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Enzymatic synthesis of bile acid derivatives and biological evaluation against *Trypanosoma cruzi*

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ABSTRACT

Enzyme catalysis was applied to synthesize derivatives of three bile acids and their biological activity was evaluated as growth inhibitors of the protozoan *Trypanosoma cruzi*. Twelve mono-, diacetyl and ester derivatives of deoxycholic, chenodeoxycholic and lithocholic acid, seven of them new compounds, were obtained through lipase-catalyzed acetylation, esterification and alcoholysis reactions in very good to excellent yield and a highly regioselective way. Among them, acetylated ester products, in which the lipase catalyzed both reactions in one-pot, were obtained. The influence of various reaction parameters in the enzymatic reactions, such as enzyme source, acylating agent/substrate ratio, enzyme/substrate ratio, solvent and temperature, was studied. Some of the evaluated compounds showed a remarkable activity as *Trypanosoma cruzi* growth inhibitors, obtaining the best results with ethyl chenodeoxycholate 3-acetate and chenodeoxycholic acid 3,7-diacetate, which showed IC_{50}: 8.6 and 22.8 μM, respectively. In addition, in order to shed light to bile acids behavior in enzymatic reactions, molecular modeling was applied to some derivatives. The advantages showed by the enzymatic methodology, such as mild reaction conditions and low environmental impact, make the biocatalysis a convenient way to synthesize these bile acid derivatives with application as potential antiparasitic agents.

1. Introduction

Bile acids are widely distributed in nature as oxygenated metabolites of steroids. The most common bile acids are: chenodeoxycholic acid (CDCA, **1a**), deoxycholic acid (DCA, **1b**) lithocholic (LCA, **1c**) and cholic acid (CA, **1d**) (Fig. 1).[1]

Bile acids are the end products of cholesterol catabolism in mammals and play a critical role in biological processes such as digestion, secretion and the regulation of cholesterol metabolism. They are of great interest by acting as powerful biological detergents that help in dissolving and absorption of fats, fatty acids and lipid soluble vitamins[2] and because they exhibit various pharmacological activities, such as potent and selective farnesoid X receptor (FXR) antagonism,[3] antimicrobial[4] and anticancer activities.[5]

Moreover, all bile acids and their conjugates form bile salts which, as amphiphilic compounds, are widely used in drug formulations as absorption promoters[6] and as excipients that can influence gastrointestinal solubility, absorption and chemical or enzymatic stability of drugs,[7] enhance transdermal penetration,[8] etc.

The application of enzymes and whole cells of microorganisms in the synthesis of pharmaceuticals derivatives has received considerable attention in recent years.[9,10] It is well recognized that enzymes are capable of accepting a wide array of substrates, and catalyze several reactions in a regio-, chemo- and enantioselective way. As a result, biocatalysts allow carrying out different chemical transformations without the need for tedious protection and deprotection steps, especially in compounds with several functional groups.[11] Particularly, the application of lipases in non-aqueous media has been widely used for several synthetic reactions such as esterification, transesterification, aminolysis, polymerization.[12–14] leading to the synthesis of pharmaceuticals and biologically active compounds.[15] Several studies on the esterification and transesterification of a variety of substrates have shown that lipases are also useful in the steroid field, playing an important role in the mild and selective interconversion of functional groups via regio- and stereoselective transformation.[16,17] Taking into account these properties, in our laboratory we have modified pregnanes

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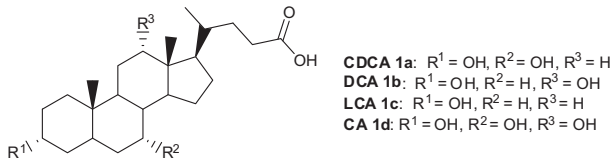


Figure 1. Structure of bile acids.

and androstanes,[18] synthesized fatty acid derivatives of dehydroepiandrosterone, hydrocortisone and 3,17-β-estradiol, a series of novel 20-succinates of pregnanes and acetyl cortexolone.[19]

In the present paper, we want to report the results obtained through lipase-catalyzed esterification, acylation and alcoholysis reactions in the preparation of derivatives of three bile acids: chenodeoxycholic acid (CDCA, **1a**), deoxycholic acid (DCA, **2a**) and lithocholic acid (LCA, **3a**).

Although the pharmacological activity of bile acid derivatives has been extensively studied, there is no evaluation of their biological activity as antiparasite so far. American trypanosomiasis (Chagas' disease) is among the most prevalent parasitic diseases worldwide.[20] It has been estimated that around 18–20 million people are infected and over 40 million individuals are at risk of infection by the hemoflagellated protozoan *Trypanosoma cruzi*, the responsible agent of this disease.[21] As other kinetoplastid parasites, *T. cruzi* has a complex life cycle possessing four main morphological forms.[22] Transmission via the placenta or by blood transfusion is the responsible mechanism for the occurrence of Chagas' disease in developed countries where it is not endemic.[23] The existing chemotherapy for Chagas' disease is still deficient and mainly based on old and empirically discovered drugs, often showing unwanted side effects. Therefore, the development of a safe and effective chemotherapy involving new antiparasitic agents, is essential.[24]

Recently, it was established that the sterol biosynthesis pathway is an important metabolic pathway in members of the Trypanosomatidae family.[25] Also in the steroid field, some examples of hybrids based on a steroidal framework have been reported as potential antiparasitic agents.[26]

Considering the role of steroids in trypanosome metabolism, our research in the field of steroids and antiparasitic activity of organic compounds, we are also presenting the evaluation of the biological activity of the bile acid derivatives synthesized in this work, as growth inhibitors of the protozoan *Trypanosoma cruzi*.

2. Results and discussions

Bile acids exhibit a hydrophobic side (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 bile acids self-associate to form multimers above a critical concentration. Hydrogen bonding plays a key role in these multimers.

The ratio of the hydrophobic to the hydrophilic surface area of bile acids determines their broad biological activity. Sometimes, their potential use 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 planned to modify the structure of some bile acids. For example, the presence of an acyl group at the C-3, C-7 or C-12 positions or esterification of the acid moiety could make them more lipophilic. Taking this into account, in the following section we are going to describe the results obtained by lipase-catalyzed esterification, acetylation and alcoholysis reactions of CDCA, DCA and LCA.

2.1. Enzymatic esterification

According to literature, bile acid esters have been obtained by various procedures. For example, chenodeoxycholic methyl ester was prepared using methanol and *p*-toluenesulfonic acid[27] or methanesulfonic acid[28] and also applying these conditions under microwave irradiation.[29] CDCA ethyl ester was described as an intermediate in the synthesis of chenodiol by reduction of (5β,7α)-7-hydroxy-3-oxocholan-24-oic acid ethyl ester.[30] Triethylamine and EEDQ were used as catalysts in the synthesis of ethyl ester of deoxycholic acid,[31] and ethyl ester of lithocholic acid was biosynthesized by rat intestinal microflora and its identity was confirmed by combined gas–liquid chromatography–chemical ionization mass spectrometry.[32]

With the aim to apply enzymes as an alternative procedure, we carried out the esterification of CDCA, DCA and LCA with ethanol and lipases as catalysts (Scheme 1).

We studied the behavior of various lipases and some reaction parameters such as solvent, temperature, enzyme/substrate ratio (E/S) and nucleophile (ethanol)/substrate ratio (Nu/S) to achieve the optimal conditions. In every case CDCA (**1a**) was used as bile acid model.

2.1.1. Enzyme screening and solvent effect

Three commercial lipases were evaluated in the esterification reaction of **1a** with ethanol: *Candida rugosa* lipase (CRL), *Candida antarctica* lipase B (CAL B) and Lipozyme, lipase from the fungus *Rhizomucor miehei* (LIP). The solvents tested were acetonitrile, hexane, diisopropyl ether and toluene and the reaction without co-solvent using ethanol both as nucleophile and solvent. Reactions were carried out at 55 °C using an enzyme/substrate ratio E/S: 10, ethanol/substrate ratio: 5 and the time necessary to achieve maximum conversion (Table 1). In the absence of biocatalyst no product was obtained and it was observed that enzyme activity was variable, giving CAL B the most satisfactory results in hexane: product **2a** at 100% conversion in 24 h of reaction (Table 1, entry 2). This lipase was also active in toluene and DIPE, but to a lesser extent (Table 1, entries 3 and 4). In hexane, LIP was also active but it showed a lower performance than CAL B showing 70% conversion after 96 h, whereas with CRL the enzyme activity was very low. Working without co-solvent, in neat ethanol, CAL B also afforded the desired product with 100% conversion but at a longer reaction time (72 h) (Table 1, entry 1).

2.1.2. Effect of enzyme/substrate ratio

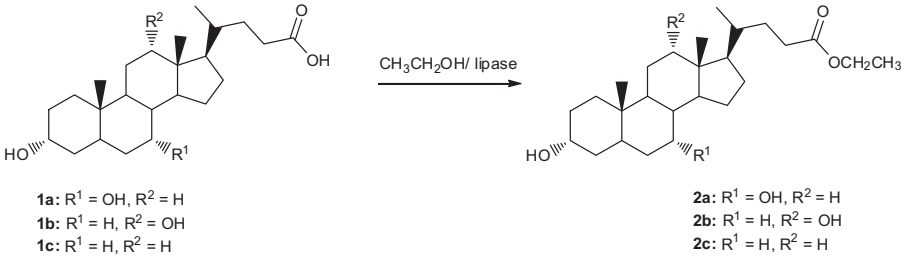
The influence of the enzyme/substrate ratio in the enzymatic esterification was evaluated at 24 h, using ethanol as nucleophile at Nu/S ratio of 5, hexane as co-solvent at 55 °C and variable amounts of CAL B. From the results (Table 1, entries 12–15), it can be concluded that a ratio E/S of 10 (Table 1, entry 2) is the most satisfactory.

2.1.3. Effect of nucleophile/substrate ratio

The influence of the Nu/S ratio on esterification yield was evaluated at 24 h in hexane using CAL B (E/S = 10) at 55 °C and variable amounts of nucleophile. From the results (Table 1, entries 16–18), it can be concluded that a ratio E/S of 5 is the most satisfactory.

2.1.4. Influence of temperature

Investigating the influence of temperature on the enzymatic esterification, we performed the reaction at 25, 35 and 55 °C. The other reaction parameters were settled to their optimal values (CAL B, hexane, E/S: 10 and Nu/S: 5). The results (Table 2, entries 2, 19 and 20) show an increase in yield with the increase in temperature. Therefore we selected 55 °C as the reaction temperature.



1a: R^1 = OH, R^2 = H
1b: R^1 = H, R^2 = OH
1c: R^1 = H, R^2 = H

2a: R^1 = OH, R^2 = H
2b: R^1 = H, R^2 = OH
2c: R^1 = H, R^2 = H

Scheme 1. Enzymatic synthesis of ethyl esters of bile acids CDCA (**1a**), DCA (**1b**) and LCA (**1c**).

Table 1
Optimization of reaction parameters for lipase-catalyzed esterification of CDCA

Entry	Lipase	Solvent	*T* (°C)	E/S[a]	Nu/S	*t* (h)	Conversion
Lipase and solvent							
1	CAL B	Ethanol	55	10	5	72	100
2	**CAL B**	**Hexane**	**55**	**10**	**5**	**24**	**100**
3	CAL B	DIPE	55	10	5	72	40
4	CAL B	Toluene	55	10	5	72	60
5	CAL B	MeCN	55	10	5	96	15
6	LIP	Ethanol	55	10	5	96	60
7	LIP	Hexane	55	10	5	96	70
8	LIP	DIPE	55	10	5	96	25
9	LIP	Toluene	55	10	5	96	30
10	CRL	Ethanol	55	10	5	96	15
11	CRL	Hexane	55	10	5	96	20
E/S							
12	CAL B	Hexane	55	15	5	24	100
13	CAL B	Hexane	55	5	5	24	70
14	CAL B	Hexane	55	2	5	24	40
15	CAL B	Hexane	55	1	5	24	35
Nu/S							
16	CAL B	Hexane	55	10	10	24	100
17	CAL B	Hexane	55	10	2	24	72
18	CAL B	Hexane	55	10	1	24	60
Temperature							
19	CAL B	Hexane	25	10	5	24	30
20	CAL B	Hexane	35	10	5	24	56

Alcohol: ethanol.
Bold type means optimal reaction conditions.
[a] E/S: enzyme amount in mg/substrate amount in mg.

Considering the previously mentioned experiments, the following standard conditions for the enzymatic esterification of chenodeoxycholic acid were chosen: CAL B as biocatalyst, temperature: 55 °C, E/S: 10, Nu/S: 5 and hexane as solvent.

2.1.5. Lipase-catalyzed esterification of DCA and LCA

Using the optimal conditions described for chenodeoxycholic acid in the previous section, we applied the enzymatic strategy in the esterification of deoxycholic (**1b**) and lithocholic (**1c**) acids. All ethyl esters were obtained in excellent yield (Ethyl CDCA: 99%, Ethyl DCA: 89% and Ethyl LCA: 93%), although the reaction time was higher for obtaining **2b** and **2c** (72 h).

2.2. Enzymatic acetylation

At our best knowledge, the preparation of monoacetyl derivatives of CDCA, DCA and LCA from the corresponding free acids have not been reported, all related data involve the methyl esters as starting materials. For example, chenodeoxycholic acid methyl ester was acetylated using acetic anhydride, pyridine and DMAP at room temperature during eight hours to lead the methyl ester-7-acetate derivative, whereas without DMAP in sixteen hours the methyl ester-3-acetate derivative was obtained.[33]

The peracetylated methyl chenodeoxycholate 3,7-diacetate was prepared from the tosylhydrazone of its methyl ester in the presence of acetic acid and sodium borohydride at 60 °C.[34] Regarding DCA, the reaction with ethyl acetate in *p*-toluenesulfonic acid and water at reflux afforded ethyl-DCA-3-acetate in 75% yield.[35]

The presence of the two hydroxyl groups in CDCA makes this compound an interesting model for studying the selectivity of the enzymatic acetylation. Therefore, at the beginning we study the behavior of lipases in the acetylation of this bile acid. Then, we applied the optimal reaction conditions to the other two bile acids and their corresponding ethyl esters (Scheme 2).

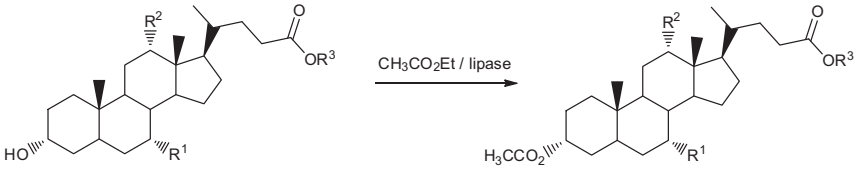
2.2.1. Enzyme screening and solvent effect

Again three commercial lipases were tested in the acetylation reaction of CDCA using ethyl acetate as acylating agent: *Candida rugosa* lipase (CRL), *Candida antarctica* lipase B (CAL B) and Lipozyme (LIP). The solvents tested were acetonitrile, hexane, diisopropyl ether and toluene and the reaction without co-solvent using ethyl acetate both as nucleophile and solvent. Reactions were carried out at 55 °C using an enzyme/substrate ratio E/S: 10, nucleophile/substrate ratio: 5 and the time necessary to achieve maximum conversion (Table 2). In the absence of biocatalyst no product was obtained and it was observed that enzyme activity was variable, giving CAL B the most satisfactory results in hexane: product chenodeoxycholic acid 3-acetate (**3a**) at 100%

Table 2
Optimization of reaction parameters for lipase-catalyzed acetylation of CDCA

Entry	Lipase	Solvent	*T* (°C)	E/S[a]	Nu/S[b]	*t* (h)	Conversion
Lipase and solvent							
1	CAL B	Ethyl acetate	55	10	—	120	100
2	CAL B	Hexane	55	10	5	48	100
3	CAL B	DIPE	55	10	5	96	40
4	CAL B	Toluene	55	10	5	96	60
5	CAL B	MeCN	55	10	5	120	15
6	LIP	Ethyl acetate	55	10	—	96	80[c]
7	LIP	Hexane	55	10	5	96	53[c]
8	LIP	DIPE	55	10	5	120	25[c]
9	LIP	Toluene	55	10	5	120	30[c]
10	CRL	Ethyl acetate	55	10	—	120	40
11	CRL	Hexane	55	10	5	120	50
E/S							
12	**CAL B**	**Hexane**	**55**	**5**	**5**	**48**	**95**
13	CAL B	Hexane	55	2	5	96	90
14	CAL B	Hexane	55	1	5	120	85
Nu/S							
15	CAL B	Hexane	55	5	10	48	90
16	CAL B	Hexane	55	5	2	48	80
17	CAL B	Hexane	55	5	1	48	65
Temperature							
18	CAL B	Hexane	25	5	5	48	51
19	CAL B	Hexane	35	5	5	48	76

Bold type means optimal reaction conditions.
[a] E/S: enzyme amount in mg/substrate amount in mg.
[b] Entries 1, 6 and 10: ethyl acetate: nucleophile and solvent.
[c] Product: Ethyl chenodeoxycholate.



1a: R^1 = OH, R^2 = H, R^3 = H
1b: R^1 = H, R^2 = OH, R^3 = H
1c: R^1 = H, R^2 = H, R^3 = H
2a: R^1 = OH, R^2 = H, R^3 = CH_2CH_3
2b: R^1 = H, R^2 = OH, R^3 = CH_2CH_3
2c: R^1 = H, R^2 = H, R^3 = CH_2CH_3

3a: R^1 = OH, R^2 = H, R^3 = H
3b: R^1 = H, R^2 = OH, R^3 = H
4a: R^1 = OH, R^2 = H, R^3 = CH_2CH_3
4b: R^1 = H, R^2 = OH, R^3 = CH_2CH_3
4c: R^1 = H, R^2 = H, R^3 = CH_2CH_3

Scheme 2. Enzymatic synthesis of acetylated derivatives of bile acids CDCA (**1a**), DCA (**1b**) and LCA (**1c**).

conversion in 48 h of reaction (Table 2, entry 2). This lipase was also active in toluene and DIPE, but to a lesser extent (Table 2, entries 3–5). CRL also showed some activity but lower than CAL B (Table 2, entries 10 and 11). Working without co-solvent with ethyl acetate as nucleophile and solvent, CAL B also afforded the desired product with 100% conversion, but at 120 h of reaction (Table 2, entry 1).

The enzymatic reaction allowed us to obtain the CDCA-3 acetate derivative in quantitative yield and under simple and mild reaction conditions. In this case, the lipase behavior was highly regioselective since the hydroxyl moiety on carbon 7 did not react at all under the mentioned conditions. Since the reaction with ethyl acetate only afforded the monoacetylated product in the position 3, we decided to try the enzymatic acetylation using vinyl acetate and isopropenyl acetate as acylating agents, well-known as active reagents in lipase-catalyzed acetylations. In this case, the same product, **3a**, was also obtained, but at a shorter reaction time (24 h).

It is important to note that LIP, with ethyl acetate without any other solvent, exhibited a good performance, showing 80% conversion after 96 h, but surprisingly, the product obtained was the ethyl ester (**2a**) and not the acetylated derivative **3a** (Table 2, entries 6–9). This interesting result suggests that, in the presence of LIP, esterification of the carboxyl group was preferred even with the small amount of ethanol from the hydrolysis of the acylating agent. As it is well-known, in enzymatic activity the solvent influence is remarkable.[12] Although LIP did not show to be the best biocatalyst in the esterification reaction of CDCA (Table 1), using ethyl acetate as solvent afforded good results.

2.2.2. Effect of enzyme/substrate ratio

The influence of E/S in the enzymatic acetylation was evaluated using Nu/S: 5, hexane as solvent at 55 °C and variable amounts of CAL B. From the obtained results, it was observed that E/S: 10 is the best (Table 2, entry 2). However, as working at E/S: 5, conversion is slightly lower but considering that it is preferable to use the lowest amount of enzyme (Table 2, entry 12), E/S: 5 was the ratio of choice.

2.2.3. Influence of temperature

Temperatures of 25, 35 and 55 °C were tested, keeping the reaction parameters at their optimal values (CAL B, hexane, Nu/S: 5, E/S: 5). Conversions of 51% at 25 °C, 76% at 35 °C and 100% at 55 °C were observed, showing the influence of temperature in yield for the catalyzed reactions (Table 2, entries 12, 18 and 19). So we decided to perform the reaction at 55 °C.

Taking into account these studies, we have chosen as optimal conditions for the enzymatic acetylation of the bile acids: CAL B as biocatalyst, ethyl acetate as acylation agent, hexane as solvent, temperature: 55 °C, E/S: 5 and Nu/S: 5.

2.2.4. Acetylation of DCA and LCA

Once the experimental conditions were optimized, we applied them to the acetylation of bile acids DCA **1b** and LCA **1c**. The results are summarized in Table 3. As it can be observed, the acetylation of DCA led to the monoacetylated derivative in C3 (**3b**), regioselectively and at shorter time than **1a**. By contrast, in the case of lithocholic acid **1c**, ethyl lithocholate 3-acetate (**4c**) was obtained as the only product. This unexpected result is of great interest since both: acetylation and esterification reactions were performed in only one step. With the aim to obtain exclusively the acetylation product from lithocholic acid we tried to favor the enzymatic acetylation over the esterification. Then, the reaction was carried out with activated acylating agents such as isopropenyl acetate and vinyl acetate and CAL B as biocatalyst. Again, it was obtained **4c** as the only product but in a shorter reaction time (16 h).

With LIP it was not possible to obtain acetylation products from **1b** or **1c**, only the corresponding ethyl esters **2b** and **2c** were obtained in very good yield.

The standard conditions were also applied to acetylation of ethyl esters **2a**–**c**. In all cases, acetylation of hydroxyl group of 3-position was observed, obtaining the corresponding ethyl esters 3-acetates **4a**–**c**.

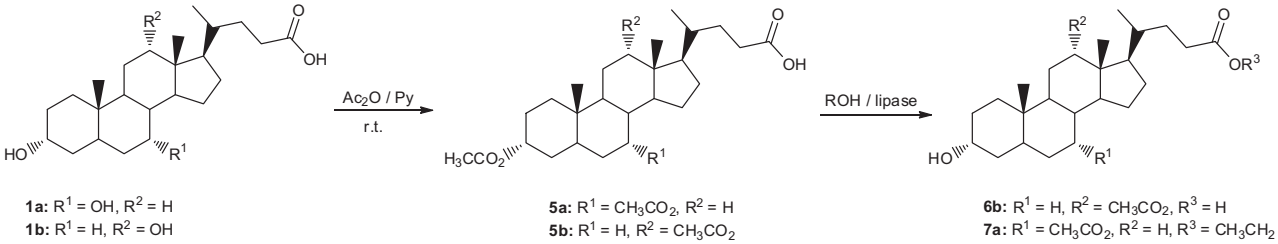
2.3. Enzymatic alcoholysis

The good regioselectivity of CAL B in catalyzing the acetylation of 3 hydroxyl group of the bile acids, prompted us to study if this lipase showed the same behavior in the alcoholysis reaction on the peracetylated chenodeoxycholic acid (**5a**) and deoxycholic acid (**5b**) (Scheme 3).

Table 3
Enzymatic acetylation of bile acids and ethyl esters

Bile acid	Lipase	Acylating agent	*t*	Product yield (%)							
				2b	**2c**	**3a**	**3b**	**3c**	**4a**	**4b**	**4c**
CDCA	CAL B	EtOAct	48			90					
DCA	CAL B	EtOAct	24				99				
LCA	CAL B	EtOAct	24								100
DCA	LIP	EtOAct	96	91							
LCA	LIP	EtOAct	24		93						
Et-CDCA	CAL B	EtOAct	48						97		
Et-DCA	CAL B	EtOAct	96							90	
Et-LCA	CAL B	EtOAct	96								85
LCA	CAL B	VinOAc	16								98
LCA	CAL B	*i*-PrOAc	16								97

Experimental conditions: Solvent: hexane; Temperature: 55 °C; E/S: 5:1; Nu/S: 5:1, *t*: time in hours. VinOAc: vinyl acetate, *i*-PrOAc: isopropenyl acetate.



Scheme 3. Chemical synthesis and enzymatic alcoholysis of peracetylated derivatives of CDCA and DCA.

The substrates for the enzymatic alcoholysis **5a** and **5b**, were obtained in 95% and 90% yield, respectively, by treatment of the corresponding acids **1a** and **1b** with acetic anhydride and pyridine at room temperature during 16 h.

At this point, we attempted to carry out the enzymatic alcoholysis of **5a**–**b** using CAL B as biocatalyst, ethanol, *n*-butanol and *n*-octanol as nucleophiles and acetonitrile, diisopropyl ether and hexane as solvents. The results are presented in Table 4. The best results were achieved with ethanol and hexane as solvent. Higher alcohols such as *n*-butanol and *n*-octanol did not show good performance. Again the lipase showed a regioselective behavior acting only at 3-position. Under these conditions, it was possible to obtain ethyl chenodeoxycholate acid 7-acetate (**7a**) from **5a** in quantitative yield, and deoxycholic acid 12-acetate (**6b**) from **5b** in good yield (Table 4, entries 1, 2 and 5). The site of alcoholysis in every case was unambiguously established by NMR spectroscopic analysis. These results prove that in the alcoholysis the enzyme maintains the same regioselectivity than in acetylation reactions, offering a good alternative to remove acyl groups on carbon 3 while keeping unaltered the other positions. This fact is in agreement with previous work performed in our laboratory using pyridoxine, terpene and steroids as substrates.[15,18,19]

It is interesting to observe that the reaction with the diacetylated derivative of chenodeoxycholic acid **5a** afforded a 7-monoacetylated ethyl ester (**7a**), working ethanol as alcoholysis and esterifying agent at the same time.

2.4. Molecular modeling

To shed light to the molecular determinants of the enzymatic reactions on bile acids, we examined the enzymatic acetylation using molecular modeling. This approach helps to understand the structural relation between the enzymatic pocket and the characteristic steroidal framework, which accounts for the selectivity of the biological behavior in this family of natural compounds. Then, molecular docking studies have been performed on chenodeoxycholic acid, deoxycholic acid and lithocholic acid with CAL B and the ability of the catalytic pocket to accommodate these substrates was evaluated. The analysis of the results led to the selection of a possible conformation for each bile acid, using the interaction with the amino acids of the catalytic site (Asp187-His224-Ser105), binding energy and the population of the clusters as selection criteria. CDCA, DCA and LCA interact with the active site (Ser105) of the enzyme through C3-OH showing distances of 1.94, 1.95 and 1.95 Å and binding energies of 5.36, 7.13 and 6.13 kcal/mol, respectively. The main favorable conformations correspond to those in which the hydroxyl group on carbon 3 points toward the catalytic triad.

In the case of CDCA and DCA, the distances of the hydroxyl groups in position 7 and 12, respectively, located in the steroid B ring, were 4.38 and 4.73 Å. These hydroxyl groups are farther from the catalytic serine than C3-OH justifying their lack of reactivity.

The docking representations of CAL B with CDCA, DCA and LCA are presented in Figure 2. In every case it can be observed the hydrophobic tunnel through which the substrates enter to reach the catalytic triad of the lipase.

We also used molecular modeling to explain the different products obtained by reaction of bile acids with ethyl acetate in hexane catalyzed by CAL B.

Under the same reaction conditions CDCA and DCA afforded the 3-acetyl derivatives. Instead LCA gave ethyl lithocholate 3-acetate, indicating that the lipase catalyzed both reactions: acetylation and esterification.

In previous work involving a combination study of docking simulations and QSAR analysis it was reported that in CAL B, among the explored five molecular interaction fields, the contribution of the electrostatic field to the reactivity was the most significant.[36] Taking this into account, hydrogen bond interactions between polar residues in CAL B and the hydroxyl groups in the steroid B ring in CDCA (C-7) and DCA (C-12), would result in a higher stabilization of these substrates in the enzyme pocket than LCA. As a consequence only the acetylation reaction was catalyzed with CDCA and DCA while ethyl-lithocholate 3-acetate, through lipase-catalyzed acetylation and esterification, was obtained from LCA under the same reaction conditions.

It could be assumed that acetylation could occur prior to esterification, leading to the formation of lithocholic acid 3-acetate (3Aco-LCA) in the first step of the process. Then, CAL B catalyzed the esterification of 3AcO-LCA with the ethanol released during the acetylation with ethyl acetate.

In Figure 3, a docking study performed on 3AcO-LCA shows the entrance of the acetylated substrate to the catalytic pocket by the end of the carboxylic acid group. It can be observed a distance of 3.14 Å from carboxylic carbon to the catalytic serine and a binding energy of 6.47 kcal/mol.

Table 4
CAL B-catalyzed alcoholysis of CDCA-3,7-diacetate (**5a**) and DCA-3,12-diacetate (**5b**)

Entry	Substrate	Solvent	Nu	Nu/S	*t* (h)	Conversion[a] (%)	
						6b	**7a**
1	**5a**	Hexane	EtOH	1.2	120		100
2	**5a**	Hexane	EtOH	5	48		100 (93)
3	**5a**	MeCN	EtOH	1.2	96		n.d.
4	**5a**	MeCN	EtOH	5	96		10
5	**5a**	DIPE	EtOH	1.2	120		10
6	**5a**	DIPE	EtOH	5	120		30
7	**5a**	Hexane	BuOH	5	96		n.d.
8	**5a**	Hexane	OctOH	5	120		n.d.
9	**5b**	Hexane	EtOH	1.2	120	25	
10	**5b**	Hexane	EtOH	5	120	73(65)	
11	**5b**	MeCN	EtOH	1.2	120	n.d.	
12	**5b**	MeCN	EtOH	5	48	n.d.	
13	**5b**	DIPE	EtOH	1.2	96	10	
14	**5b**	DIPE	EtOH	5	120	40	
15	**5b**	Hexane	BuOH	5	48	n.d.	
16	**5b**	Hexane	OctOH	2	48	n.d.	

Experimental conditions: enzyme: CAL B; solvent: hexane; temperature: 55 °C; E/S: 5:1; Nu/S: 5:1.
n.d.: non detected.
[a] Entries 2 and 10: % yield in brackets.

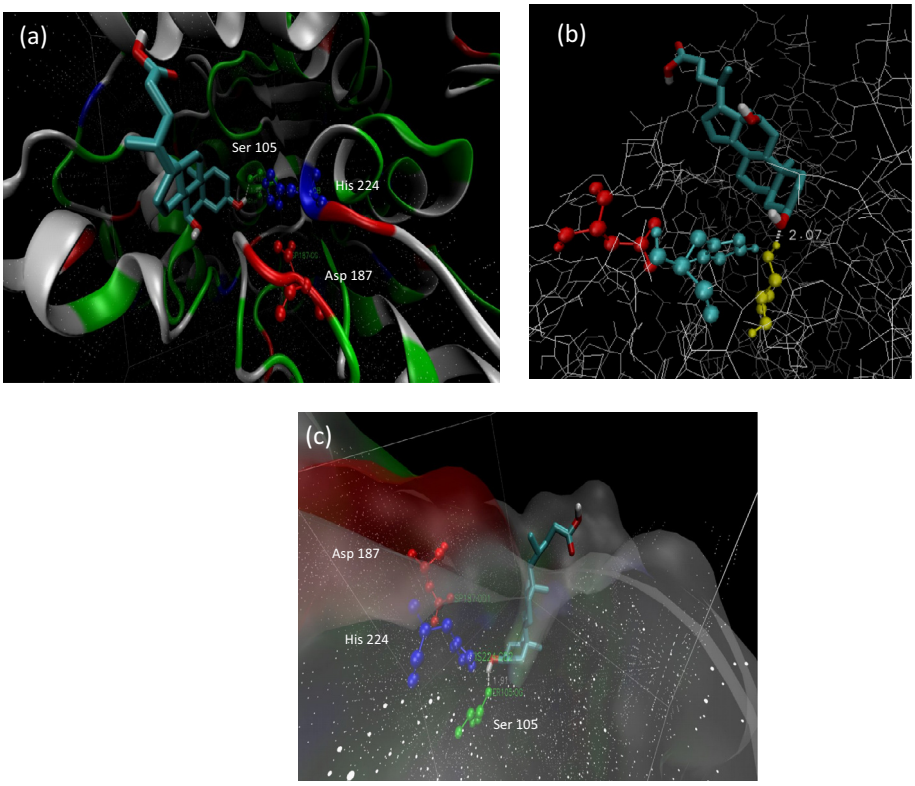


Figure 2. Docking of CAL B with: (a) CDCA, (b) DCA and (c) LCA.

2.5. Biological evaluation

Lipids have essential roles in biological membranes and are sources of stored energy. Recently, it was established that the sterol biosynthesis pathway is an important metabolic pathway in members of the Trypanosomatidae family.[25] Unlike mammals, which synthesize cholesterol, epimastigote form of *T. cruzi* produce mostly ergosterol. The sterol biosynthesis pathway is therefore considered one of the most attractive targets for the specific treatment of Chagas disease, and several enzymes from this pathway have been studied as possible treatment targets.[37]

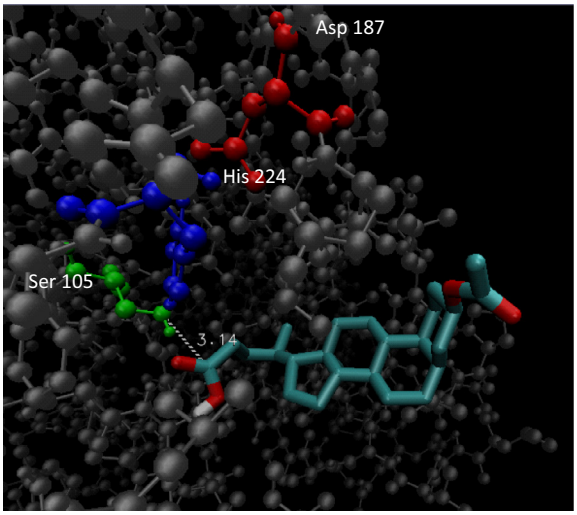


Figure 3. Docking of lithocholic acid 3-acetate.

Moreover, different azasterols have been evaluated as structural analogues of steroids in trypanosomes. They function as inhibitors of key pathways in the synthesis of steroids, having the enzyme sterol 24-methyltransferase as molecular target in *Leishmania* species and *Trypanosoma cruzi*.[38]

Taking into account that bile acids are considered as lipid metabolism regulators, the reported bile acid derivatives could be inhibitors of *T. cruzi* sterol biosynthesis. Therefore, all synthesized compounds were evaluated as growth inhibitors against the epimastigote form of *Trypanosoma cruzi*. The assays were performed at concentrations ranging from 300 to 0.14 μM in three independent assays. Axenic parasites were incubated with different concentrations of bile acid derivatives to obtain a dose–response curve that allowed calculation of the IC_{50} values. Benznidazole (Bz), the only one drug used for Chagas' treatment, was used as a positive control. Table 5 shows the results of only active compounds. A remarkable inhibitory effect on *T. cruzi* growth was observed in the case of the compounds ethyl chenodeoxycholate 3-acetate (**4a**) and chenodeoxycholic acid 3,7-diacetate (**5a**), exhibiting IC_{50} values of 8.6 and 22.8 μM, respectively. It is interesting to note that compound **4a** resulted two fold more potent than benznidazole and compound **5a** showed a potency of the same order.

Figures 4 and 5 show the growth curve of epimastigote *T. cruzi* in the presence of compounds **4a** and **5a**, respectively. Compounds **1a**, **2c** and **7a** showed inhibition but at lower extent and the rest of the tested compounds were inactive even at a concentration of 300 μM.

It can be observed that CDCA derivatives are the most active products and CDCA was the only bile acid that showed activity. Particularly in this case, it seems that two key factors improve the biological activity of the bile acid derivatives: the presence of an acetate group in 3-position of ring A and the ethyl group esterifying the acid moiety in the side chain. Both of them substantially

Table 5
IC_{50} values for *Trypanosoma cruzi* growth inhibition by bile acids derivatives

Compounds	1a	2c	4a	5a	7a	Bz
IC_{50} (μM)	236.1 ± 3.1	266.3 ± 3.9	**8.6 ± 0.2**	**22.8 ± 1.3**	295 ± 5.2	**18.8 ± 0.3**

Bold type means optimal reaction conditions.

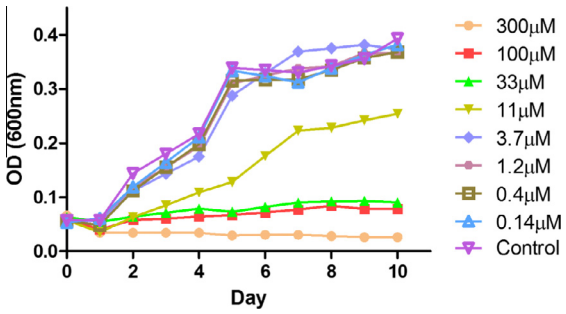


Figure 4. Effect of ethyl chenodeoxycholate 3-acetate (**4a**) concentration on *Trypanosoma cruzi* growth.

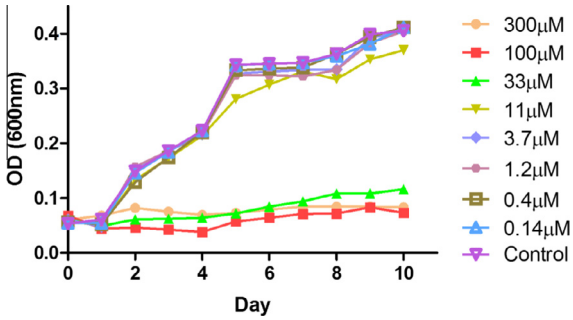


Figure 5. Effect of chenodeoxycholic acid 3,7-diacetate (**5a**) concentration on *Trypanosoma cruzi* growth.

increase the inhibitory activity displayed by CDCA, showing an IC_{50} value of about 30 times lower. It is also interesting to note that the presence of only one of these two groups has the opposite effect because both ethyl ester (**2a**) and 3-acetate (**3a**) did not exhibited antiparasitic activity. Furthermore, the presence of an acetate group in the 7 position maintaining the ester function, did not improved the activity of CDCA (**7a** vs **4a**). In addition, two acetate groups at 3- and 7-positions also caused an increase in the growth inhibition, being 5–10 times more potent than **1a**. It could be assumed that the presence of an acetate group in the 3-position is essential for activity. Moreover, it is also required the presence of another group, acetate or ethyl ester that increases the lipophilicity of the compound, which clearly improve the percent inhibition. The same applies for the case of LCA, showing no activity, but with the ethyl ester showing a slight inhibition although at a high concentration. In the case of DCA, none of the derivatives was active, probably due to the presence of OH or AcO group in the position 12, which would not allow an adequate interaction of the compound with the corresponding molecular target.

In summary, the observed results indicate that the presence of functional groups in positions 3 and 7 of the steroid skeleton favors the antiparasitic activity of bile acid derivatives. In particular, the presence of acetyl group in position 3 rather than position 7 increases the inhibitory activity exhibited by CDCA.

3. Conclusions

This work describes the application of enzymes to the synthesis of derivatives of the three bile acids CDCA, DCA and LCA in a highly regioselective way. Scheme 4 shows the performed reactions and the obtained products.

Among the tested enzymes, *Candida antarctica* B lipase gave the best results in esterification, acylation and alcoholysis reactions. To begin, CDCA, DCA and LCA ethyl esters were obtained as the only reaction products. The three esters were completely characterized by spectroscopic methods and, in the case of Ethyl CDCA and Ethyl DCA, obtained in higher yield than those reported by chemical synthesis (89% vs 78% and 93% vs 19%, respectively). Regarding Ethyl LCA, in previous work was not isolated and its identity was only determined by GC–MS.

Lipase-catalyzed acetylation allowed us to obtain two new monoacetyl bile acid derivatives in the position 3 of ring A in the steroid skeleton: 3-AcO-CDCA, 3-AcO-DCA. The reaction was highly regioselective, neither 7-OH in CDCA nor 12-OH in DCA was transformed. Under the same reaction conditions LCA afforded the acetyl derivative of the ethyl ester by catalysis of CAL B for acetylation and esterification reactions in one pot.

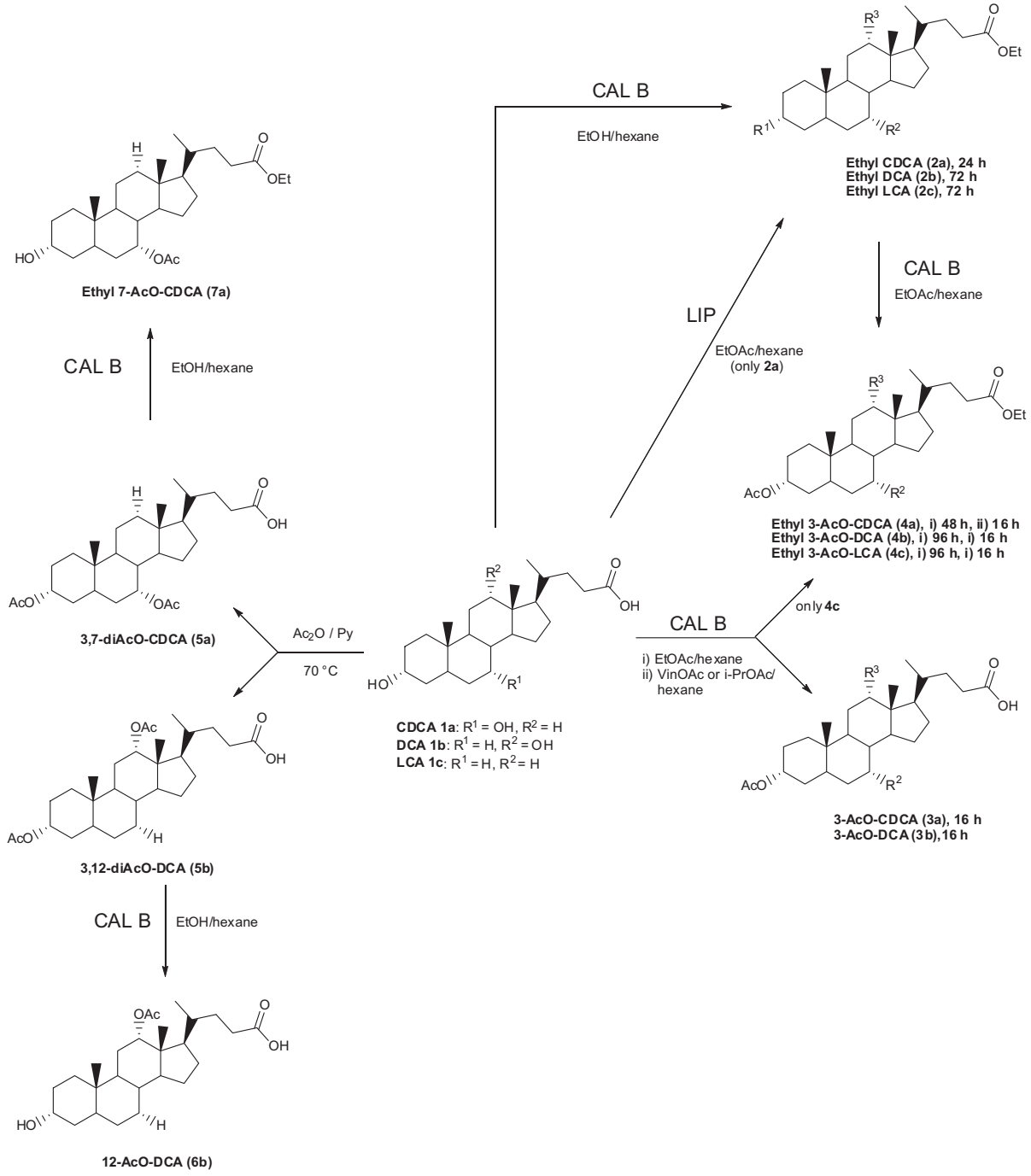
The enzymatic acetylation of the ethyl esters also afforded the 3-monoacetylated products. Now two new products were obtained, ethyl 3-AcO-CDCA and ethyl 3-AcO-LCA. Finally, it is interesting to remark that the treatment of CDCA with Lipozyme under acetylation conditions afforded the corresponding ethyl ester instead of the acetylation product.

By lipase-catalyzed alcoholysis of the peracetylated derivatives of CDCA and DCA it was possible to obtain two new products: the complementary acetates 7-AcO in CDCA and 12-AcO in DCA. Here, it is also interesting that in the case of CDCA, both alcoholysis of acetyl group and esterification of the carboxyl group gave the acetylated ester ethyl 7-AcO-CDCA.

In summary, the enzymatic reactions offer a good alternative to synthesize ester and monoacetylated derivatives from bile acids. Although the synthesis of these compounds performed by chemical methods is not difficult, it has the disadvantage of using hazardous reagents such as acetic anhydride, pyridine, and strong acidic media. The lipase-catalyzed procedure uses ethanol as reagent for esterification and alcoholysis reactions and ethyl acetate as acylating agent. The enzymatic approach also shows other interesting advantages: 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 friendly to the environment than 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 and acetylation of CDCA, CAL B retained 90% and 80% activity after five reaction cycles, respectively.

Molecular modeling was applied to rationalize the selective behavior of CAL B and the one-pot acetylation and esterification in the case of LCA.

All of the compounds were evaluated against epimastigote *Trypanosoma cruzi*. Compounds **4a** and **5a** showed to be very potent as inhibitors of parasite growth with IC_{50} = 8.6 and 22.8 μM (benznidazole: 18.8 μM). Considering this data, these



Scheme 4. Reactions and products from bile acids: CDCA, DCA and LCA.

products can be useful as potential drugs for chemotherapy of Chagas' disease.

4. Experimental

4.1. 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) was 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; 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), using EtOAc/hexane 3/7 as developing solvent 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 × 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 from 100 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 and stable at this temperature for 10 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 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.

4.2. Enzymatic esterification. General procedure

CAL B (1 g) was added to a solution of the corresponding bile acid (100 mg) in hexane (10 mL) and ethanol (0.15 mL). The suspension was shaken (200 rpm) at 55 °C and the progress of the reaction monitored by TLC/GC. 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 (9:1–1:1). Reuse experiments: the filtered and washed enzyme was used in the next enzymatic esterification under the same reaction conditions.

4.3. Enzymatic acetylation. General procedure

CAL B (500 mg) was added to a solution of the corresponding bile acid (100 mg) in hexane (10 mL) and ethyl acetate (0.25 mL). The suspension was shaken (200 rpm) at 55 °C and the progress of the reaction monitored by TLC/GC. 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 (9:1–1:1). Reuse experiments: the filtered and washed enzyme was used in the next enzymatic acetylation under the same reaction conditions.

4.4. Chemical peracetylation

In a typical procedure, a mixture of the corresponding bile acid (400 mg, 1 mmol) acetic anhydride (0.5 mL, 5 mmol) and pyridine (1 mL) was stirred at room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was partitioned between saturated solution ammonium chloride (10 mL) and methylene chloride (10 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 × 10 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 (9:1–7:3).

4.5. Enzymatic alcoholysis. General procedure

To a solution of the peracetylated bile 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 55 °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 (9:1–1:1).

4.5.1. Ethyl chenodeoxycholate (2a)

Yield 99%; colorless oil; $[\alpha]_D^{25}$ +6.4° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.12 (2H, q, *J* = 6.8 Hz, –OC*H*$_2$CH$_3$), 3.84 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 3.46 (1H, tt, *J* = 11.3, 4.2 Hz, H-3), 2.33 (1H, m, H-23b), 2.20 (1H, m, H-23a), 1.25 (3H, t, *J* = 6.7 Hz, OCH$_2$C*H*$_3$), 0.92 (3H, d, *J* = 6.4 Hz, H-21), 0.90 (3H, s, H-19), 0.65 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) d 174.4 (C-24), 72.0 (C-7), 68.5 (C-3), 60.2 (OC*H*$_2$CH$_3$), 31.0 (C-23), 22.8 (C-19), 18.3 (C-21), 14.2 (–OCH$_2$C*H*$_3$), 11.8 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{26}H_{44}NaO_4$ 443.3137. Found: $C_{26}H_{44}NaO_4$ 443.3132.

4.5.2. Ethyl deoxycholate (2b)

Yield 89%; colorless oil; $[\alpha]_D^{25}$ +33.6° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.12 (2H, q, *J* = 6.7 Hz, –OC*H*$_2$CH$_3$), 3.97 (1H, dd, *J* = 2.5, 3.0 Hz, H-12), 3.60 (1H, tt, *J* = 11.0, 4.0 Hz, H-3), 2.34 (1H, m, H-23b), 2.22 (1H, m, H-23a), 1.24 (3H, t, *J* = 6.7 Hz, OCH$_2$C*H*$_3$), 0.96 (3H, d, *J* = 6.5 Hz, H-21), 0.90 (3H, s, H-19), 0.67 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) d 174.3 (C-24), 73.1 (C-12), 71.8 (C-3), 60.2 (OC*H*$_2$CH$_3$), 30.9 (C-23), 23.1 (C-19), 17.3 (C-21), 14.2 (–OCH$_2$C*H*$_3$), 12.7 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{26}H_{44}NaO_4$ 443.3137. Found: $C_{26}H_{44}NaO_4$ 443.3141.

4.5.3. Ethyl lithocholate (2c)

Yield 93%; white solid, mp 87–88 °C; $[\alpha]_D^{25}$ +6.4° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.11 (2H, q, *J* = 6.6 Hz, OC*H*$_2$CH$_3$), 3.61 (1H, tt, *J* = 11.2, 4.2 Hz, H-3), 2.31 (1H, m, H-23b), 2.19 (1H, m, H-23a), 1.25 (3H, t, *J* = 6.6 Hz, –OCH$_2$C*H*$_3$), 0.91 (3H, d, *J* = 6.4 Hz, H-21), 0.89 (3H, s, H-19), 0.63 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.4 (C-24), 71.8 (C-3), 60.2 (OC*H*$_2$CH$_3$), 31.0 (C-23), 23.3. (C-19), 18.2 (C-21), 14.2 (OCH$_2$C*H*$_3$), 12.0 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{26}H_{44}NaO_3$ 427.3188. Found: $C_{26}H_{44}NaO_3$ 427.3191.

4.5.4. Chenodeoxycholic acid 3-acetate (3a)

Yield 90%; white solid, mp 108.5–109 °C; $[\alpha]_D^{25}$ +9.6° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.58 (1H, tt, *J* = 11.3, 4.4 Hz, H-3), 3.85 (1H, dd, *J* = 3.0, 2.5 Hz, H-7), 2.38 (1H, m, H-23b), 2.25 (1H, m, H-23a), 2.01 (1H, s, COC*H*$_3$), 0.93 (3H, d, *J* = 6.4 Hz, H-21), 0.91 (3H, s, H-19), 0.66 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 179.2 (C-24), 170.8 (*C*OCH$_3$), 74.4 (C-7), 68.5 (C-3), 30.8 (C-23), 22.7 (C-19), 21.5 (*C*H$_3$CO–), 18.2 (C-21), 11.8 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{26}H_{42}NaO_5$ 457.2930. Found: $C_{26}H_{42}NaO_5$ 457.2925.

4.5.5. Deoxycholic acid 3-acetate (3b)

Yield 99%; colorless oil; $[\alpha]_D^{25}$ +41.6° (*c* 1.0, $CHCl_3$). ^{1}H NMR (CDCl3, 500 MHz) δ 4.72 (1H, tt, *J* = 11.0 Hz, H-3), 4.00 (1H, dd, *J* = 2.5, 3.0 Hz, H-12), 2.33 (1H, m, H-23b), 2.24 (1H, m, H-23a), 2.03 (3H, s, COC*H*$_3$), 0.98 (3H, s, H-19), 0.93 (3H, d, *J* = 6.4 Hz, H-21), 0.69 (3H, s, H-18); 13C NMR (CDCl3, 500 MHz) δ 174.4 (C-24), 170.8 (*C*OCH$_3$), 74.4 (C-12), 72.5 (C-3), 31.0 (C-23), 23.2 (C-19), 17.5 (C-21), 12.9 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{26}H_{42}NaO_5$ 457.2930. Found: $C_{28}H_{46}NaO_5$ 457.2927.

4.5.6. Ethyl chenodeoxycholate 3-acetate (4a)

Yield97%; white solid, mp 90–90.5 °C; $[\alpha]_D^{25}$ +36.8° (*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, OC*H*$_2$CH$_3$), 3.85 (1H, dd, *J* = 2.5, 3.0 Hz, H-7), 2.33 (1H, m, H-23b), 2.20 (1H, m, H-23a), 2.00 (1H, s, COC*H*$_3$), 1.25 (3H, t, *J* = 6.7 Hz, –OCH$_2$C*H*$_3$), 0.92 (3H, d,

J = 6.4 Hz, H-21), 0.91 (3H, s, H-19), 0.66 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.3 (C-24), 170.8 ($COCH_3$), 74.4 (C-7), 68.5 (C-3), 60.2 (OCH_2CH_3), 31.0 (C-23), 22.7 (C-19), 21.5 (CH_3CO-), 18.3 (C-21), 14.3 (OCH_2CH_3), 11.8 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{28}H_{46}NaO_5$ 485.3243. Found: $C_{26}H_{44}NaO_4$ 485.3238.

4.5.7. Ethyl deoxycholate 3-acetate (4b)

Yield 89%; colorless oil; $[\alpha]_D^{25}$ +41.6° (c 1.0, $CHCl_3$). ^{1}H NMR (CDCl3, 500 MHz) δ 4.71 (1H, tt, J = 11.3, 4.5 Hz, H-3), 4.12 (2H, q, J = 6.5 Hz, OCH_2CH_3), 3.98 (1H, dd, J = 2.5, 3.0 Hz, H-12), 2.33 (1H, m, H-23b), 2.23 (1H, m, H-23a), 2.02 (3H, s, $COCH_3$), 1.25 (3H, t, J = 6.7 Hz, –OCH_2CH_3), 0.97 (3H, d, J = 6.5 Hz, H-21), 0.92 (3H, s, H-19), 0.68 (3H, s, H-18); ^{13}C NMR (CDCl3, 500 MHz) δ 174.3 (C-24), 170.7 ($COCH_3$), 74.3 (C-12), 73.2 (C-3), 60.2 (OCH_2CH_3), 30.9 (C-23), 23.1 (C-19), 21.5 (CH_3CO–), 17.4 (C-21), 14.3 (OCH_2CH_3), 12.7 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{28}H_{46}NaO_5$ 485.3243. Found: $C_{26}H_{44}NaO_4$ 485.3240.

4.5.8. Ethyl lithocholate 3-acetate (4c)

Yield 75%; white solid, mp 151–151.5 °C; $[\alpha]_D^{25}$ +24.4° (c 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.70 (1H, tt, J = 11.2, 4.2 Hz, H-3), 4.10 (2H, q, J = 6.7 Hz, OCH_2CH_3), 2.32 (1H, m, H-23b), 2.19 (1H, m, H-23a), 2.02 (1H, s, $COCH_3$), 1.24 (3H, t, J = 6.7 Hz, –OCH_2CH_3), 0.91 (3H, d, J = 6.4 Hz, H-21), 0.89 (3H, s, H-19), 0.63 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.3 (C-24), 170.7 ($COCH_3$), 74.4 (C-3), 60.2 (OCH_2CH_3), 31.0 (C-23), 23.3 (C-19), 21.4 (CH_3CO–), 18.3 (C-21), 14.2 (OCH_2CH_3), 12.0 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{28}H_{46}NaO_4$ 469.3294. Found: $C_{28}H_{46}NaO_4$ 469.3289.

4.5.9. Chenodeoxycholic acid 3,7-di-acetate (5a)

Yield 90%; white solid, mp 96–97 °C (lit.[39] mp 99 °C (dec)); $[\alpha]_D^{25}$ +8.5° (c 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.88 (1H, dd, J = 3.0, 2.5 Hz, H-7), 4.58 (1H, tt, J = 11.2, 4.3 Hz, H-3), 2.38 (1H, m, H-23b), 2.27 (1H, m, H-23a), 2.03 (3H, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 0.93 (3H, d, J = 6.4 Hz, H-21), 0.93 (3H, s, H-19), 0.65 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 179.2 (C-24), 170.7 ($COCH_3$), 170.5 ($COCH_3$), 74.2 (C-7), 71.3 (C-3), 30.8 (C-23), 22.7 (C-21), 21.6 (CH_3CO-), 21.5 (CH_3CO–), 18.2 (C-19), 11.7 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{28}H_{44}NaO_6$ 499.3036. Found: $C_{28}H_{44}NaO_6$ 499.3032.

4.5.10. Deoxycholic acid 3,12-diacetate (5b)

Yield 90%; white solid, mp 125–126 °C (lit.[40] colorless oil); $[\alpha]_D^{25}$ +65.5° (c 1.0, $CHCl_3$) (lit 68.6°). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.07 (1H, dd, J = 3.0, 2.5 Hz, H-12), 4.69 (1H, tt, J = 11.0, 4.2 Hz, H-3), 2.37 (1H, m, H-23b), 2.24 (1H, m, H-23a), 2.09 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 0.90 (3H, d, J = 6.4 Hz, H-21), 0.89 (3H, s, H-19), 0.71 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 179.7 (C-24), 170.6 ($COCH_3$), 170.5 ($COCH_3$), 75.9 (C-12), 74.2 (C-3), 30.6 (C-23), 23.0 (C-19), 21.4 (CH_3CO–), 21.3 (CH_3CO–), 17.5 (C-21), 12.4 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{27}H_{44}NaO_6$ 499.3036. Found: $C_{27}H_{44}NaO_6$ 499.3041.

4.5.11. Deoxycholic acid 12-acetate (6b)

Yield 60%; colorless oil; $[\alpha]_D^{25}$ +40.1° (c 1.0, $CHCl_3$). ^{1}H NMR (CDCl3, 500 MHz) δ 5.07 (1H, t, J = 2.6 Hz, H-12), 3.62 (1H, tt, J = 11.0, 4.1 Hz, H-3), 2.38 (1H, m, H-23b), 2.23 (1H, m, H-23a), 2.08 (3H, s, $COCH_3$), 0.89 (3H, s, H-19), 0.82 (3H, d, J = 6.4 Hz, H-21), 0.72 (3H, s, H-18); 13C NMR (CDCl3, 500 MHz) δ 178.9 (C-24), 170.6 ($COCH_3$), 75.9 (C-12), 71.8 (C-3), 30.8 (C-23), 23.1 (C-19), 17.5 (C-21), 12.4 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{26}H_{42}NaO_5$ 457.2930. Found: $C_{28}H_{46}NaO_5$ 457.2934.

4.5.12. Ethyl chenodeoxycholate acid 7-acetate (7a)

Yield 93%; white solid, mp 88–89 °C; $[\alpha]_D^{25}$ +6.5° (c 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.86 (1H, dd, J = 3.0, 2.5 Hz, H-7), 4.11 (2H, q, J = 6.5 Hz, OCH_2CH_3), 3.49 (1H, tt, J = 11.2, 4.3 Hz, H-3), 2.31 (1H, m, H-23b), 2.21 (1H, m, H-23a), 2.04 (3H, s, $COCH_3$), 1.24 (3H, t, J = 6.5 Hz, –OCH_2CH_3), 0.92 (3H, d, J = 6.5 Hz, H-21), 0.91 (3H, s, H-19), 0.64 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.3 (C-24), 170.6 ($COCH_3$), 71.8 (C-7), 71.3 (C-3), 60.2 (OCH_2CH_3), 30.8 (C-23), 22.7 (C-19), 21.6 (CH_3CO-), 18.3 (C-21), 14.2 (OCH_2CH_3), 11.7 (C-18). HRMS: $[M+Na]^+$ Calcd. $C_{28}H_{46}NaO_5$ 485.3243. Found: $C_{28}H_{46}NaO_5$ 485.3238.

4.6. Drug screening

Trypanosoma cruzi epimastigotes from clone CL Brener were grown at 28 °C in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mg/ml of hemin, 100 μg/ml of streptomycin and 100 U/ml of penicillin. Epimastigotes were seeded at a final concentration of 5×10^6 cells/mL and were incubated with different concentrations of the compounds ranging from 0.14 to 300 μM during 12 days at 28 °C. All drugs tested and benznidazole were dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in LIT never exceeded 0.5% (v/v). Control conditions of parasites without drug (100% growth) and medium without parasites were included. Parasite viability was determined at 600 nm in ELISA reader everyday.

4.7. Molecular 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.[41] The Autodock 4.2 method was applied considering a pair of rotatable bonds for steroids 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.

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Supplementary data

Supplementary data (spectral data for compounds **2**–**7**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.05.035.

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