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Chemoenzymatic synthesis of new derivatives of glycyrrhetinic acid with antiviral activity. Molecular docking study

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ABSTRACT

We present an efficient approach to the synthesis of a series of glycyrrhetinic acid derivatives. Six derivatives, five of them new compounds, were obtained through chemoenzymatic reactions in very good to excellent yield. In order to find the optimal reaction conditions, the influence of various parameters such as enzyme source, nucleophile:substrate ratio, enzyme:substrate ratio, solvent and temperature was studied. The excellent results obtained by lipase catalysis made the procedure very efficient considering their advantages such as mild reaction conditions and low environmental impact. Moreover, in order to explain the reactivity of glycyrrhetinic acid and the acetylated derivative to different nucleophiles in the enzymatic reactions, molecular docking studies were carried out. In addition, one of the synthesized compounds exhibited remarkable antiviral activity against TK + and TK- strains of Herpes simplex virus type 1 (HSV-1), sensitive and resistant to acyclovir (ACV) treatment.

1. Introduction

The pentacyclic triterpenoid glycyrrhetinic acid (GA) is the major bio-active constituent isolated from the roots of *Glycyrrhiza glabra*, which is a sweet-tasting material and has greater sweetening power than sugar, making it a widely used as additive in the food industry [1–3]. GA has been used as a lead compound to search more potent derivatives with different pharmacological properties. Glycyrrhetinic acid and some derivatives have been shown to exhibit anti-inflammatory [4,5] and anti-viral [6,7] activities. In addition, it has been reported derivatives of GA as inhibitors of cholinesterases [8] and 11β-HSD1 and 11β-HSD2 [9–11]. Despite these reports, few potential applications of GA derivatives for the development of new pharmacological agents have been investigated.

The application of enzymes as biocatalysts in the synthesis and transformation of different substrates with engaging properties has widely expanded in the last years [12–14]. Enzymes catalyze diverse type of reactions in a highly regio, chemo, and enantioselective way using a wide ranging of compounds. Therefore, biocatalysts constitutes a good alternative to obtaining of compounds through a Green Chemistry approach carrying out chemical transformations more easly, particularly in substrates with several functional groups [15–19]. In our laboratory we have studied the application of lipases in diverse reactions like esterifications, transesterifications and aminolysis of multiple substrates, obtaining a wide diversity of new compounds, which show interesting biological activities [20,21]. Recently, we have reported the synthesis of numerous compounds with potential applications as antiparasitic [22,23], antitumoral [24] and antiviral agents [25].

Continuing with our project on enzymatic transformations, we report here the results from the application of lipases in the preparation of a series of glycyrrhetinic acid derivatives.

In addition, all synthesized compounds were biologically evaluated as potential antiviral agents against TK+ and TK− strains of Herpes simplex virus type 1 (HSV-1), sensitive and resistant to acyclovir (ACV) treatment, respectively.

HSV-1 is a member of the Herpesviridae family and infects a high proportion of human population, causing a range of diseases from mild uncomplicated mucocutaneous to more serious infections, such as keratitis and encephalitis. HSV-1 is the causal agent of cold sore and encephalitis [26]. The current gold standard treatment for HSV-1 infections is ACV, a guanosine analogue antiviral drug. With the emergence of Herpesvirus strains resistant to nucleoside analogues, including ACV, there is an urgent need for new and more effective treatments for HSV infections [27]. In this work,

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we evaluated the antiviral capacity of five glycyrrhetinic acid (GA) derivatives against HSV-1.

2. Results and discussions

Many glycyrrhetinic acid derivatives were chemically synthesized and displayed a wide spectrum of applications in different areas. Some of them, with modifications in C-3, C-11 and C-29 positions, were used in the treatment of metabolic diseases [28]. Other derivatives with modifications at C-3 and C-30, showed a selective inhibition of the enzyme BChE [8]. Some chemically prepared alkanolamides, amides and esters derived from glycyrrhetinic acid had a high anti-inflammatory activity [11].

In this section we are going to describe the results obtained by applying lipases as catalysts for obtaining derivatives of glycyrrhetinic acid (GA, **1**).

Our initial experiments to obtain the ester derivatives were performed using **1** as substrate and ethanol as nucleophile. We studied the esterification catalyzed by lipases from several sources: from *Candida antarctica* B (CAL B), from *Candida rugosa* (CRL), Lipozyme from *Rhizomucor miehei* (LIP), *Rhizopus oryzae* lipase (ROL), Lipozyme from *Thermomyces lanuginosus* (TLL) and from *Carica papaya* (CPL), which is the remaining solid fraction of papaya latex after wash off of proteases, in a variety of solvents of different polarity (acetonitrile, acetone, diisopropyl ether, dioxane, hexane and toluene). Unfortunately, none of the enzymes tested catalyzed the esterification reaction of **1** with ethanol.

Then, in order to obtain amides derivatives, we tried the reaction of **1** with *n*-butylamine as model nucleophile. Several different conditions (diverse lipases and solvents) were applied but the same unsatisfactory results were observed.

Additionally, taking into account the previous successful results in the enzymatic esterification reaction of acetylated bile acids [21,22], we examined the possibility of acetylate the OH group of **1** using ethyl acetate as acylating agents and different lipases as biocatalysts at several conditions but no reaction was observed. The reaction with activated acylating agents such as vinyl or isopropenyl acetate or other acylating agents like ethyl caproate or diethyl succinate conducted to the same unsuccessful results.

From these results and considering the work previously reported [22], we carried out the chemical acetylation of **1** obtaining 3-acetyl glycyrrhetinic acid (**2**) in excellent yield (98%). Then, we studied the esterification (with ethanol) and amidation (with butylamine) reactions of **2** using several lipases in various solvents at different temperatures, but again no reaction was observed. In summary, under all reaction conditions, neither **1** nor **2** proved to be good substrates for nucleophilic agents such as ethanol or *n*-butylamine.

Lastly, we decided to study the reaction of **1** and **2** with alkanolamines as nucleophiles. We carried out the lipase catalyzed reaction with ethanolamine (**3a**) applying the optimal conditions previously determined for other similar substrates: CAL B as biocatalyst (E/S: 10), hexane as solvent, temperature: 55 °C, a Nu/S: 5. In this case, we observed that while **1** did not reacted, **2** led to the corresponding ethanolamide (**4a**) (Scheme 1). Therefore, considering this result, in order to optimize the reaction conditions, we performed different experiments using **2** as substrate and varying the lipase, solvent, enzyme:substrate ratio (E/S), nucleophile:-substrate ratio (Nu/S) and temperature.

2.1. Amidation of 3-acetyl glycyrrhetinic acid (2)

Six commercial lipases from different sources were evaluated for enzymatic amidation with ethanolamine: from the yeasts *Candida rugosa* (CRL), *Candida antarctica* B (CAL B) and *Candida antarctica* lipase B (CAL B immo plus); from the fungus *Rhizomucor miehei* (LIP), *Rhizopus oryzae* lipase (ROL) and *Thermomyces lanuginosus* (TLL).

Several solvents were also assayed: hexane, diisopropyl ether, acetonitrile and acetone. As it was mentioned, the reactions were carried out at 55 °C using E/S: 10 and Nu/S: 5. It was observed that enzyme activity was variable, giving CAL B the most satisfactory results in acetonitrile; CAL B was also active in DIPE and hexane, but to a lesser extent (Fig. 1). LIP in DIPE and hexane has also showed activity but with a lower performance than CAL B showing conversion 53% at 96 h and 46% at 72 h, respectively, whereas with CRL, ROL, TLIM the enzyme activity were very low. In case of CAL B immo plus, no product was observed under the conditions tested. In the absence of biocatalyst no product was detected.

It is important to note the chemoselective behavior of CAL B since the ethanolamide was the only obtained product; the isomeric aminoester was not detected. Under chemical conditions, alkanolamines are susceptible for acylation both at the amino and hydroxy group. These results are in coincidence with studies which report the same chemoselective behavior of lipase, solely affording the amide [20,24,29–31].

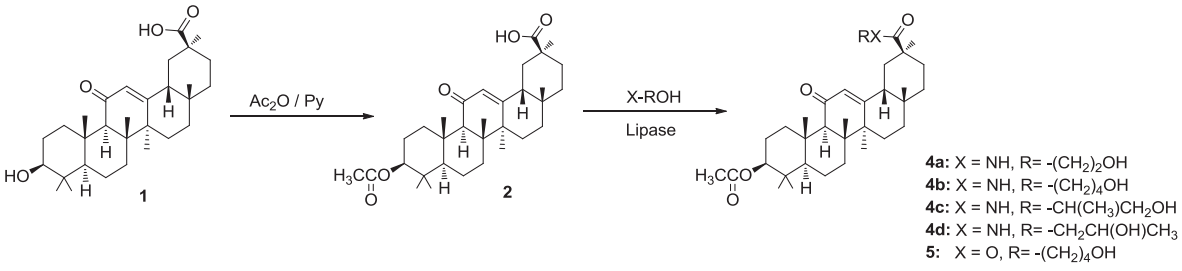
Once determined the appropriate lipase and solvent, the influence of the enzyme:substrate ratio on this reaction was evaluated at 48 h, using a Nu/S ratio of 5, acetonitrile as solvent at 55 °C and variable amounts of CAL B. From the results, it can be concluded that a ratio E/S of 10 is the most acceptable (Fig. 2).

Similarly, the influence of the Nu/S ratio over amidation yield was evaluated at 48 h in acetonitrile using CAL B (E/S = 10) at 55 °C and variable amounts of nucleophile. As it was observed, a ratio Nu/S of 5 is enough to afford the best conversion (Fig. 3).

Finally, we performed the reaction at different temperatures setting the other reaction parameters to their optimal determined values (CAL B, acetonitrile, E/S: 10 and Nu/S: 5). Results showed higher yield with the increase in the temperature. Therefore we selected 55 °C as the best reaction temperature.

Considering the previously mentioned experiments, the determined conditions were applied for enzymatic reactions of **2** with different alkanolamines (**3a-d**) obtaining only the products of amidation (**4a-d**) in very good yields (Table 1).

As it was exhibited, the acetylated glycyrrhetinic acid (**2**) lacked reactivity to both alcohols and amines, whereas amidation reaction



Scheme 1. Chemoenzymatic synthesis of glycyrrhetinic acid derivatives.

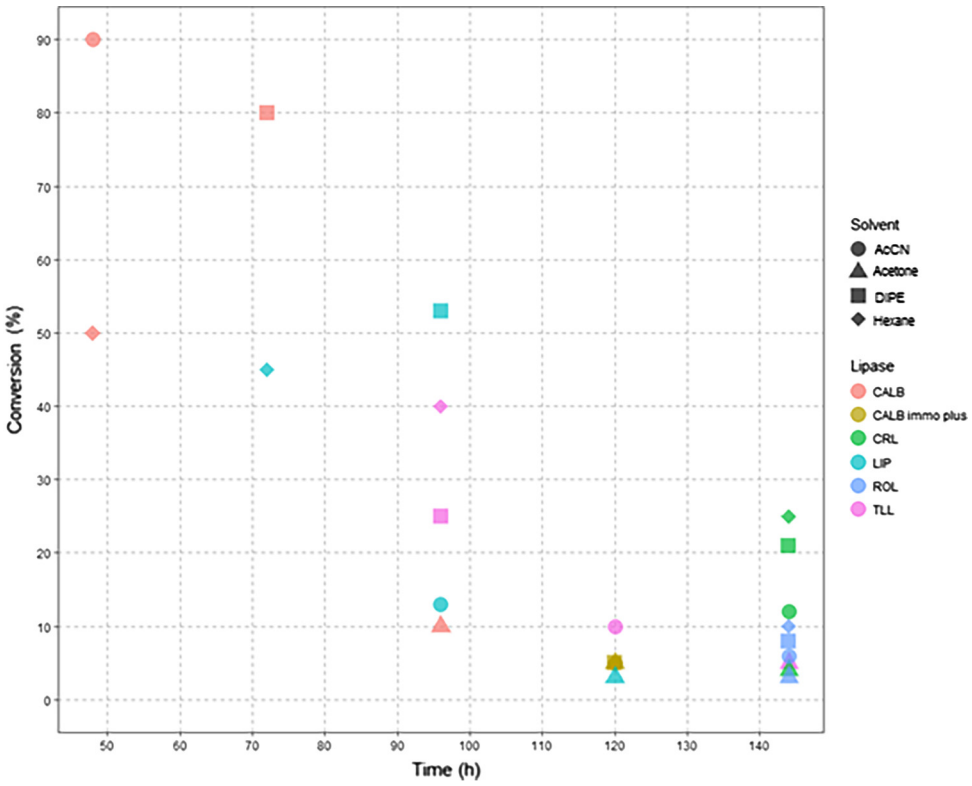


Fig. 1. Screening of biocatalyst and solvent for reaction of **2** with ethanolamine at 55 °C using E/S: 10 and Nu/S: 5.

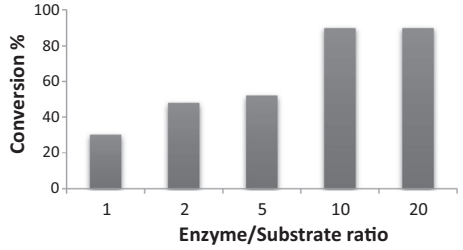


Fig. 2. Influence of E/S ratio on reaction of **2** with ethanolamine. Reaction conditions: Enzyme: CAL B; Solvent: Acetonitrile; Temperature: 55 °C; Nu/S: 5; time: 48 h.

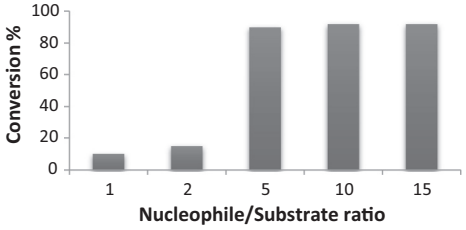


Fig. 3. Influence of Nu/S ratio on reaction of **2** with ethanolamine. Reaction conditions: Enzyme: CAL B; Solvent: Acetonitrile; Temperature: 55 °C; E/S: 10; time: 48 h.

was observed in the case of the reaction with alkanolamines. In view of these experimental results, new questions arose regarding the chemical structure of the nucleophile used. Therefore, it was of interest to study the reaction of **2** with diols and diamines, using 1,4-butanediol and 1,4-diaminobutane as model nucleophiles. The reaction conditions employed were those optimized for the amidation reaction of **2** with ethanolamine: CAL B as biocatalyst, temperature: 55 °C, E/S: 10, Nu/S: 5 and acetonitrile as solvent.

Table 1
Enzymatic synthesis of 3-acetylglycyrrhetinic acid derivatives.

Nucleophile	Product[a]	Yield (%)[b]
$H_2NCH_2CH_2OH$	**4a**	83
$H_2N(CH_2)_4OH$	**4b**	76
$H_2NCH(CH_3)CH_2OH$	**4c**	68
$H_2NCH_2CH(OH)CH_3$	**4d**	73
$HO(CH_2)_4OH$	**5**	86

[a] Reaction conditions: Enzyme: CAL B; Solvent: AcCN; Temperature: 55 °C; E/S: 10; Nu/S: 5; t: 48 h.
[b] Isolated yield after column chromatography.

We observed that in the case of reaction with 1,4-butanediol, the corresponding hydroxyester (**5**) was obtained in 86% yield at 48 h reaction (Table 1). However with 1,4-diaminobutane no product was observed. These results are very interesting and suggest that the presence of an additional hydroxyl group in the nucleophile is essential for the reaction to occur.

2.2. Molecular modeling

In order to comprehend the lack of reactivity of **1** to the studied reactions, as well as the reactivity of **2** to specific nucleophiles, we applied computational analysis. Using molecular modeling we were able to understand the structural and functional relationship between the active site of the lipase and the ligand, giving an idea of the selective biological behavior of the enzyme on this family of compounds. In addition, the differences observed for substrates **1** and **2** and the different reactivity of acetylated glycyrrhetinic acid (**2**) with alcohols, amines, alkanolamines, diols and diamines were explained.

Taking into account the interaction distances with the aminoacids of the catalytic site (Asp187-His224-Ser105), the binding energy and the population of the clusters, we have selected an optimal interaction conformation between the enzyme and each substrate. Initially, we carried out docking between CAL B and substrates **1** and **2**. In the case of compound **2**, an appropriate interaction conformation was chosen, on which a second computational study was carried out to evaluate the different nucleophiles used in the reactions.

Docking evaluations were performed for glycyrrhetinic acid (**1**) and acetylated glycyrrhetinic acid (**2**) generating two hundred conformers for each one using the Lamarckian genetic algorithm. In the case of **1**, a single cluster was obtained, in which the glycyrrhetinic acid get into the active site through the hydroxyl group, located at 2.92 Å from the catalytic serine and having a binding energy of −3.05 kcal/mol (Fig. 4). For compound **2**, four clusters were found; the binding energies and the corresponding frequencies are shown in Fig. 5. The interaction conformations of these clusters in the active site of lipase and its arrangement with respect to catalytic triad are shown in Fig. 6.

According to the results obtained, docking of **1** with CAL B shows that although this substrate is located at a short distance from the active site, it has higher binding energy than **2**. Consequently, the reaction is less favorable. Besides, **1** gets into the active site of the lipase through the hydroxyl group, while **2** enters through the carboxylic acid group, as it is observed on the obtained clusters with binding energies between −7.60 and −6.45 kcal/mol (Fig. 5).

Finally we have evaluated the effect of the different nucleophiles in the reactions with **2**. We have chosen cluster 2, the most populated since it was the most similar to the experimentally acquired conformation. In order to explain the experimental results, a second docking study was carried out between CAL B-cluster 2 and the five tested type of nucleophiles: alcohol (ethanol), amine (butylamine), alkanolamine (ethanolamine), diol (1,4-butanediol) and diamine (1,4-diaminobutane).

Results showed four clusters for ethanol, three clusters for ethanolamine, five clusters for butylamine and a only cluster for 1,4-butanediol and 1,4-diaminobutane. In all cases the most populous cluster corresponds to the lowest energy and corresponds to the interaction conformations of greater similarity to those adopted experimentally.

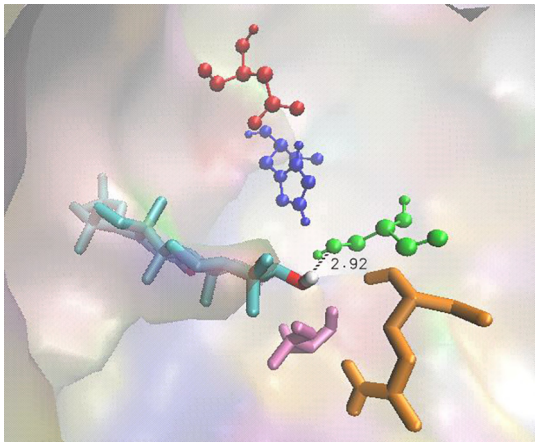


Fig. 4. Docking of glycyrrhetinic acid (**1**) with CAL B. Interaction conformation obtained for the single cluster found at the active site of lipase. Catalytic triad: Serine 105 (green), Histidine 224 (blue), Aspartic acid 187 (red). Oxyanion hollow: Glutamine 106 (brown), Threonine 40 (pink).

It has been observed that in the cases of docking with ethanol, butylamine and 1,4-diaminobutane, nucleophiles are far away from the catalytic serine in the most likely interaction conformation what could explain the lack of reactivity (Fig. 7). When ethanolamine was used as nucleophile, the hydroxyl group of the nucleophile is very close to the oxygen of the carbonyl group of the acylated glycyrrhetinic acid interacting through hydrogen bond. Therefore, the amino group is placed at an appropriate distance to react. This would allow a greater stabilization of the tetrahedral intermediate allowing this nucleophile to approximate enough to the acyl enzyme complex and to react (Fig. 8A). In addition, the same hydrogen bond stabilization occurs in the case of 1,4-butanediol finding both hydroxyl groups of nucleophile very close to carbonyl moiety of **2**, which might explain the observed reactivity (Fig. 8B).

2.3. Biological evaluation

We first evaluated cytotoxicity of GA and compounds **1, 2** and **4a-d** at concentrations ranging from 1 to 200 μM. Each one was dissolved in dimethylsulfoxide (DMSO) and diluted in culture medium. The maximum concentration of DMSO tested (1%) exhibited no cytotoxicity for Vero cells. After 24 h incubation at 37 °C, cell viability was determined (Fig. 9A).

The derivatives showed a CC_{50} value higher than 200 μM, except for compound **4d** which exhibited a CC_{50} value of 190.2 μM (Fig. 8A). All compounds exhibited CC_{50} values higher than that corresponding to GA (61 μM). Hence, all compounds tested were no cytotoxic for Vero cells.

Then, to evaluate a potential antiviral action of all compounds, first we performed a qualitative screening. Compounds **2**, **4a-c** and **5** showed no anti-HSV-1 activity. As expected, 50 μM GA inhibited HSV-1-induced cytopathic effect in a 75% with respect to infected untreated controls, whereas no protection of cellular damage was observed in the presence of 10 μM.[32] Derivative **4d** showed a similar reduction of cytopathic effect at 50 μM and, besides, 10 μM of compound **4d** protected 25% of infected cells, concentration at which GA had already lost the antiviral activity.

To evaluate whether this protection correlated with a decrease in viral infectivity, viral yields belonging to those wells where protection was observed were harvested and titrated in Vero cells. Compound **4d** was able to prevent HSV-1 multiplication when added after infection in a dose-dependent manner, exhibiting an EC_{50} of 4.95 μM. In this case, the selectivity index ($SI = CC_{50}/EC_{50}$) was 38.38 (Fig. 10). Taking into account that compound **4d** was tested as a mixture of the stereoisomers, we performed the racemic resolution of (±)-1-amino-2-propanol.[33,34]

Once the two pure enantiomers of (±)-1-amino-2-propanol were obtained, the reaction of **2** with (*S*)-(+)-1-amino-2-propanol and (*R*)-(-)-1-amino-2-propanol was carried out. The corresponding stereoisomeric alkanolamides, which denominated **4d**(*S*) and **4d**(*R*), were obtained with 75% yield.

In order to investigate if the antiviral activity would be ascribed to one of the two stereoisomers of the molecule, after Vero cell infection with HSV-1 (m.o.i. = 0.1), different concentrations of the stereoisomeric mixture, **4d**(*S*) and **4d**(*R*) were added and, after 24 h incubation at 37 °C, supernatants were titrated in Vero cells. As expected, the stereoisomeric mixture followed a dose–response curve with a EC_{50} of 6.2 μM (Fig. 10A). Noteworthy, **4d**(*R*) slightly restrained viral replication starting from a concentration of 25 μM, while **4d**(*S*) was the stereoisomer that gathered most of the anti-HSV-1 activity, with an EC_{50} value of 1.92 μM and a SI > 104. 19. None of both stereoisomers were cytotoxic for Vero cells (Fig. 10B).

ACV and related nucleoside analogues can successfully mitigate HSV infections, but the emergence of HSV-1 populations resistant

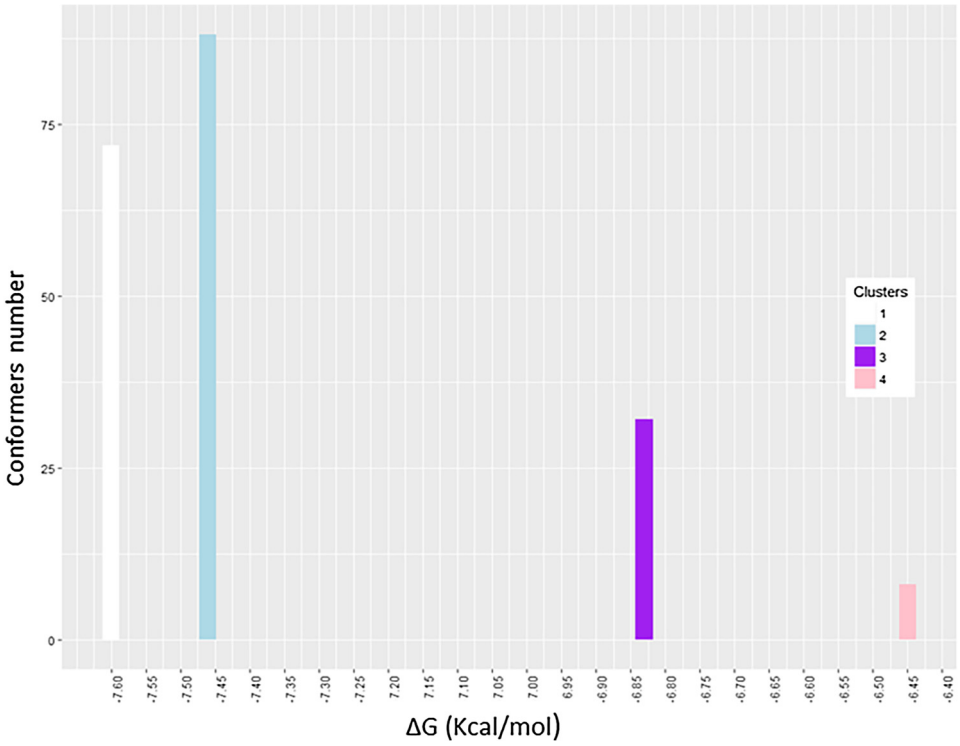


Fig. 5. Histogram corresponding to the docking of acetylated glycyrrhetinic acid (**2**) with CAL B.

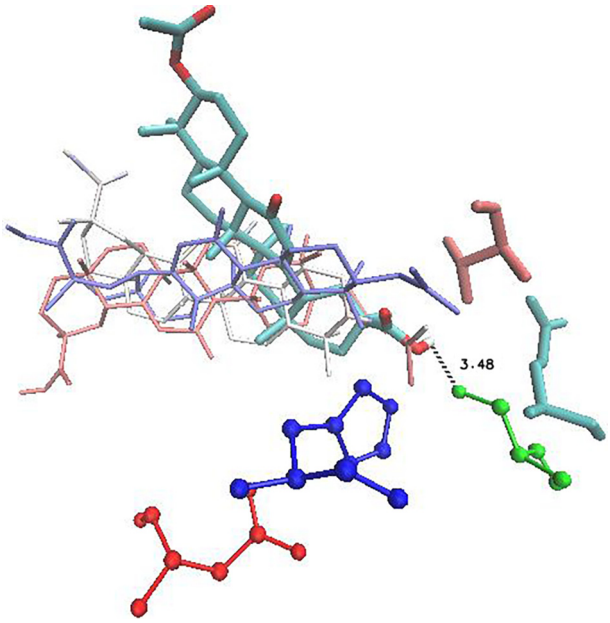


Fig. 6. Docking of acetylated glycyrrhetinic acid (**2**) with CAL B. Conformation interaction acquired for each cluster at the active site of lipase. Cluster 1 (white); cluster 2 (light blue); cluster 3 (violet); cluster 4 (pink). Catalytic triad: Serine 105 (green), Histidine 224 (blue), Aspartic acid 187 (red). Oxyanion hollow: Glutamine 106 (light blue), Threonine 40 (pink).

to ACV has created a barrier for treatment, especially in the case of immunocompromised patients. Thus, we evaluated the antiviral action of the stereoisomeric mixture and the two stereoisomers against HSV-1 ACV-resistant strain (B2006). We observed the same profiles of dose-dependent inhibition, with an EC_{50} of 8.67 μM for compound **4d** and 9.89 μM for **4d**(*S*), and a slight inhibition for the **4d**(*R*) (Fig. 10B).

3. Materials and methods

3.1. General

Solvents and Chemicals were purchased from Merck Argentina and Sigma-Aldrich 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), Lipase from *Rhizomucor miehei* (LIP): Lipozyme® RM 1 M, Novozymes (7800 U/g), *Candida antarctica* lipase B (CAL B immo plus): Purolite (9550 PLU/g) and lipase from *Thermomyces lanuginosus* (TLL): Sigma Aldrich (3000 U/g) were generous gifts of Novozymes Spain; lipase from *Rhizopus oryzae* (ROL) is a heterologous lipase, which was produced by Chemical Engineering Department of Barcelona Autonomous University; Carica papaya lipase (CPL) is the remaining solid fraction of papaya latex, after wash off of proteases using distilled water. CPL is a naturally immobilized enzyme and was a generous gift of Dr. Georgina Sandoval, CIATEJ, Mexico. E/S: enzyme amount in mg/substrate amount in mg. Enzymatic reactions were carried out on MaxQ 4000 Thermo Scientific Co. digital incubator shaker, at the corresponding temperature and 200 rpm. To monitor the reaction progress aliquots were withdrawn and analyzed by TLC performed on commercial 0.2 mm aluminum-coated silica gel plates (F254) and visualized by immersion in an aqueous solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.04 M), $Ce(SO_4)_2$ (0.003 M) in concentrated H_2SO_4 (10%).% Conversion was determined using a Waters 1515 HPLC equipped with an isocratic pump, manual injector and Waters 2414 refractive index detector was used. The reactions were monitored employing a C-18 Phenomenex Phenogel column 5 μM 10E5A, 300 x 7.8 mm and eluting with AcCN:H_2O 60:40-acetic acid 1% 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. ^1H NMR and ^{13}C NMR spectra were

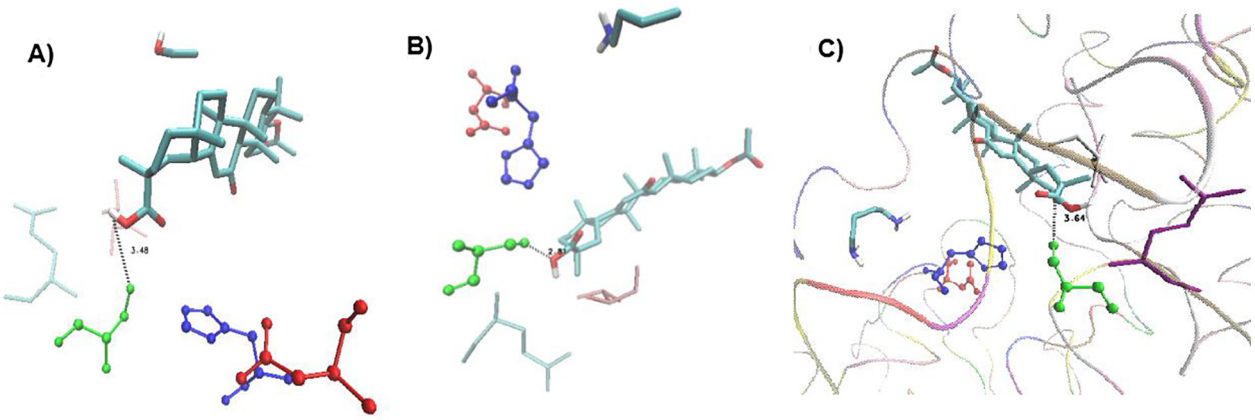


Fig. 7. Docking between **2** (conformation 2) and: (A) ethanol, (B) butylamine, (C) 1,4-diaminobutane.

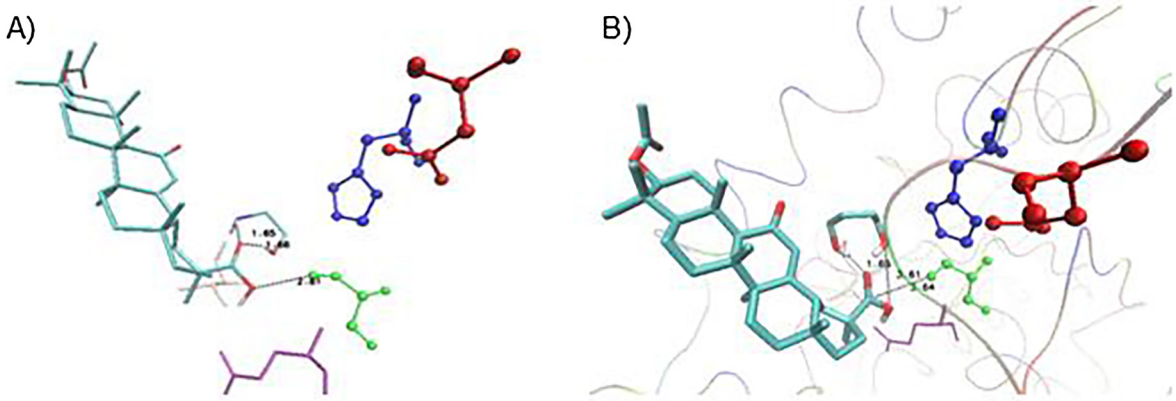


Fig. 8. Docking between **2** (conformation 2) and: A) ethanolamine, B) 1,4-butanediol.

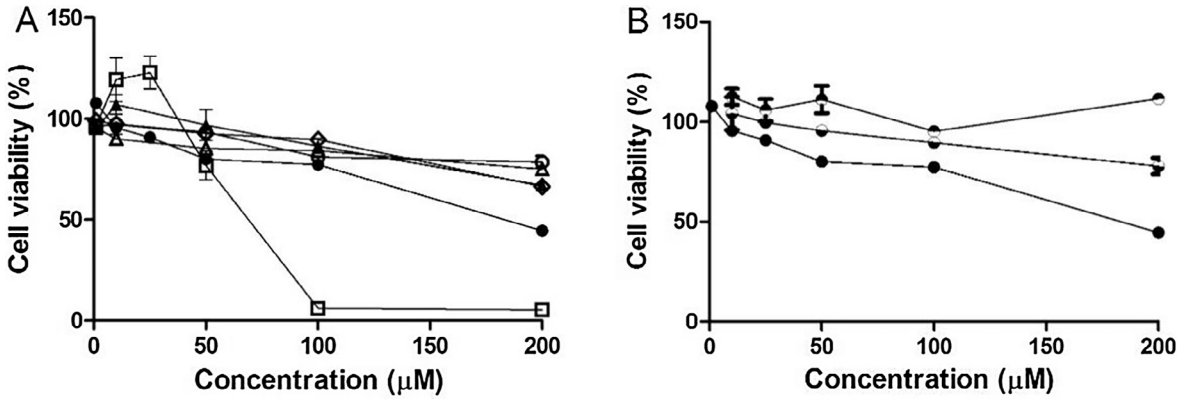


Fig. 9. Cytotoxicity of: **(A)** glycyrrhetinic acid (GA) (□) and compounds **2** (△), **4a** (○), **4b** (▲), **4c** (◇) and **4d** (●); **(B)** stereoisomers of **4d: 4d**(***S***) (◒) and **4d**(***R***) (◓). Vero cells were grown in 96-well cell plates and treated or not with the compounds (1–200 μM). After 24 h incubation at 37 °C, cell viability was measured by means of a MTT colorimetric assay.

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 ^1H and ^{13}C respectively. The ^1H 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. High Resolution Mass Spectrometry was recorded with Thermo Scientific EM/DSQ II – DIP. The results were within ±0.02% of the theoretical values.

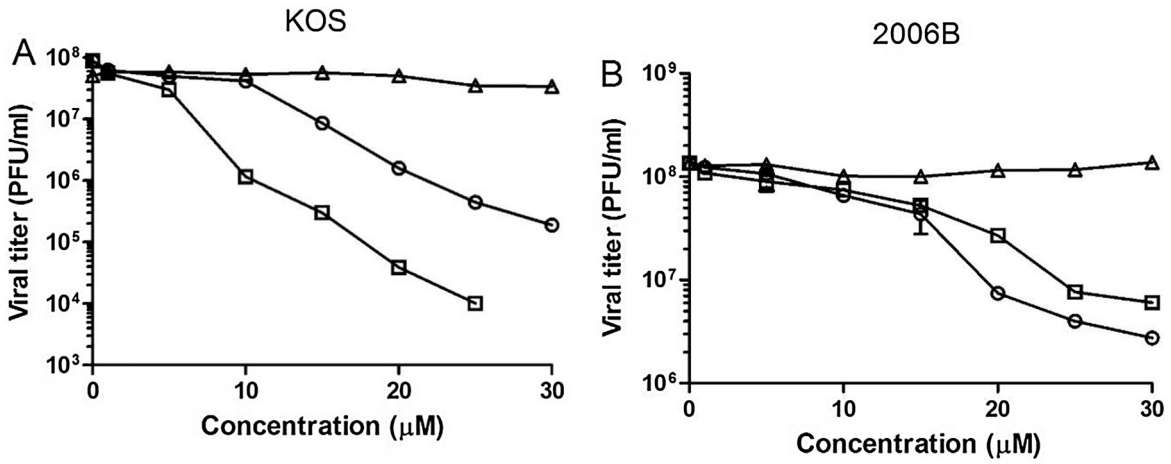


Fig. 10. Antiviral activity of compounds **4d** (○), **4d**(*S*) (□) and **4d**(*R*) (△). Vero cells grown in 96-well plates were infected with a m.o.i = 0.1 of HSV-1 KOS strain (**A**) and HSV-1 B2006 strain (**B**), and treated or not with the three compounds. After 24 h of incubation at 37 °C, virus was collected and titrated in Vero cells.

3.2. Chemical acetylation

In a typical procedure, glycyrrhetinic acid (100 mg) was heated at room temperature with acetic anhydride (2 mL) and pyridine (1 mL) for 16 h. 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 (MgSO4), and the solvent was evaporated. The residue was purified by column chromatography (silica gel) employing mixtures of hexane/EtOAc as eluent (7:3–1:9). Yield 98%.

3.3. Enzymatic reaction with alkanolamines. General procedure

To a solution of 3-acetylglycyrrhetinic acid (**2**) (50 mg) in acetonitrile (5 mL), CAL B (500 mg) and the corresponding alkanolamine (1.2 eq) was added. The mixture was shaken at 200 rpm and 55 °C. Once the reaction was finished (48 h), the enzyme was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography (silica gel) employing mixtures of hexane-EtOAc as eluent (4:1–1:4). Reuse experiments: the filtered and washed enzyme was used in the next enzymatic esterification under the same reaction conditions.

3.3.1. 3-Acetylglycyrrhetinic acid (*2*)

Yield 98% of pure compound as a white solid; mp 311–312 °C (Ref. [28]: 310–313 °C); $[\alpha]_D^{25}$ +162.8° (*c* 1.0, $CHCl_3$) (Ref. [28]: $[\alpha]_D^{25}$ + 163.3° (*c* 1.0, $CHCl_3$)). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.70 (s, 1H, H-12), 4.52 (dd, *J* = 4.6, 11.7 Hz, 1H, H-3), 2.80 (dt, *J* = 3.8, 13.8 Hz, 1H, H-1a), 2.37 (s, 1H, H-9), 2.18 (dd, *J* = 3.4, 12.8 Hz, 1H, H-18), 2.05 (s, 3H, —OCOC*H*$_3$), 1.37 (s, 3H, CH_3-29), 1.23 (s, 3H, CH_3-26), 1.16 (s, 3H, CH_3-25), 1.13 (s, 3H, CH_3-28), 0.88 (s, 6H, CH_3-23, CH_3-24), 0.83 (s, 3H, CH_3-27); ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 200.7 (C-11), 179.2 (C-30), 171.4 (C-31), 170.4 (C-13), 128.0 (C-12), 80.8 (C-3), 61.6 (C-9), 54.8 (C-5), 48.2 (C-18), 45.4 (C-20), 43.6 (C-14), 43.1 (C-8), 41.0 (C-19), 38.6 (C-10), 37.9 (C-1), 37.6 (C-4), 36.8 (C-22), 32.5 (C-7), 31.7 (C-17), 30.9 (C-21), 28.3 (C-16), 28.2 (C-15), 27.9 (C-29), 26.3 (C-27), 26.2 (C-2), 23.4–23-1 (C-23, C-24), 21.1 (C-32), 18.5 (C-28), 17.2 (C-6), 16.5 (C-26), 16.3 (C-25). HRMS: $[M + Na]^+$ Calcd. $C_{32}H_{48}NaO_5$ 535.3399. Found: $C_{32}H_{48}NaO_5$ 535.3405.

3.3.2. N-(3-acetylglycyrrhetinoyl)ethanolamine (*4a*)

Yield 83% of pure compound as a white solid; mp 255–256 °C; $[\alpha]_D^{25}$ 158.8 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.61 (s, 1H, H-12), 4.46 (dd, *J* = 4.8, 11.7 Hz, 1H, H-3), 3.61 (m, 3H, -HNCH_aH$_b$CH_2OH), 3.40 (dt, *J* = 5.5, 14.0 Hz, 1H, —HNCH$_a$H$_b$CH$_2$-OH), 2.72 (dt, *J* = 3.8, 13.8 Hz, 1H, H-1a), 2.33 (s, 1H, H-9), 2.15 (dd, *J* = 3.5, 12.6 Hz, 1H, H-18), 2.01 (s, 3H, —OCOC*H*$_3$), 1.33 (s, 3H, CH_3-29), 1.13 (s, 3H, CH_3-26), 1.11 (s, 3H, CH_3-25), 1.08 (s, 3H, CH_3-28), 0.83 (s, 6H, (CH_3-23, CH_3-24), 0.77 (s, 3H, CH_3-27); ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 200.7 (C-11), 172.2 (C-30), 171.1 (C-31), 170.6 (C-13), 127.7(C-12), 80.7 (C-3), 62.3 (—HNCH$_2$CH_2-OH), 61.8 (C-9), 55.1 (C-5), 48.9 (C-18), 45.6 (C-20), 43.5 (C-14), 42.4 (C-8), 40.4 (C-19), 39.5 (—HNCH_2CH$_2$OH), 38.9 (C-10), 38.3 (C-1), 38.1 (C-4), 37.1 (C-22), 32.8 (C-7), 31.9 (C-17), 30.4 (C-21), 28.9 (C-16), 28.5 (C-15), 28.1 (C-29), 26.6 (C-27), 26.3 (C-2), 23.6 (C-23), 23.4 (C-24), 21.4 (C-32), 18.5 (C-28), 17.4 (C-6), 16.8 (C-26), 16.5 (C-25). HRMS: $[M + Na]^+$ Calcd. $C_{34}H_{53}NNaO_5$ 578.3821 Found: $C_{34}H_{53}NNaO_5$ 578.3817.

3.3.3. N-(3-acetylglycyrrhetinoyl)butanolamine (*4b*)

Yield 76.0% of pure compound as a white solid; mp > 300 °C; $[\alpha]_D^{25}$ +160.3° (c 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.65 (s, 1H, H-12), 4.49 (dd, *J* = 4.6, 11.7 Hz, 1H, H-3), 3.61 (t, *J* = 5.9 Hz, 3H, -HNCH_aH$_b$(CH$_2$)$_2$CH_2OH), 3.26 (q, *J* = 5.9 Hz, 1H, —HNCH$_a$H$_b$(CH$_2$)$_2$CH$_2$OH), 2.75 (dt, *J* = 3.8, 13.6 Hz, 1H, H-1a), 2.36 (s, 1H, H-9), 2.22 (dd, *J* = 3.0, 12.0 Hz, 1H, H-18), 2.04 (s, 3H, –OCOC*H*$_3$), 1.35 (s, 3H, CH_3-29), 1.13 (s, 3H, CH_3-26), 1.10 (s, 3H, CH_3-25), 1.07 (s, 3H, CH_3-28), 0.86 (s, 6H, s, CH_3-23, CH_3-24), 0.77 (s, 3H, CH_3-27); ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 201.1 (C-11), 172.1 (C-30), 171.0 (C-31), 170.4 (C-13), 127.6 (C-12), 80.6 (C-3), 62.1 (—HN(CH$_2$)$_3$CH_2OH), 61.7 (C-9), 55.0 (C-5), 48.8 (C-18), 45.5 (C-20), 43.3 (C-14), 42.3 (C-8), 40.2 (C-19), 39.3 (HNCH$_2$(CH$_2$)$_3$OH), 38.8 (C-10), 38.2 (C-1), 38.0 (C-4), 37.0 (C-22), 32.7 (C-7), 31.8 (C-17), 30.3 (C-21), 29.7 (—HNCH$_2$CH$_2$CH_2CH$_2$OH), 29.1 (C-15), 28.8 (C-16), 28.0 (C-29), 26.5 (C-27), 26.4 (C-2), 26.1 (—HNCH$_2$CH_2-CH$_2$CH$_2$OH), 23.5 (C-23), 23.2 (C-24), 21.3 (C-32), 18.4 (C-28), 17.3 (C-6), 16.7 (C-26), 16.4 (C-25). HRMS: $[M + Na]^+$ Calcd. $C_{36}H_{57}NNaO_5$ 606.4134 Found: $C_{36}H_{57}NNaO_5$ 606.4130.

3.3.4. N-(3-acetylglycyrrhetinoyl)-1-amino-2-propanol (*4c*)

Yield 68%; white solid, mp 249–251 °C; $[\alpha]_D^{25}$11.323(*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.65 (s, 1H, H-12), 4.50 (dd, *J* = 4.6, 11.7 Hz, 1H, H-3), 3.49–3.58 (m, 3H, CH$_3$C*H*(NH)C*H*$_2$OH—), 2.76 (dt, *J* = 3.7, 13.6 Hz, 1H, H-1a), 2.35 (s, 1H, H-9), 2.18 (d, *J* = 4.6, 12.3, 1H, Hz, H-18), 2.03 (s, 3H, —OCOC*H*$_3$), 1.35 (s, 3H,

CH_3-29), 1.23 (s, 3H, CH_3-26), 1.13 (s, 3H, CH_3-25), 1.11 (d, J = 7.1 Hz, 3H, $CH_3CH(CH_2OH)NH$—), 0.86 (s, 6H, CH_3-23, CH_3-24), 0.78 (s, 3H, CH_3-27); ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 200.8 (C-11), 181.8 (C-30), 177.1 (C-31), 171.1 (C-13), 127.9 (C-12), 80.6 (C-3), 66.5 ($CH_3CH(CH_2OH)NH$—), 61.7 (C-9), 54.9 (C-5), 48.5 (C-18), 45.4 (C-20), 44.2 ($CH_3CH(CH_2OH)NH$–), 43.3 (C-8, C-14), 41.7 (C-19), 38.7 (C-10), 38.0 (C-1), 37.9 (C-4), 36.9 (C-22), 32.6 (C-7), 31.8 (C-17), 31.4 (C-21), 29.7 28.7 (C-16), 28.6 (C-15), 28.0 (C-29), 26.5 (C-27), 26.4 (C-2), 23.5 (C-23), 23.2 (C-24), 22.6 ($CH_3CH(CH_2OH)NH$—), 21.3 (C-32), 18.6 (C-28), 17.3 (C-6), 16.6 (C-26), 16.4 (C-25). HRMS: $[M + Na]^+$ $C_{36}H_{57}NNaO_5$ 606.4134 Found: $C_{36}H_{57}NNaO_5$ 606.4139.

3.3.5. N-(3-acetylglycyrrhetinoyl)-2-amino-1-propanol (**4d**)

Yield 73%; white solid, mp 253–254 °C; $[\alpha]_D^{25}$ 15.135 (c 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$, 500 MHz) δ 5.69 (s, 1H, H-12), 4.51 (dd, J = 4.6, 11.7 Hz, 1H, H-3), 3.92 (m, 2H, $CH_3CH(OH)CH_aH_bNH$-), 3.46 (dd, J = 3.0, 1H, 6.7 Hz, $CH_3CH(OH)CH_aH_bNH$-), 2.80 (dt, J = 3.7, 13.6 Hz, 1H, H-1a), 2.36 (s, 1H, H-9), 2.18 (dd, J = 3.4, 12.2 Hz, 1H, H-18), 2.05 (s, 3H, —$OCOCH_3$), 1.36 (s, 3H, CH_3-29), 1.21 (s, 3H, CH_3-26), 1.16 (s, 3H, CH_3-25), 1.13 (d, J = 7.1 Hz, 3H, $CH_3CH(CH_2OH)NH$—), 0.88 (s, 6H, CH_3-23, CH_3-24), 0.82 (s, 3H, CH_3-27); ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 201.1 (C-11), 183.4 (C-30), 172.1 (C-31), 171.5 (C-13), 127.3 (C-12), 80.8 (C-3), 66.0 ($CH_3CH(OH)CH_2NH$—), 61.5 (C-9), 54.7 (C-5), 48.6 (C-18), 46.6 ($CH_3CH(OH)CH_2NH$—), 45.3 (C-20), 44.7 (C-14), 43.2 (C-8), 42.0 (C-19), 38.5 (C-10), 37.9 (C-1), 37.8 (C-4), 36.7 (C-22), 32.4 (C-7), 31.6 (C-17), 31.4 (C-21), 28.7 (C-16), 28.4 (C-15), 27.7 (C-29), 26.3 (C-27), 26.2 (C-2), 23.2 (C-23), 23.0 (C-24), 21.3 (C-32), 20.1 ($CH_3CH(OH)CH_2NH$—), 18.4 (C-28), 17.1 (C-6), 16.3 (C-26), 16.1 (C-25). HRMS: $[M + Na]^+$ $C_{36}H_{57}NNaO_5$ 606.4134 Found: $C_{36}H_{57}NNaO_5$ 606.4128.

3.3.6. N-(3-acetylglycyrrhetinoyl)-4-hydroxy-1-butanol (**5**)

Yield 86%; white solid, mp 293 °C; $[\alpha]_D^{25}$ + 152.3° (c 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$, 500 MHz) δ 5.69 (s, 1H, H-12), 4.52 (dd, J = 4.6, 11.7 Hz, 1H, H-3), 4.18 (t, J = 6.4 Hz, 2H, —$COOCH_2(CH_2)_2CH_2OH$), 3.72 (t, J = 6.5 Hz, 2H, —$COOCH_2(CH_2)_2CH_2OH$), 2.79 (dt, J = 3.5, 13.6 Hz, 1H, H-1a), 2.36 (s, 1H, H-9), 2.18 (dd, J = 3.0, 12.0 Hz, 1H, H-18), 2.05 (s, 3H, —$OCOCH_3$), 1.37 (s, 3H, CH_3-29), 1.22 (s, 3H, CH_3-26), 1.16 (s, 3H, CH_3-25), 1.13 (s, 3H, CH_3-28), 0.88 (s, 6H, s, CH_3-23, CH_3-24), 0.83 (s, 3H, CH_3-27); ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 200.1 (C-11), 174.7 (C-30), 171.0 (C-31), 169.2 (C-13), 128.2 (C-12), 80.6 (C-3), 64.7 (—$COOCH_2(CH_2)_2CH_2OH$), 62.5 (—$COOCH_2(CH_2)_2CH_2OH$), 61.7 (C-9), 55.0 (C-5), 48.2 (C-18), 45.4 (C-20), 43.7 (C-14), 43.2 (C-8), 40.9 (C-19), 38.8 (C-10), 38.1 (C-1), 37.7 (C-4), 36.9 (C-22), 32.7 (C-7), 31.9 (C-17), 31.8 (—$COOCH_2CH_2CH_2CH_2OH$), 31.0 (C-21), 29.6 (—$COOCH_2CH_2CH_2CH_2OH$), 28.5 (C-16), 28.4 (C-15), 28.1 (C-29), 26.5 (C-27), 26.4 (C-2), 23.6 (C-23), 23.4 (C-24), 21.3 (C-32), 18.7 (C-28), 17.4 (C-6), 16.7 (C-26), 16.4 (C-25). HRMS: $[M + Na]^+$ Calcd. $C_{36}H_{56}NaO_6$ 607.3975 Found: $C_{36}H_{56}NaO_6$ 607.3981.

3.4. 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 [35]. The Autodock 4.2 method was applied considering all the rotatable bonds for substrates **1** and **2,** while all the protein 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.

3.5. Drug screening

3.5.1. Cytotoxicity assay

The cell viability was determined by using the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide). The absorbance of each well was measured on a Eurogenetics MPR-A 4i microplate reader. Results were expressed as a percentage of absorbance of treated cells with respect to untreated ones. The CC_{50} was defined as the concentration of compound that caused 50% reduction in cell viability.

3.5.2. Antiviral qualitative screening

Vero cells grown in 96-well plates were infected with HSV-1 (KOS strain) with a multiplicity of infection (m.o.i) of 0.1 and, after virus adsorption, cells were treated or not with different concentrations of the synthesized derivatives, in a range where no cytotoxicity was observed. After 24 h of incubation at 37 °C in 5% CO_2 atmosphere, cytopathic effect was evaluated under microscope and the protective effect of each compound was established as a percentage with respect to infected untreated cells.

3.5.3. Viral titration

Vero cells grown in 24-well plates were infected with serial 10-fold dilutions of viral yields and incubated for 1 h at 37 °C. Residual inocula were replaced by maintenance medium with 0.7% of methylcellulose. After 72 h of incubation at 37 °C, cells were fixed with formaldehyde 10%, stained with Crystal Violet, and plaque forming units were counted. The EC_{50} was defined as the concentration of compound that caused 50% reduction in viral yield.

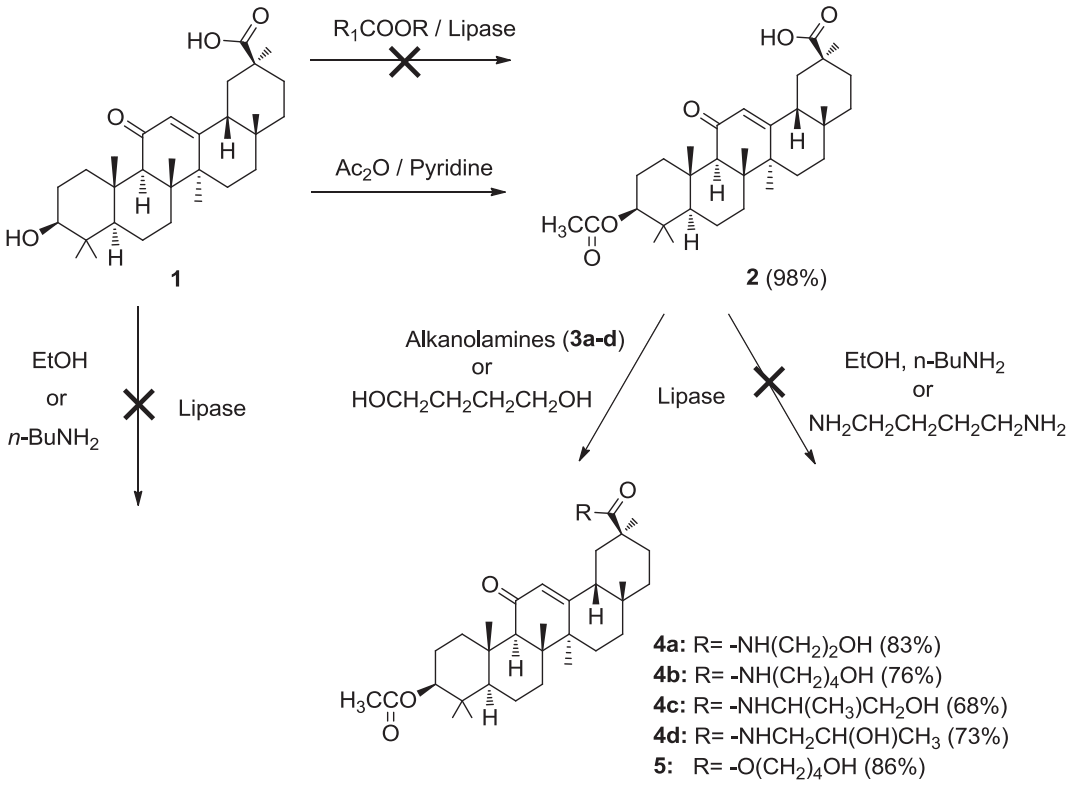
4. Conclusions

In this work we described the chemoenzymatic synthesis of new derivatives of glycyrrhetinic acid. Scheme 2 shows the performed reactions and the obtained products. The CAL B lipase showed selective activity catalyzing the synthesis of alkanolamides (**4**) and hydroxyesters (**5**) only from 3-acetyl glycyrrhetinic acid (**2**), which was previously obtained through a chemical method. Lipase catalysis was not observed in the reaction of neither glycyrrhetinic acid (**1**) with any nucleophile nor **2** with alcohols, amines and diamines. The enzymatic approach shows interesting advantages: the reaction is simple, it is worked at low temperature, the products are easily isolated by filtration and solvent evaporation and shows low environmental impact.

In order to explain the experimental results, molecular modeling studies were also carried out. We have determined that the presence of an additional hydroxyl group in the nucleophile is essential to reactivity. The hydrogen bond interaction between the nucleophile and the acyl enzyme complex favors the reaction as in the case of alkanolamines and diols.

In addition, we have evaluated the effect of the synthesized derivatives as antiviral agents.

There is an urgent need to explore new and effective antiviral drugs. The present work has investigated the antiviral effect of five synthesized GA derivatives. From all compounds assayed, only compound **4d** showed antiviral activity against HSV-1 and, furthermore, against an HSV-1 ACV-resistant strain, in vitro conditions. We observed that most of the activity was present in the **4d(*S*)** estereoisomer, while the **4d(*R*)** estereoisomer alone did not produced a significant effect on viral replication.



Scheme 2. Reactions and products from glycyrrhetinic acid.

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