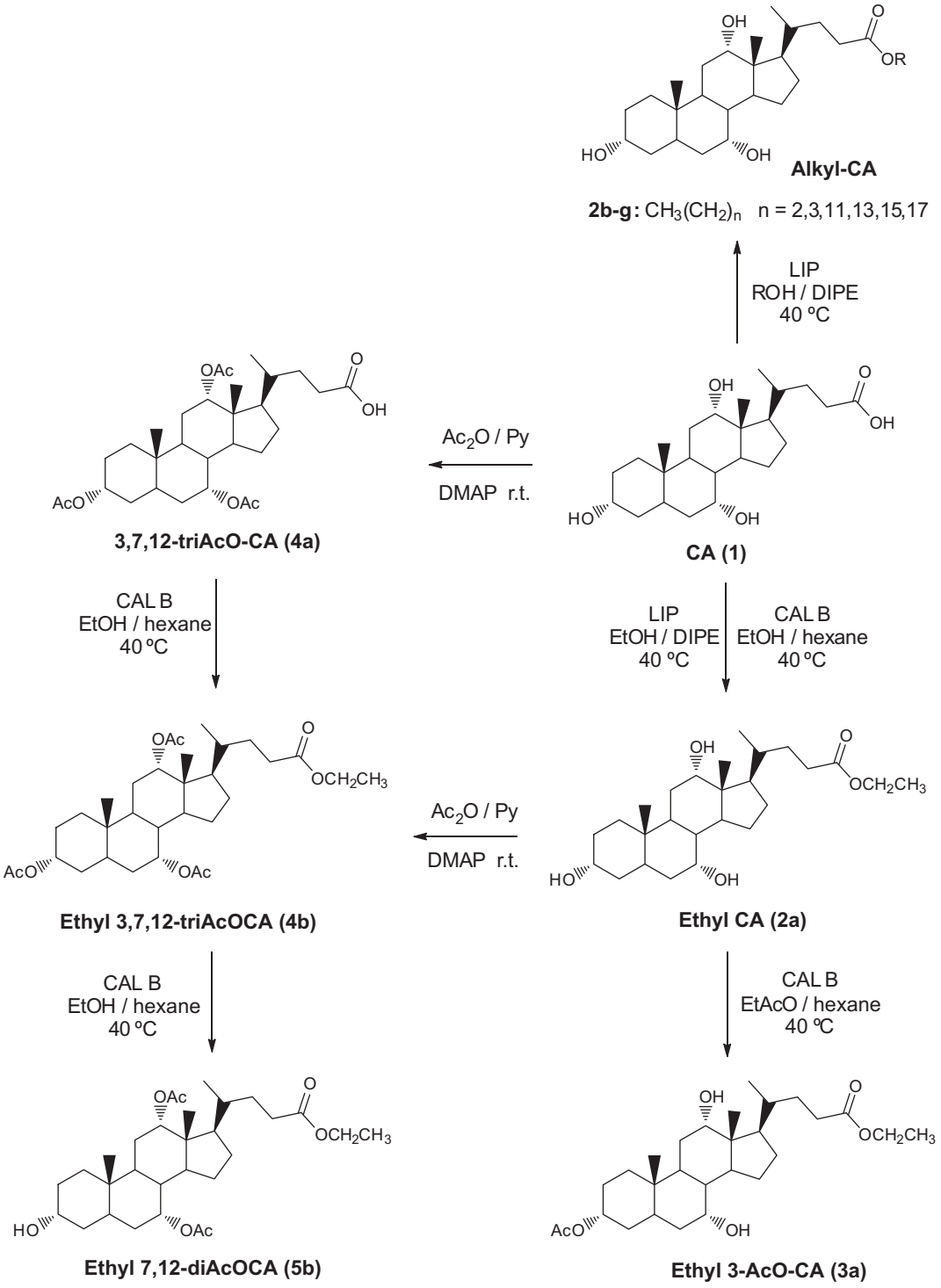


Fig. 2. Docking of CALB with ethyl ester CA (a) and lauryl ester CA (b).



Scheme 4. Reactions and products from CA.

at an optimal binding distance to the catalytic serine (2.07 Å) (Fig. 2a).

The negative result in the acetylation for **2d**, the lauryl ester of CA, was also clarified by molecular modeling. It was noted that this substrate cannot be properly accommodated in the hydrophobic pocket of the enzyme. As the alkyl chain is bent, the OH groups are too far to be able to interact with the catalytic triad. The nearest hydroxyl group is that on C-12, with a distance of 9.24 Å to the catalytic serine for the most representative conformer (Fig. 2b).

In a previous work we performed molecular docking studies on chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LA) with CAL B [13]. Table 5 shows the data corresponding to the distance COOH-Ser105 and OH-Ser105 for these three bile acids, CA and some derivatives.

The comparison of these results, which are in accordance with the lipase activity and regioselectivity experimentally observed, allowed us to draw some conclusions.

The four bile acids showed low values of the distance COOH-Ser105. They could be esterified by lipase catalysis affording the

ethyl ester, in excellent yield for CDCA, DCA and LA. CA yield was poor with CAL B, but increased to 80% with Lipozyme.

For the four bile acids, acetylation was regioselective and only the 3-AcO was obtained. Docking showed that the main favorable conformations correspond to those in which the hydroxyl group on carbon 3 points toward the catalytic triad. It seems that the hydrophilic α face in CA, containing the three hydroxyls and the carboxyl group, prevents it from entering the active site by the end of any of the three hydroxyl groups, therefore no product of acetylation from CA was obtained. The ethyl group in the ethyl ester of CA decreases the polarity of the molecule allowing the interaction with the active site and affording the product in very good yield.

In most cases, there is a correlation between the distance showed by the bile acids and their derivatives with the activity and regioselectivity displayed by the lipase (Table 5). The molecular modeling study confirmed the experimental results showing that CA behavior in the enzymatic reactions is completely different from the other three bile acids (CDCA, DCA and LA) previously studied.

4. Conclusions

In this work, we describe the application of enzymes to the synthesis of derivatives of cholic acid. Scheme 4 shows the performed reactions and the obtained products.

The influence of various reaction parameters on the results was analyzed. Among the tested enzymes, *R. miehei* lipase gave the best results in esterification reactions, affording alkyl esters as the only reaction products, six of them being new compounds.

It was not possible to obtain acetylation products from CA by any of the lipases tested.

According to literature, only the triacetylated derivative was obtained through chemical procedures. However, ethyl ester CA **2a** was a suitable substrate and *C. antarctica* B lipase was the catalyst of choice allowing us to obtain the ethyl 3α-acetyl cholate **3a** in excellent yield (87%). This derivative was chemically obtained in 75% yield [15]. The reaction was highly regioselective, neither 7-OH nor 12-OH was transformed. Under the same reaction conditions the lauryl ester of cholic acid not was acetylated.

By lipase-catalyzed alcoholysis of the peracetylated cholic acid the corresponding ethyl ester was obtained; no products of deacetylation were observed. Instead, by alcoholysis of peracetylated ethyl ester of cholic acid it was possible to obtain a ethyl 7α,12α-diacetoxy-3α-hydroxy-5β-cholanate as a new product.

In summary, the enzymatic catalysis offers a good alternative to synthesize a variety of esters and mono- and diacetyl derivatives from cholic acid, under mild reaction conditions.

The lipase-catalyzed procedure uses a series of alcohols as reagent for esterification, ethanol for alcoholysis and ethyl acetate as acylating agent. Moreover, the reaction is simple, it is performed at low temperature and the products are isolated by simple filtration and solvent evaporation. The lipase is biodegradable and consequently more compatible to the environment than the chemical catalysts. In addition, because the enzyme is insoluble in the reaction medium, it is easily removed by filtration and can be reused. In the esterification reaction LIP retained 80% activity after three reaction cycles and in the acetylation reaction, CAL B retained 88% activity after five reaction cycles.

In addition, molecular modeling was applied to rationalize the selective behavior of CAL B in the acetylation reactions and helped to explain the lack of reactivity of CA through the particular interaction between the lipase active site and this bile acid. A comparison between the behavior of cholic acid and chenodeoxycholic, deoxycholic and lithocholic acid is also presented.

Acknowledgments

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Appendix A. Supplementary data

Spectral data for compounds **2a–g**, **3a**, **4a**, **b** and **5b**, associated with this article are supplied. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2015.12.014.

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