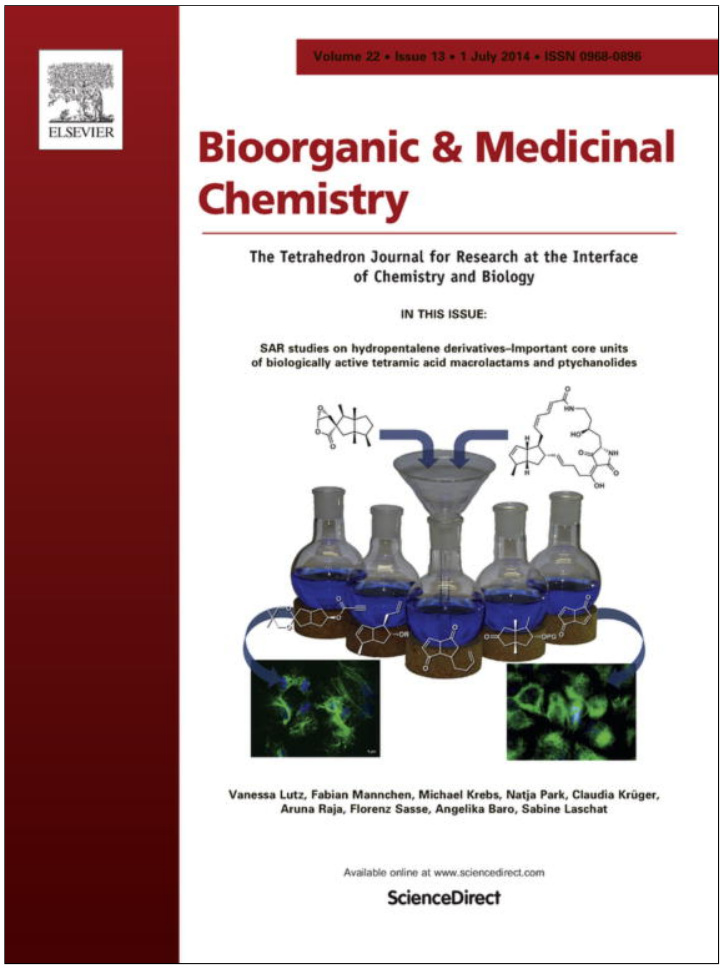

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Preparation, anticholinesterase activity and molecular docking of new lupane derivatives

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ABSTRACT

A set of twenty one lupane derivatives (**2**–**22**) was prepared from the natural triterpenoid calenduladiol (**1**) by transformations on the hydroxyl groups at C-3 and C-16, and also on the isopropenyl moiety. The derivatives were tested for their inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and some structure–activity relationships were outlined with the aid of enzyme kinetic studies and docking modelization. The most active compound resulted to be 3,16,30-trioxolup-20(29)-ene (**22**), with an IC_{50} value of 21.5 μM for butyrylcholinesterase, which revealed a selective inhibitor profile towards this enzyme.

1. Introduction

Triterpenoids are naturally occurring compounds with ubiquitous distribution and a wide range of biological activities.[1–3] Pentacyclic triterpenoids provide privileged structures for further modifications and structure activity relationship (SAR) studies.[4–6] Lupanes in particular, have attracted attention since they exhibit a broad range of biological and pharmacological properties such as antitumor, anti-inflammatory, anti-HIV, anticholinesterase, insecticidal and antimalarial activities.[2,3,7–15]

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with memory impairment and cognitive deficit. It is characterized by low levels of the neurotransmitter acetylcholine (ACh) in the brain of AD patients. The inhibition of acetylcholinesterase (AChE), the enzyme that catalyzes ACh hydrolysis, is the most used therapeutic strategy used to treat AD. AChE inhibitors can alleviate AD symptoms by improving cholinergic functions in AD patients. In the healthy brain, butyrylcholinesterase (BChE), another enzyme, is involved in the metabolic degradation of ACh. BChE activity increases as AD progresses. Therefore, the concurrent inhibition of both AChE and BChE should provide additional benefits in the treatment of AD.[16–19]

Our interest in bioactive triterpenes, prompted us to synthesize a series of derivatives from natural calenduladiol (**1**), isolated from *Chuquiraga erinacea* D. Don. subsp. *erinacea* (Asteraceae).[11–13,20] Calenduladiol (**1**) is a pentacyclic triterpenoid belonging to the lupane type (Fig. 1). In a previous work, we observed the enhancement of the inhibitory activity against AChE of **1** by the introduction of sulfate groups when it was treated with the sulfating reagent $Me_3N{\cdot}SO_3$ and the analogue **2** was obtained.[13] In this paper we report the preparation of 21 lupane derivatives (**2**–**22**) from compound **1** and their ability to inhibit AChE and BChE. Furthermore, we have studied the kinetic of the AChE inhibition for the most active derivative (**10**) and the key binding interactions between this compound and AChE through docking modelization.

2. Results and discussion

2.1. Chemistry

In order to analyze the role of the hydroxyl groups at C-3 and C-16, and also the importance of the isopropenyl moiety in the anticholinesterase activity, we have carried out the transformations shown in Schemes 1 and 2.

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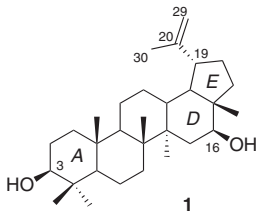


Figure 1. Structure of calenduladiol (**1**).

The starting calenduladiol **1**, was obtained in good yield from the ethanolic extract of *C. erinacea* subsp. *erinacea*, as previously reported.[13] Allylic oxidation of **1** with 2.5 equiv of SeO_2 afforded the corresponding α,β-unsaturated aldehyde **3** in very good yield while the treatment of **1** with a 0.5 equiv of SeO_2 rendered the allylic alcohol **4** as the major product. Reduction of the double bond of **1** was carried out by catalytic hydrogenation yielding the derivative **5** (Scheme 1).

Treatment of diols **1**, **3** and **5** with 8 equiv of trimethylamine–sulfur trioxide complex ($Me_3N{\cdot}SO_3$) for 7 min at 150 °C under MW irradiation afforded the corresponding ammonium sulfates, which were transformed via ion exchange into the disodium salts **2**, **7** and **10**, respectively (Scheme 1). The ^1H and ^{13}C NMR spectra of compounds **2**, **7**, and **10**, confirmed that sulfate groups were located at C-3 and C-16. Resonances showing H-3α at δ_H 3.91 ppm (dd, J = 4.2, 11.4 Hz) and H-16α at δ_H 4.32 ppm (t, J = 8.0 Hz) for compound **2**, H-3α at δ_H 3.92 ppm (dd, J = 4.3, 11.5 Hz) and H-16α at δ_H 4.49 ppm (t, J = 7.8 Hz) for compound **7** and H-3α at δ_H 3.93 ppm (dd, J = 4.2, 11.5 Hz) and H-16α at δ_H 4.30 ppm (dd, J = 6.2, 9.7 Hz) for compound **10** were characteristic of the presence of two sulfate groups at C-3 and C-16, both of them in β orientation. This was in accordance with the chemical shifts observed for C-3 (δ_C 87.6 ppm (**2**), 87.9 ppm (**7**) and 87.8 ppm (**10**)) and C-16 (δ_C 85.8 ppm (**2**), 85.9 ppm (**7**) and 86.3 ppm (**10**)) which were unequivocally assigned from the HSQC and HMBC spectra.

Compounds **1** and **2** were converted into the epoxy derivatives **6** and **11**, respectively, by treatment with MCPBA. The 20-(*S*) configuration of these compounds was assigned on the basis of our previous work with similar 20,29-epoxylupanes using VCD.[12]

The trisulfated derivative **8** was obtained by sulfation of triol **4** with $Me_3N{\cdot}SO_3$ (12 equiv) (Scheme 1). The downfield shift of 8.5 ppm for C-3, 8.4 ppm for C-16 and 6 ppm for C-30 in the ^{13}C NMR spectrum of compound **8**, compared to compound **4**, confirmed that the sulfate groups were located at C-3, C-16 and C-30. Considering that this synthetic route gave an overall yield of 4%, from compound **1** in two steps, we decided to try an alternative path by the reduction of aldehyde **7**. Although this route involved four steps, the overall yield was 22% from **1**. Intermediate alcohol **9** was obtained by treatment of **7** with $NaBH_4$ and $EuCl_3$ without affecting sulfate groups attached to C-3 and C-16.[21] A subsequent reaction of **9** with $Me_3N{\cdot}SO_3$ (4 equiv) rendered the trisulfated **8** (Scheme 1).

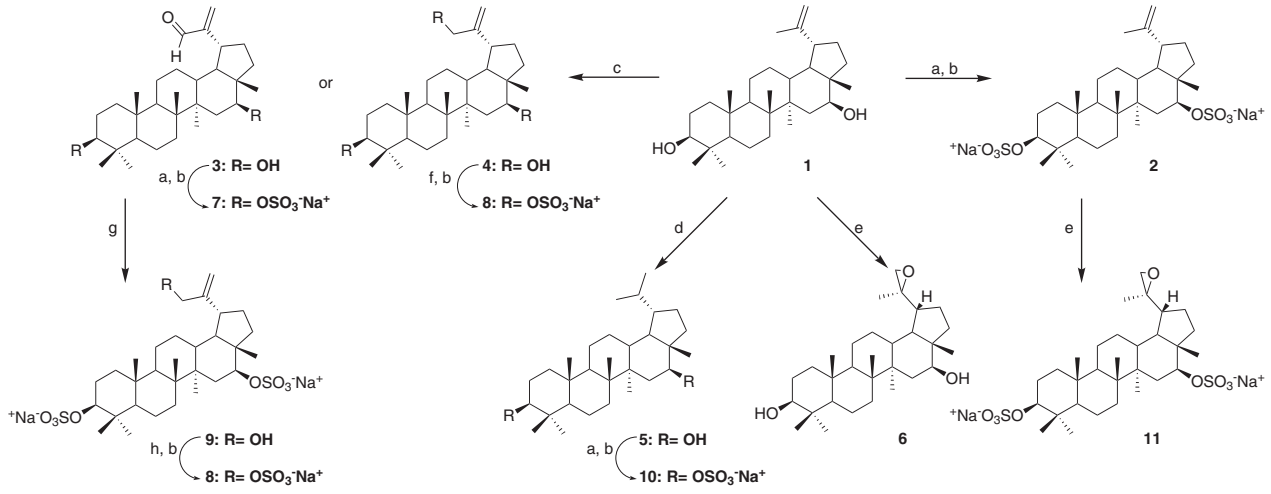
Diols **1** and **3** were treated with an excess of acetic anhydride in pyridine to yield the diacetylated compounds **12** and **15**, respectively, while the triacetylated compound (**16**) was obtained from triol **4** using the same reaction conditions (Scheme 2). The ^1H and ^{13}C NMR spectra of compounds **12**, **15**, and **16**, confirmed that two acetoxy groups were located at C-3 and C-16, both of them with a β orientation. ^1H and ^{13}C signals were unequivocally assigned with the analyses of HSQC and HMBC correlations.

When **1** was treated with 1 equiv of acetic anhydride, a 1:1 mixture of the monoacetates **13** and **14** was obtained. Both monoacetylated derivatives were separated and purified by flash chromatography. The spectroscopic data of **13** revealed that the 16β hydroxyl group remained free while the acetoxy group was attached to C-3. ^1H and ^{13}C NMR data of compound **14** confirmed that, in this case, the acetoxy group was attached to C-16.

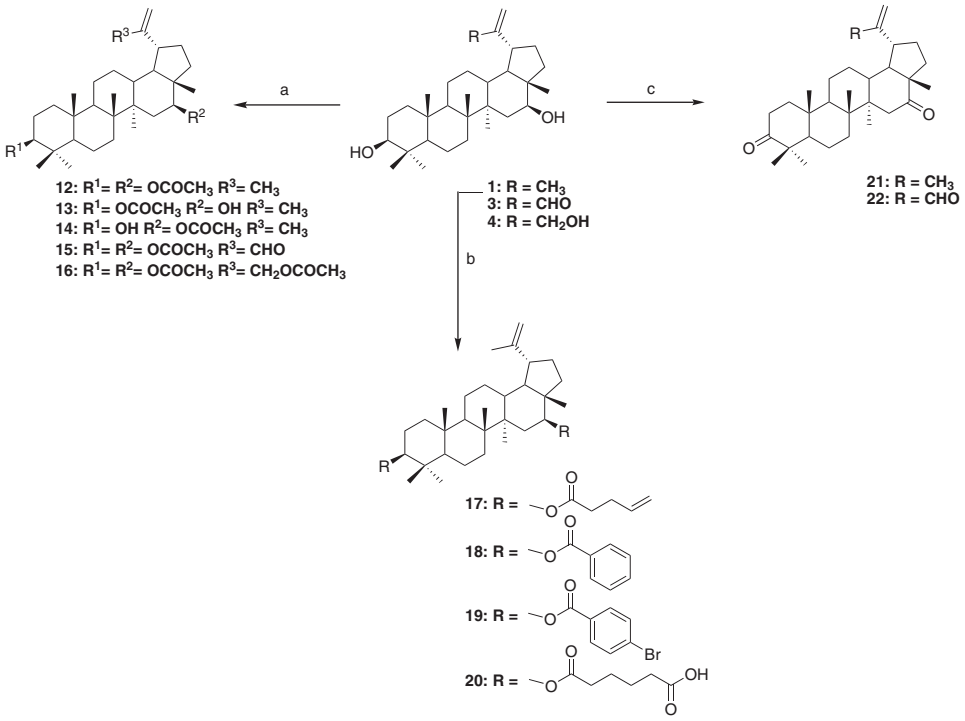
Treatment of diol **1** with the corresponding acyl chlorides in pyridine and DMAP, afforded the esters **17**, **18** and **19**. The diacid **20** was prepared by reaction of **1** with adipoyl chloride and subsequent hydrolysis of the intermediate acyl chloride (Scheme 2). Finally, diketones **21** and **22** were prepared from **1** and **3**, respectively, by oxidation with Jones reagent in acetone.

2.2. In vitro inhibition studies on AChE and BChE

The AChE inhibitory activity of compounds **3**–**22** was evaluated and compared to that of natural triterpenoid **1** and analogue **2**, previously prepared by our group.[13] The AChE inhibition was determined by Ellman's method with eserine and tacrine as reference compounds.[22] In a preliminary assay the inhibition percentage at a fixed concentration was determined for all the derivatives. Compounds **3**–**6**, **8**–**11**, **20** and **22** showed better inhibition than **1**, under the same experimental conditions. The concentration required for 50% AChE inhibition (IC_{50}) was then determined for those compounds. The results for AChE inhibition are summarized in Table 1.



Scheme 1. Preparation of compounds **2**–**11**. Reagents and conditions: (a) 8 equiv $Me_3N{\cdot}SO_3$, DMF, MW, 7 min, 150 °C; (b) Amberlite CG-120 (MeOH); (c) SeO_2, EtOH, reflux; (d) H_2, Pd/C, EtOAc; (e) MCPBA, $NaHCO_3$, DCM; (f) 12 equiv $Me_3N{\cdot}SO_3$, DMF, MW, 9 min, 150 °C; (g) $NaBH_4$, $EuCl_3$, MeOH; (h) 4 equiv $Me_3N{\cdot}SO_3$, DMF, MW, 7 min, 150 °C.



Scheme 2. Preparation of compounds **12**–**22**. Reagents and conditions: (a) Ac_2O, Py, DMAP, DCM; (b) RCOCl or ArCOCl, Py, DMAP, DCM; (c) Jones reagent, acetone.

Table 1
Inhibition of AChE and BChE activity and selectivity index

Compounds	AChE[a]		BChE[b]		Selectivity index[c]
	% Inhibition at 200 μM	IC_{50} (μM)	% Inhibition at 200 μM	IC_{50} (μM)	
1	8.1 ± 0.2	>200	42.0 ± 0.8	>200	
2	71.0 ± 1.7	190.0 ± 3.0[d]	78.5 ± 1.3	64.3 ± 1.2	2.95
3	43.5 ± 1.1	>200	42.0 ± 4.4	—	
4	40.4 ± 0.7	>200	32.0 ± 0.9	—	
5	24.3 ± 1.8	>200	14.3 ± 1.9	—	
6	24.3 ± 1.4	>200	19.4 ± 1.3	—	
7	6.1 ± 1.4	—	18.4 ± 2.3	—	
8	12.9 ± 0.1	>200	50.2 ± 6.9	>200	
9	10.8 ± 2.6	>200	56.8 ± 0.5	188.4 ± 1.1	>1.70
10	98.9 ± 2.9	58.8 ± 1.4	87.8 ±1.8	104.2 ± 1.2	0.56
11	13.7 ± 1.2	>200	43.7 ± 1.3	>200	
12	3.3 ± 1.0	—	35.2 ± 3.0	—	
13	1.9 ± 1.0	—	46.2 ± 3.2	>200	
14	5.6 ± 1.3	—	51.2 ± 2.2	>200	
15	6.8 ± 0.9	—	45.8 ± 2.2	>200	
16	7.2 ± 0.8	—	-	—	
17	6.4 ± 0.1	—	29.3 ± 3.5	—	
18	3.9 ± 0.6	—	39.9 ± 2.1	—	
19	n.i.[e]	—	18.4 ± 1.0	—	
20	20.2 ± 1.0	>200	89.2 ± 1.6	80.6 ± 1.3	>2.48
21	6.4 ± 0.3	—	33.4 ± 0.5	—	
22	21.7 ± 1.2	>200	86.5 ± 2.7	21.5 ± 1.2	>9.30
Eserine	—	0.011 ± 0.001	—	0.014 ± 0.001	
Tacrine	—	0.029 ± 0.002	—	0.004 ± 0.001	

[a] AChE from electric eel.
[b] BChE from horse serum.
[c] Selectivity index = IC_{50} (AChE)/IC_{50} (BChE).
[d] From Ref. 13.
[e] n.i. no inhibition.

Most of the tested compounds were observed to elicit a weak AChE inhibition, with IC_{50} values higher than 200 μM. In general, when the hydroxyl groups were acylated (**12**–**19**) the inhibition of ACE was of the same order than that of the starting compound **1**. Also, sulfation of those groups was observed to render better ACE inhibitors, as long as the side chain was not oxidized (**2** vs **1**, **10** vs **5**). On the other hand, for non-sulfated compounds, when the side chain was oxidized a higher inhibition was observed compared to the natural triterpenoid (**3**, **4**, **6** vs **1**). Compound **10**, with two sulfate groups at C-3 and C-16 and an isopropyl group attached to C-19, showed the most potent inhibition for AChE with an IC_{50} value of 58.8 μM. Even if **10** was found to be less active than

the reference compounds, it was able to inhibit the enzyme more effectively than **2** (IC_{50} = 190.0 μM), showing the importance of the isopropyl group instead of the isopropenyl moiety in the activity. Derivative **10** was selected for the kinetic study of AChE inhibition and also for molecular docking.

Compounds **2**–**22** were also evaluated as potential BChE inhibitors in a preliminary assay (percentage of BChE inhibition at a fixed concentration) and compared to **1** (Table 1). Most of the derivatives exhibited higher inhibition of BChE than that observed for AChE. The IC_{50} values were determined only for those analogues that exhibited better inhibition than **1** in the preliminary assay (**2**, **8**–**11**, **13**–**15**, **20** and **22**). As shown in Table 1, compounds **2**, **9**, **10**, **20** and **22** exhibited BChE inhibition with IC_{50} values lower than 200 μM. Again, the sulfation of the hydroxyl groups proved to be a successful strategy to increase enzyme inhibition of these triterpenoids (**2** vs **1**, **10** vs **5**). The most active compound resulted to be **22**, 3,16,30-trioxolup-20(29)-ene, with an IC_{50} value of 21.5 μM, which revealed a selective inhibitor profile towards BChE. This result is interesting because BChE has the ability of delaying the onset and decreasing the rate of Aβ fibril formation in vitro, a central event in the pathogenesis of AD.[23,24]

2.3. Kinetic characterization of AChE inhibition

Disodium 3,16-disulfate with a saturated lateral chain **10** was identified as the most potent AChE inhibitor. Thus, it was chosen for the determination of the inhibitor type kinetic study. Enzyme activity was evaluated at different fixed substrate concentrations and increasing inhibitor concentrations and the data obtained were used to elucidate the enzyme inhibition mechanism. The results are illustrated in the form of Lineweaver–Burk plots (Fig. 2). The double-reciprocal plots show that both K_m and V_{max} values are enhanced with increasing concentration of **10**, but the ratio of K_m/V_{max} is still unchanged. The slopes are independent of the concentration of the inhibitor, which indicate that this compound is an uncompetitive inhibitor of the enzyme. Compound **10** does not bind to the free enzyme but binds reversibly to the enzyme–substrate complex, yielding an inactive complex.

Replots of the $1/v$ versus concentration of compound **10** gave an estimate of the inhibition constant αK_i of 144.4 μM.

2.4. Molecular modeling study

Molecular docking studies were performed to obtain more information about the binding mode and the interactions between the enzyme and compound **10**—the most active of the tested group—and to gain a structural insight into the inhibition mechanism. The docking studies were performed with the AChE complexed with acetylcholine according to the enzyme inhibition mechanism of **10**. Table 2 summarizes the docking results of the derivative **10**. Two hundred and fifty-six docking runs with **10** generated 256 conformers that were clustered according to their similarity, rendering four clusters. The best results of the docking were the conformations of cluster N°1, the cluster with the lowest energy, and cluster N°3, the largest one because solutions that are found many times in reiterated docking experiments typically correspond to compounds with better free energy of binding.[25,26]

The conformation adopted in cluster N°1 is shown in Figure 3A. Part of the triterpenoid is buried into the aromatic gorge, explaining the acompetitive inhibition mechanism of the AChE. It penetrates the peripheral site through *A* ring and binds the enzyme at the entrance of the gorge near the enzyme surface leaving rings *D* and *E* out of the pocket. The main hydrophobic interactions between the hydrocarbon skeleton of the inhibitor and the protein were observed with the residues: GLN74, PHE290, PHE331 and TYR334 (Fig. 4A). The docking simulation also showed that the affinity of **10** for the complex enzyme–substrate is favored by hydrogen bonding interactions, which involve the sulfate group at ring *A*. Sulfate group at C-3 come close to TYR121. The distance between the sulfate oxygen of the inhibitor and hydroxyl group of the TYR121 is 2.41 Å.

The conformation of cluster N°3 is shown in Figure 3B. The triterpenoid is located at the entrance of the gorge exposing its aliphatic side to the enzyme. The main hydrophobic interactions between the hydrocarbon skeleton of the inhibitor **10** and the protein were observed with the residues: GLN74, TRP279, ILE287, PHE290, TYR334 (Fig. 4B). Binding is also assisted by a hydrogen bond between the sulfate group oxygen at C-3 and the hydrogen of the amide group side chain of GLN74 (1.87 Å).

In both cases the major interactions are hydrophobic due to the many aromatic residues located at the peripheral site. These results agree with those recently reported by our group for a disulfated steroidal inhibitor of the AChE.[27] This study revealed that the 2β,3α-dihydroxy-5α-cholestan-6-one disulfate penetrates the gorge of the AChE through its side chain due to its high hydrophobic character whereas ring *A* substituted with two sulfate groups is placed out of the pocket. This compound mainly showed hydrophobic interactions between the side chain and rings *C* and *D* with the aromatic residues of the enzyme. The disulfated cholestane, that also showed an acompetitive mechanism of action, is buried into the peripheral site, such as compound **10**.

The docking studies allowed us to establish the orientation of the inhibitor **10** relative to the AChE as well as its conformation when bound to each other. This study allowed identifying hydrophobic interactions inside the aromatic gorge and hydrogen bonding interactions acting as stabilizing factors in the enzyme–substrate–inhibitor complex. Further molecular dynamics studies of this complex as starting point are necessary to check the complex inhibitor–enzyme stability, to determinate if the enzyme

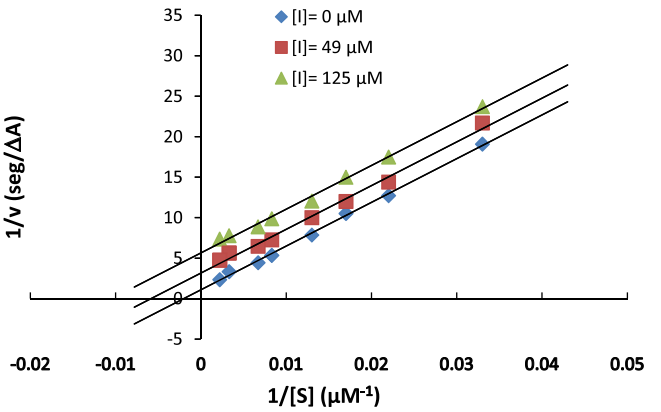


Figure 2. Lineweaver–Burk plots of the inhibition of AChE by compound **10** with acetylthiocholine (*S*) as substrate. Linear regression equations: $y = 539.81x + 1.0878$ ($R^2 = 0.9975$); $y = 539.92x + 3.1565$ ($R^2 = 0.9913$); $y = 539.89x + 5.6472$ ($R^2 = 0.9951$) for 0, 49 and 125 μM, respectively.

Table 2
Summary of the docking results of disodium 3β,16β-dihydroxy-20,29-dihydrolupane disulfate (**10**)

Compound	Total number of cluster	Docking statistics		Energy (kcal/mol)
		Cluster rank	Number of runs in the cluster	
10	4	1	71	−4.54
		2	25	−4.16
		3	159	−4.10
		4	1	−3.46

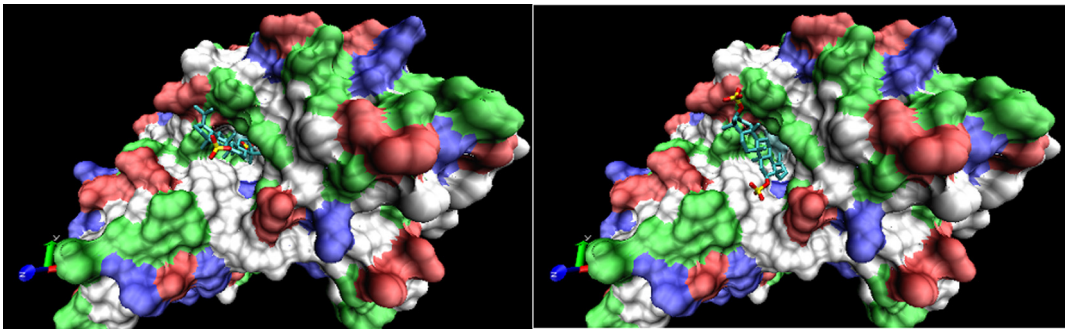


Figure 3. Docking results for compound **10**: (A) Cluster N° 1 (left). (B) Cluster N° 3 (right). Blue: basic residues, red: acid residues, green: polar residues.

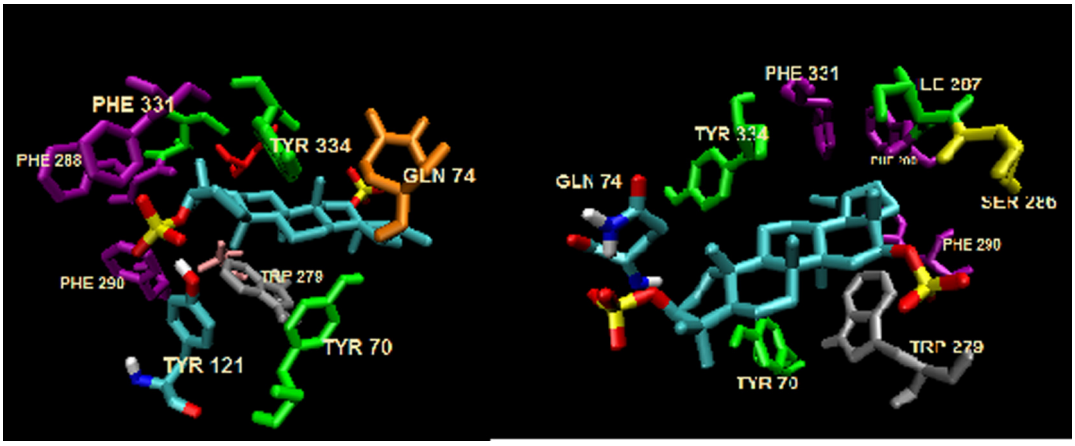


Figure 4. Docking of compound **10** showing the interactions with AChE: (A) Cluster N° 1 (left). (B) Cluster N° 3 (right).

undergoes structural rearrangements and verify the distances and an angles observed in the interactions are within a suitable range.

3. Conclusion

In summary, a set of lupane derivatives (**2**–**22**) has been synthesized from calenduladiol (**1**), a triterpenoid with the uncommon feature of being hydroxylated at C-16, which is not commercially available. These compounds have been prepared by simple reactions with moderate to good yields, rendering twenty one triterpenoids, seventeen new and four known compounds. All of them were tested for in vitro anticholinesterase activity against AChE and BChE. Compound **10** was identified as the most effective AChE inhibitor. A kinetic study of inhibition of AChE and molecular modeling indicated that **10** was able to bind to the complex enzyme–substrate with hydrophobic and hydrogen bonding interactions acting as stabilizing factors in the enzyme–substrate–inhibitor complex. On the other hand, compound **22**, resulted to be the most active against BChE showing also selectivity towards this enzyme, an interesting results considering that the role of BChE is more relevant as the disease progresses. The findings of the present study suggest that this trioxolupane may provide a useful template for the development of new lupane derivatives with improved and selective BChE inhibition.

4. Experimental section

4.1. Chemistry

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. NMR measurements, including COSY, HSQC, HMBC experiments, were carried out on Bruker ARX300, Bruker Avance 400, Bruker AMK 500 and/or Bruker AMK 600 spectrometers. NMR spectra were recorded in $CDCl_3$, MeOD or DMSO-d_6. Chemical shifts are given in ppm (δ) with TMS as an internal standard. High- and low-resolution mass spectra were obtained on a VG Autospec spectrometer and a LCT Premier XE (Waters) spectrometer. UV spectra were recorded on a JASCO V-630BIO spectrophotometer. Microwave assisted reactions were carried out in a CEM Discover reactor.

Silica gel 60 (0.2–0.63 mm, Merck) was used for column chromatography. Silica gel 60 (200–425 mesh, Aldrich) was used for flash chromatography. Analytical TLC was performed on Silicagel 60 F_{254} sheets (0.2 mm thickness, Merck). *p*-Anisaldehyde-acetic acid spray reagent and UV light (254 and 366 nm) were used for detection.

All chemicals and solvents were analytical grade and solvents were purified by general methods before being used. The commercially available trimethylamine–sulfur trioxide complex ($Me_3N{\cdot}SO_3$) was purchased from Aldrich. AChE from electric eel (type VI-S), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), tacrine and eserine were purchased from Sigma. BChE (horse serum) was purchased from MP Biomedicals. Calenduladiol (**1**), used as starting material for the preparation of compounds **2**–**22**, was extracted from aerial parts of *C. erinacea* subsp. *erinacea* as previously described.[13] ^1H and ^{13}C NMR spectra of **1** can be found in the Supplementary data.

All derivatives were rigorously characterized by NMR spectroscopy and mass spectrometry. The NMR data of derivatives **2**, **3** and **4** were identical to those previously reported.[13,21] In the case of compounds **3**, **4** and **12** we have completed the NMR data available in the literature.[21,28] Compounds **5**–**11**, **13**–**22** are described here for the first time and bidimensional NMR spectra (COSY, HMBC, HSQC) were used for the unequivocal assignments of all carbons

and representative protons. Selected NMR spectra are included in the Supplementary data.

4.2. Preparation of 3β,16β-dihydroxylup-20(29)-en-30-al (3)

A solution of **1** (60.0 mg, 0.14 mmol) in EtOH (5 mL) was treated with SeO_2 (38.5 mg, 0.35 mmol). The reaction mixture was heated under reflux until the disappearance of the starting material was confirmed by TLC (24 h). Then, the reaction mixture was cooled and EtOH was removed under reduced pressure. The crude was treated with water (20 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column flash chromatography on silica gel with hexane/AcOEt (7:3) to afford 60.0 mg of compound **3** (97%). Compound **3** showed identical spectroscopic data to those previously reported.[21] ^1H and ^{13}C NMR spectra of **3** can be found in the Supplementary data.

4.3. Preparation of lup-20(29)-en-3β,16β,30-triol (4)

A solution of **1** (86.0 mg, 0.19 mmol) in EtOH (10 mL) was treated with SeO_2 (10.8 mg 0.10 mmol). The reaction mixture was heated under reflux for 24 h. Then, the reaction mixture was cooled and EtOH was removed under reduced pressure. The crude was treated with water (20 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column flash chromatography on silica gel with hexane/AcOEt (6.5:3.5) to afforded 17.8 mg of compound **4** (20.2%) as an amorphous white solid, together with unreacted starting compound **1**. Compound **4** showed identical spectroscopic data to those previously reported.[21] EIMS *m*/*z* (%): 458 $[M]^+$ (40), 440 (100), 425 (14), 382 (21), 207 (66), 189 (58); HR-EIMS *m*/*z*: 458.3753 (calcd for $C_{30}H_{50}O_3$ $[M]^+$ 458.3760). ^1H and ^{13}C NMR spectra of **4** can be found in the Supplementary data.

4.4. Preparation of 20,29-dihydrolupan-3β,16β-diol (5)

A solution of **1** (40.0 mg, 0.09 mmol) in dry AcOEt (5 mL) was hydrogenated in the presence of catalytic amount of Pd/C 10%. The reaction mixture was stirred until the disappearance of the starting material was confirmed by TLC (24 h). After elimination of the solvent the residue obtained was submitted to column flash chromatography on silica gel with hexane/AcOEt (8.5:1.5) to afford 26.1 mg (65%) of compound **5** as a white amorphous solid, mp 250–252 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 0.75 (3H, d, J = 5.1 Hz, H-30), 0.76 (6H, s, H-24, H-28), 0.84 (3H, s, H-26), 0.85 (3H, d, J = 7.1 Hz H-29), 0.97 (6H, s, H-23, H-25), 1.04 (3H, s, H-27), 3.19 (1H, dd, J = 4.8, 11.2 Hz, H-3), 3.56 (1H, dd, J = 4.8, 11.0 Hz, H-16); ^{13}C NMR (100 MHz, $CDCl_3$) δ 79.1 (C-3), 77.5 (C-16), 55.4 (C-5), 49.9 (C-9), 48.8 (C-17), 47.3 (C-18), 44.5 (C-19), 44.3 (C-14), 41.1 (C-8), 39.0 (C-4), 39.0 (C-1), 38.2 (C-22), 37.2 (C-10), 37.2 (C-13), 36.9 (C-15), 34.5 (C-7), 29.3 (C-20), 28.1 (C-23), 27.5 (C-2), 26.6 (C-21), 23.1 (C-30), 22.2 (C-12), 21.0 (C-11), 18.5 (C-6), 16.2 (C-25, C-26), 16.1 (C-27), 15.5 (C-29), 15.2 (C-24), 12.2 (C-28); EIMS *m*/*z* (%): 444 $[M]^+$ (68), 429 (4), 426 (42), 411 (10), 207 (100), 189 (56); HR-EIMS *m*/*z*: 444.3982 (calcd for $C_{30}H_{52}O_2$ $[M]^+$ 444.3967).

4.5. Preparation of 20*S*,29-epoxylupan-3β,16β-diol (6)

MCPBA (98.4 mg, 0.57 mmol) and 6 mL of a 10% Na_2CO_3 solution were added to a solution of **1** (169.0 mg, 0.38 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred vigorously at 5 °C for 4 h, and then the aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic extracts were washed successively with 5% Na_2SO_3 solution, saturated $NaHCO_3$ solution and water, dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was purified by column flash chromatography on silica gel using hexane/AcOEt (7.5:2.5) to give 101.6 mg (58%) of compound **6** as an amorphous white solid, mp 195–197 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 0.68 (3H, s, H-28), 0.70 (3H, s, H-24), 0.78 (3H, s, H-26), 0.91 (3H, s, H-23), 0.92 (3H, s, H-25), 0.97 (3H, s, H-27), 1.18 (3H, s, H-30), 2.56 (1H, d, J = 4.8 Hz, H-29b), 2.60 (1H, d, J = 4.8 Hz, H-29a) 3.12 (1H, dd, J = 5.5, 10.4 Hz, H-3), 3.51 (1H, dd, J = 4.5, 10.9 Hz, H-16); ^{13}C NMR (75 MHz, $CDCl_3$) δ 78.8 (C-3), 76.7 (C-16), 60.1 (C-20), 57.1 (C-29), 55.3 (C-5), 49.8 (C-9), 49.0 (C-17), 48.9 (C-19), 46.0 (C-18), 43.9 (C-14), 40.9 (C-8), 38.8 (C-1), 38.8 (C-4), 37.5 (C-22), 37.1 (C-10), 36.6 (C-15), 36.5 (C-13), 34.2 (C-7), 28.0 (C-23), 27.3 (C-2), 26.4 (C-21), 26.2 (C-12), 20.9 (C-11), 18.3 (C-6), 18.3 (C-30), 16.1 (C-26), 16.1 (C-27), 16.0 (C-25), 15.4 (C-24), 11.7 (C-28); EIMS *m*/*z* (%): 458 $[M]^+$ (17), 440 (29), 425 (14), 400 (20), 382 (36), 207 (100), 189 (88); HR-EIMS *m*/*z*: 458.3749 (calcd for $C_{30}H_{50}O_3$ $[M]^+$ 458.3760).

4.6. General procedures for the preparation of the sulfated compounds 2, 7, 8 and 10

A solution of $Me_3N{\cdot}SO_3$ and **1**, **3**, **5** or **9** in dry DMF was placed in a microwave-special closed vial and the solution was irradiated for 7 min at 150 °C in a microwave reactor. The reaction mixture was then cooled to room temperature and quenched with water (1 mL). After evaporation to dryness the residue was eluted through Amberlite CG-120 (sodium form) with MeOH, evaporated under reduced pressure and purified by column flash chromatography on silica gel with CH_2Cl_2/MeOH mixtures as eluent to afford the sulfated compounds.

4.6.1. Disodium 3β,16β-dihydroxylup-20(29)-ene disulfate (2)

Following the general procedure, a solution of **1** (100.0 mg, 0.23 mmol) in DMF (4 mL) was treated with $Me_3N.SO_3$ (244.0 mg, 1.81 mmol). Purification of the resulting crude by flash chromatography with CH_2Cl_2/MeOH (4:1) afforded 135.9 mg (93%) of compound **2** as a white amorphous solid. Its ^1H and ^{13}C NMR data were identical to those previously reported.[13]

4.6.2. Disodium 3β,16β-dihydroxylup-20(29)-en-30-al disulfate (7)

Compound **3** (20.0 mg, 0.04 mmol) in DMF (2 mL) was treated with $Me_3N.SO_3$ (47.5 mg, 0.35 mmol) according to the general procedure. Purification of the resulting crude by flash chromatography with CH_2Cl_2/MeOH (4:1) afforded 25.8 mg (89%) of compound **7** as a white amorphous solid, mp 107–108 °C; ^1H NMR (300 MHz, CD_3OD) δ 0.80 (3H, s, H-25), 0.87 (6H, s, H-26, H-28), 1.02 (3H, s, H-23), 1.04 (3H, s, H-24), 1.07 (3H, s, H-27), 2.86 (1H, ddd, J = 5.7, 10.6, 10.7 Hz, H-19), 3.92 (1H, dd, J = 4.3, 11.5 Hz, H-3), 4.49 (1H, t, J = 7.8 Hz, H-16), 6.05 (1H, br s, H-29a), 6.42 (1H, br s, H-29b), 9.49 (1H, s, H-30); ^{13}C NMR (75 MHz, CD_3OD) δ 196.7 (C-30), 157.8 (C-20), 135.2 (C-29), 87.9 (C-3), 85.9 (C-16), 57.2 (C-5), 51.1 (C-9), 50.6 (C-18), 48.8 (C-17), 45.1 (C-14), 42.2 (C-8), 39.8 (C-1), 39.6 (C-4), 38.9 (C-15), 38.9 (C-19), 38.5 (C-13), 38.1 (C-10), 35.6 (C-7), 35.5 (C-22), 33.1 (C-21), 28.7 (C-23), 28.3 (C-12), 25.4 (C-2), 22.0 (C-11), 19.4 (C-6), 16.7 (C-27), 16.7 (C-26), 16.6 (C-25), 16.2 (C-24), 12.8 (C-28); HRMS (ESI) *m*/*z*: 637.2214 (calcd for $C_{30}H_{46}NaO_9S_2$ $[M-Na]^-$ 637.2481).

4.6.3. Trisodium 3β,16β,30-trihydroxy-lup-20(29)-ene trisulfate (8)

Compound **4** (10.0 mg, 0.02 mmol) in DMF (1 mL) was treated with $Me_3N.SO_3$ (35.6 mg, 0.26 mmol) according to the general procedure. Purification of the resulting crude by flash chromatography

with CH_2Cl_2/MeOH (7:3) afforded 3.3 mg (20%) of compound **8** as a white amorphous solid. Alternatively, a solution of **9** (11.4 mg, 0.02 mmol) in DMF (1 mL) was treated with $Me_3N.SO_3$ (9.2 mg, 0.07 mmol) to give 8.3 mg (50%) the same product **8**. Mp 100–102 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 0.81 (3H, s, H-25), 0.85 (3H, s, H-28), 0.89 (3H, s, H-26), 1.02 (3H, s, H-23), 1.08 (6H, s, H-24, H-27), 2.41-2.53 (1H, m, H-19), 3.92 (1H, dd, J = 4.0, 11.1 Hz, H-3), 4.37 (1H, t, J = 8.2 Hz, H-16), 4.97 (1H, br s, H-29a), 5.04 (1H, br s, H-29b), 4.47 (2H, br s, H-30); ^{13}C NMR (75 MHz, CD_3OD) δ 151.0 (C-20), 110.4 (C-29), 87.6 (C-3), 85.8 (C-16), 71.3 (C-30), 57.2 (C-5), 51.3 (C-9), 45.3 (C-14), 45.2 (C-13), 42.3 (C-8), 39.6 (C-1), 39.6 (C-4), 38.8 (C-19), 38.7 (C-15), 38.2 (C-10), 35.7 (C-7), 35.7 (C-22), 30.8 (C-21), 28.7 (C-23), 27.7 (C-12), 25.4 (C-2), 22.2 (C-11), 19.4 (C-6), 16.8 (C-25), 16.7 (C-26), 16.6 (C-27), 16.3 (C-24), 12.8 (C-28); HRMS (ESI) m/z: 741.1781 (calcd for $C_{30}H_{47}Na_2O_{12}S_3$ $[M-Na]^-$ 741.2025).

4.6.4. Disodium 3β,16β-dihydroxy-20,29-dihydrolupane disulfate (10)

Compound **5** (22.2 mg, 0.05 mmol) in DMF (2 mL) was treated with $Me_3N{\cdot}SO_3$ (53.9 mg, 0.40 mmol) according to the general procedure. Purification of the resulting crude by flash chromatography with CH_2Cl_2/MeOH (8.5:1.5) afforded 22.7 mg (70%) of compound **10** as a white amorphous solid, mp 136-138 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 0.81 (6H, s, H-25, H-28), 0.87 (3H, d, J = 6.9 Hz, H-29), 0.89 (3H, d, J = 6.9 Hz, H-30), 0.90 (3H, s, H-26), 1.02 (3H, s, H-23), 1.04 (3H, s, H-24), 1.09 (3H, s, H-27), 3.93 (1H, dd, J = 4.2, 11.5 Hz, H-3), 4.30 (1H, dd, J = 6.2, 9.7 Hz, H-16); ^{13}C NMR (75 MHz, CD_3OD) δ 87.8 (C-3), 86.3 (C-16), 57.2 (C-5), 51.0 (C-1), 45.8 (C-18), 45.4 (C-19), 42.3 (C-14), 39.8 (C-8), 39.7 (C-4), 39.6 (C-1), 39.5 (C-15), 38.5 (C-10), 38.1 (C-13), 35.6 (C-22), 35.5 (C-7), 30.5 (C-20), 28.7 (Me-23), 27.7 (C-21), 25.4 (C-2), 23.3 (Me-30), 22.8 (C-12), 22.1 (C-11), 19.4 (C-6), 16.8 (C-26), 16.7 (C-25), 16.6 (C-27), 16.2 (C-29), 15.4 (C-24), 13.3 (C-28); HRMS (ESI) m/z: 625.2633 (calcd for $C_{30}H_{50}NaO_8S_2$ $[M-Na]^-$ 625.2845).

4.7. Preparation of disodium 3β,16β,30-trihydroxy-lup-20(29)-ene 3,16-disulfate (9)

31.0 mg (0.12 mmol) of $EuCl_3$ in dry MeOH (1 mL) were added to a solution of compound **7** (80.0 mg, 0.12 mmol) in dry MeOH (3 mL). Then, the mixture was added slowly to a solution of $NaBH_4$ (4.5 mg, 0.12 mmol) in MeOH (1 mL). The reaction mixture was stirred and heated under reflux until disappearance of the starting material by TLC (24 h). Then, the reaction mixture was filtered, evaporated under reduced pressure and purified by column flash chromatography with CH_2Cl_2/MeOH (7.5:2.5) to give 40.9 mg (51%) of compound **9** as a white amorphous solid, mp 130–131 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 0.81 (3H, s, H-25), 0.84 (3H, s, H-28), 0.88 (3H, s, H-26), 1.02 (3H, s, H-23), 1.07 (3H, s, H-24), 1.08 (3H, s, H-27), 2.41 (1H, ddd, J = 5.7, 10.0, 10.6 Hz, H-19), 3.91 (1H, dd, J = 3.8, 10.9 Hz, H-3), 4.34 (1H, t, J = 7.7 Hz, H-16), 4.95 (1H, br s, H-29b), 4.03, 4.06 (2H, d_{AB}, J = 13.5 Hz, H-30a,b); ^{13}C NMR (75 MHz, CD_3OD) δ 155.4 (C-20), 107.4 (C-29), 87.6 (C-3), 85.8 (C-16), 65.0 (C-30), 57.2 (C-5), 51.3 (C-9), 45.2 (C-14), 44.7 (C-13), 42.3 (C-8), 39.9 (C-1), 39.6 (C-4), 38.8 (C-19, C-15), 38.1 (C-10), 35.6 (C-7), 35.5 (C-22), 32.6 (C-21), 28.7 (C-23), 27.4 (C-12), 25.4 (C-2), 22.1 (C-11), 19.4 (C-6), 16.7 (C-25, C-26), 16.6 (C-27), 16.3 (C-24), 12.7 (C-28). HRMS (ESI) m/z: 639.2689 (calcd for $C_{30}H_{48}NaO_9S_2$ $[M-Na]^-$ 639.2637).

4.8. Preparation of disodium 3β,16β-dihydroxy-20*S*,29-epoxylupane disulfate (11)

MCPBA (21.4 mg, 0.12 mmol) and 6 mL of 10% Na_2CO_3 were added to a solution of **2** (40.0 mg, 0.06 mmol) in CH_2Cl_2 (3 mL). The reaction mixture was stirred vigorously at room temperature for 12 h, and then the aqueous layer was extracted with *n*-BuOH (3 × 15 mL). The combined organic extracts were washed successively with 5% Na_2SO_3 solution, saturated $NaHCO_3$ solution and water, dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was purified by preparative-RP TLC with MeOH/H_2O (6.5:3.5) to give 4.0 mg (10%) of compound **11** as an amorphous white solid, mp 134–135 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 0.78 (3H, s, H-25), 0.80 (3H, s, H-28), 0.88 (3H, s, H-26), 1.01 (3H, s, H-23), 1.04 (3H, s, H-24), 1.06 (3H, s, H-27), 1.23 (3H, s, H-30), 2.64 (2H, br s, H-29) 3.90 (1H, dd, J = 4.2, 11.4 Hz, H-3), 4.29 (1H, t, J = 8.0 Hz, H-16); ^{13}C NMR (75 MHz, CD_3OD) δ 87.5 (C-3), 85.6 (C-16), 61.5 (C-20), 58.0 (C-29), 57.2 (C-5), 51.1 (C-9), 50.4 (C-18), 49.4 (C-17), 47.3 (C-19), 45.2 (C-14), 42.3 (C-8), 39.8 (C-1), 39.6 (C-4), 38.7 (C-15), 38.1 (C-10), 38.0 (C-13), 35.5 (C-7), 35.4 (C-22), 28.7 (C-23), 27.7 (C-21), 27.0 (C-12), 25.4 (C-2), 22.1 (C-11), 19.4 (C-6), 18.5 (C-30), 16.7 (C-25), 16.7 (C-26), 16.5 (C-27), 16.2 (C-24), 12.9 (C-28). HRMS (ESI) m/z: 639.2695 (calcd for $C_{30}H_{48}NaO_9S_2$ $[M-Na]^-$ 639.2637).

4.9. General procedures for the preparation of the acetylated compounds 12–16

To a solution of **1**, **3** or **4** in pyridine were added a catalytic amount of DMAP and Ac_2O. The reaction mixture was stirred at room temperature for 24 h until disappearance of the starting material. Then, water was added, and the mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with a saturated solution of $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography with CH_2Cl_2 as eluent to afford the desired acetate.

4.9.1. Lup-20(29)-ene-3β,16β-diol diacetate (12)

Following the general procedure, a solution of **1** (20.0 mg, 0.05 mmol) in pyridine (1 mL) was treated with Ac_2O (1.2 mL, 12.7 mmol) to give after purification 12.1 mg (51%) of compound **12** as white amorphous solid, mp 105–107 °C. ^{1}H and ^{13}C NMR spectra of **12** are included in the Supplementary data.

4.9.2. 3β-Acetoxy-16β-hydroxy-lup-20(29)-ene (13) and 3β-hydroxy-16β-acetoxy-lup-20(29)-ene (14)

Following the general procedure, a solution of **1** (50.0 mg, 0.11 mmol) in CH_2Cl_2 (2 mL) and pyridine (0.5 mL) was treated with Ac_2O (11 μL, 0.11 mmol) to give after purification 13.7 mg (25%) of compound **13** and 13.7 mg (25%) of compound **14** as white amorphous solids. Compound **13**: mp 110–111 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.79 (3H, s, H-28), 0.83 (3H, s, H-26), 0.84 (3H, s, H-23), 0.85 (3H, s, H-24), 0.98 (3H, s, H-25), 1.03 (3H, s, H-27), 1.68 (3H, s, H-30), 2.04 (3H, s, $OCOCH_3$), 2.49 (1H, ddd, J = 6.0, 11.0, 11.0 Hz, H-19), 3.61 (1H, dd, J = 4.3, 9.8 Hz, H-16), 4.46 (1H, dd, J = 5.4, 10.9 Hz, H-3), 4.59 (1H, br s, H-29a), 4.70 (1H, br s, H-29b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2 ($OCOCH_3$), 150.1 (C-20), 110.0 (C-29), 81.1 (C-3), 77.2 (C-16), 55.6 (C-5), 50.1 (C-9), 48.8 (C-17), 47.9 (C-19), 47.8 (C-18), 44.2 (C-14), 41.1 (C-8), 38.6 (C-1), 38.0 (C-4), 37.9 (C-22), 37.4 (C-13), 37.2 (C-10), 37.0 (C-15), 34.3 (C-7), 30.0 (C-21), 28.1 (C-23), 24.9 (C-12), 23.8 (C-2), 21.5 ($OCOCH_3$), 21.0 (C-11), 19.5 (C-30), 18.3 (C-6), 16.6 (C-24), 16.3 (C-26), 16.3 (C-25), 16.1 (C-27), 11.8 (C-28); EIMS m/z: 484 $[M]^+$ (36), 466 (6), 424(57), 216 (44), 207 (50), 189 (82), 203 (41); HR-EIMS m/z: 484.3940 (calcd for $C_{32}H_{52}O_3$ $[M]^+$ 484.3916). Compound **14**: mp 110–112 °C ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.75 (3H, s, H-24), 0.82 (3H, s, H-26), 0.84 (3H, s, H-28), 0.96 (3H, s, H-23), 1.03 (3H, s, H-25), 1.04 (3H, s, H-27), 1.67 (3H, s, H-30), 2.02 (3H, s, $OCOCH_3$), 2.48 (1H, ddd, J = 6.0, 10.4, 11.3 Hz, H-19), 3.17–3.19 (1H, m, H-3), 4.59 (1H, br s, H-29a),

4.70 (1H, br s, H-29b), 4.87 (1H, dd, J = 4.6, 11.3 Hz, H-16); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.9 ($OCOCH_3$), 150.0 (C-20), 110.1 (C-29), 79.2 (C-16), 79.0 (C-3), 55.4 (C-5), 50.0 (C-9), 47.9 (C-18), 47.6 (C-19), 47.5 (C-17), 44.3 (C-14), 41.1 (C-8), 39.0 (C-4), 38.8 (C-1), 37.8 (C-22), 37.5 (C-13), 37.2 (C-10), 34.4 (C-7), 33.6 (C-15), 29.8 (C-21), 28.1 (C-23), 27.5 (C-2), 24.8 (C-12), 21.5 ($OCOCH_3$), 20.9 (C-11), 19.4 (C-30), 18.4 (C-6), 16.2 (C-26), 16.1 (C-24), 16.1 (C-25), 15.5 (C-27), 12.9 (C-28); EIMS m/z: 484 $[M]^+$ (28), 466 (26), 424(16), 216 (26), 207 (6), 203 (42), 189 (90); HR-EIMS m/z: 484.3924 (calcd for $C_{32}H_{52}O_3$ $[M]^+$ 484.3916).

4.9.3. 3β,16β-Diacetoxy-lup-20(29)-en-30-al (15)

Following the general procedure, a solution of **3** (20.0 mg, 0.04 mmol) in pyridine (1 mL) was treated with Ac_2O (1.2 mL, 12.7 mmol) to give after purification 16.6 mg (70%) of compound **15** as a white amorphous solid, mp 109-110 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.82 (3H, s, H-24), 0.83 (6H, s, H-23, H-26), 0.87 (3H, s, H-28), 1.02 (6H, s, H-25, H-27), 2.02 (3H, s, $OCOCH_3$), 2.04 (3H, s, $OCOCH_3$), 4.45 (1H, dd, J = 4.6, 11.0 Hz, H-3), 4.92 (1H, dd, J = 4.7, 11.3 Hz, H-16), 5.95 (1H, br s, H-29a), 6.30 (1H, br s, H-29b), 9.51 (1H, s, H-30); ^{13}C NMR (150 MHz, $CDCl_3$) δ 195.0 (C-30), 171.2 ($OCOCH_3$ (C-3)), 170.9 ($OCOCH_3$ (C-16)), 156.3 (C-20), 132.5 (C-29), 81.0 (C-3), 78.9 (C-16), 55.4 (C-5), 49.8 (C-9, C-18), 47.7 (C-17), 44.0 (C-14), 41.0 (C-8), 38.5 (C-1), 37.9 (C-4), 37.8 (C-22), 37.1 (C-10, C-13, C-19), 34.3 (C-7), 33.5 (C-15), 29.8 (C-21), 28.0 (C-23), 23.8 (C-2, C-12), 21.5 ($OCOCH_3$), 21.5 ($OCOCH_3$), 21.0 (C-11), 18.2 (C-6), 16.6 (C-24), 16.3 (C-26), 16.1 (C-25), 15.9 (C-27), 12.8 (C-28). HR-EIMS m/z: 540.3807 (calcd for $C_{34}H_{52}O_5$ $[M]^+$ 540.3815).

4.9.4. Lup-20(29)-ene-3β,16β,30-triol triacetate (16)

Following the general procedure, a solution of **4** (20.0 mg, 0.04 mmol) in pyridine (1 mL) was treated with Ac_2O (1.2 mL 12.7 mmol) to give after purification 18.4 mg (72%) of compound **16** as a white amorphous solid, mp 107-108 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.83 (3H, s, H-26), 0.84 (6H, s, H-23, H-28), 0.85 (3H, s, H-24), 1.04 (6H, s, H-25, H-27), 2.02 (3H, s, $OCOCH_3$), 2.04 (3H, s, $OCOCH_3$), 2.10 (3H, s, $OCOCH_3$), 4.46 (1H, dd, J = 5.2, 11.5 Hz, H-3), 4.57, 4.55 (2H, d_{AB}, J = 14.0 Hz, H-30a,b), 4.87 (1H, dd, J = 4.7, 11.2 Hz, H-16), 4.95 (1H, br s, H-29a), 4.97 (1H, br s, H-29b); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 ($OCOCH_3$ (C-3)), 170.9 ($OCOCH_3$ (C-16)), 170.8 ($OCOCH_3$ (C-30)), 148.5 (C-20), 110.9 (C-29), 81.0 (C-3), 79.0 (C-16), 68.3 (C-30), 55.4 (C-5), 49.9 (C-9), 48.6 (C-18), 47.4 (C-17), 44.2 (C-14), 41.1 (C-8), 38.8 (C-13), 38.5 (C-1), 37.9 (C-4), 37.6 (C-22), 37.4 (C-10), 37.1 (C-19), 34.3 (C-7), 33.6 (C-15), 29.8 (C-21), 28.1 (C-23), 23.9 (C-12), 23.8 (C-2), 21.5 ($OCOCH_3$), 21.5 ($OCOCH_3$), 21.2 ($OCOCH_3$ (C30)), 21.1 (C-11), 18.2 (C-6), 16.6 (C-24), 16.3 (C-26), 16.2 (C-25), 16.1 (C-27), 12.7 (C-28); EIMS m/z: 542 $[M - C_2H_2O]^+$ (4), 524 (54), 464 (30), 189 (82); HR-EIMS m/z: 542.3990 (calcd for $C_{34}H_{54}O_5$ $[M-C_2H_2O]^+$ 542.3971).

4.10. General procedure for the preparation of the acylated compounds 17–20

To a solution of **1** in dry CH_2Cl_2 (5 mL) were added the corresponding acid chloride, Et_3N and a catalytic amount of DMAP. The reaction mixture was stirred under N_2 atmosphere at the appropriate temperature. The progress of the reaction was monitored by TLC. The reaction was quenched with saturated aqueous $NaHCO_3$ solution (5 mL). The mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with a saturated solution of $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated. The crude was purified by flash chromatography on silica gel with hexane/AcOEt (9:1) to afford the desired ester.

4.10.1. Lup-20(29)-ene-3β,16β-diol di-4-pentenoate (17)

Following the general procedure, to a solution of **1** (60.0 mg, 0.14 mmol) in CH_2Cl_2 were added Et_3N (75 μL, 0.54 mmol) and 4-pentenoyl chloride (35 μL, 0.54 mmol). The reaction mixture was stirred at room temperature for 24 h to give after purification 9.9 mg (12%) of compound **17** as a white amorphous solid, mp 240–241 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.84 (6 H, s, H-23, H-26), 0.85 (3H, s, H-28), 0.86 (3H, s, H-24), 1.04 (6H, s, H-25, H-27), 1.68 (3H, s, H-30), 2.38 (4H, d, J = 6.7 Hz, H-3′, H-3″), 2.39 (4H, d, J = 5.7 Hz, H-2′, H-2″), 2.50 (1H, m, H-19), 4.48 (1H, dd, J = 5.1, 10.3 Hz, H-3), 4.60 (1H, br s, H-29a), 4.71 (1H, br s, H-29b), 4.88 (1H, dd, J= 4.9, 11.4 Hz, H-16), 4.98 (1H, br s, H-5′a), 5.02 (2H, br s, H-5′b, H-5″a), 5.08 (1H, br s, H-5″b), 5.82 (2H, m, H-4′, H-4″); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0 (C-1′(C-3)), 172.7 (C-1″(C-16)), 150.0 (C-20), 137.0 (C-4′, C-4"), 115.5 (C-5′, C-5″), 110.1 (C-29), 81.0 (C-3), 79.2 (C-16), 55.5 (C-5), 50.0 (C-9), 48.0 (C-18), 47.7 (C-19), 47.5 (C-17), 44.4 (C-14), 41.2 (C-8), 38.5 (C-1), 38.0 (C-4), 37.8 (C-22), 37.5 (C-13), 37.2 (C-10), 34.4 (C-7), 34.2 (C-2′, C-2″), 33.7 (C-15), 29.9 (C-21), 29.2 (C-3′, C-3″), 28.1 (C-23), 24.8 (C-12), 23.9 (C-2), 21.0 (C-11), 19.4 (C-30), 18.3 (C-6), 16.7 (C-24), 16.3 (C-26), 16.2 (C-25), 16.1 (C-27), 13.0 (C-28). EIMS m/z: 606 $[M]^+$ (0.1), 506 (67), 406(51), 363 (48), 216 (89), 203 (100), 189 (84). HR-EIMS m/z: 606.4668 (calcd for $C_{40}H_{62}O_4$ $[M]^+$ 606.4648).

4.10.2. Lup-20(29)-ene-3β,16β-diol dibenzoate (18)

Following the general procedure described above, to a solution of **1** (50.0 mg, 0.11 mmol) in CH_2Cl_2 were added Et_3N (62 μL, 0.45 mmol) and an excess of benzoylchloride (50 μL, 0.43 mmol). The reaction mixture was stirred at room temperature for 3 h to give after purification 30.1 mg (41%) of compound **18** as a white amorphous solid, mp 219–220 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92 (3H, s, H-26), 0.93 (3H, s, H-23), 1.00 (3H, s, H-24), 1.01 (3H, s, H-28), 1.09 (3H, s, H-25), 1.14 (3H, s, H-27), 1.72 (3H, s, H-30), 2.54 (1H, ddd, J = 5.3, 10.9, 11.0 Hz, H-19), 4.63 (1H, br s, H-29a), 4.72 (1H, dd, J = 4.9, 9.9 Hz, H-3), 4.74 (1H, br s, H-29b), 5.13 (1H, dd, J = 4.9, 11.3 Hz, H-16), 7.41–7.46 (4H, m, H-3′, H-3″, H-5′, H-5″), 7.52–7.57 (2H, m,, H-4′, H-4″), 8.02–8.05 (4H, m, H-2′, H-2″, H-6′, H-6″); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.4 (Ar*C*O (C-3)), 166.2 (Ar*C*O (C-16)), 149.9 (C-20), 132.8 (d), 131.2 (C-1′, C-1″), 129.7 (C-2′, C-2″, C-6′, C-6″), 128.4 (C-3′, C-3″, C-5′, C-5″), 110.2 (C-29), 81.7 (C-3), 80.0 (C-16), 55.6 (C-5), 50.0 (C-9), 48.1 (C-18), 47.8 (C-17), 47.7 (C-19), 44.5 (C-14), 41.2 (C-8), 38.6 (C-1), 38.3 (C-4), 37.9 (C-22), 37.6 (C-13), 37.3 (C-10), 34.4 (C-7), 33.7 (C-15), 29.9 (C-21), 28.2 (C-23), 24.8 (C-12), 23.9 (C-2), 21.1 (C-11), 19.4 (C-30), 18.3 (C-6), 16.9 (C-24), 16.3 (C-26), 16.2 (C-25), 16.1 (C-27), 13.2 (C-28). HR-EIMS m/z: 650.4365 (calcd for $C_{44}H_{58}O_4$ $[M]^+$ 650.4335).

4.10.3. Lup-20(29)-ene-3β,16β-diol di-*p*-bromobenzoate (19)

Following the general procedure, to a solution of **1** (50.0 mg, 0.11 mmol) in CH_2Cl_2 were added Et_3N (93 μL, 0.68 mmol) and *p*-bromobenzoyl chloride (50 μL, 0.34 mmol). The reaction mixture was stirred at 0 °C for 48 h to give after purification 38.5 mg (42%) of compound **19** as a white amorphous solid, mp 109.0–109.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (6H, s, H-23, H-26), 0.99 (6H, s, H-24, H-28), 1.10 (3H, s, H-25), 1.13 (3H, s, H-27), 1.71 (3H, s, H-30), 2.54 (1H, ddd, J = 4.9, 10.6, 10.8 Hz, H-19), 4.63 (1H, br s, H-29a), 4.72 (1H, dd, J = 5.4, 10.6, H-3), 4.74 (1H, br s, H-29b), 5.12 (1H, dd, J = 4.6, 11.3 Hz, H-16), 7.30 - 7.34 (2H, m, *p*-BrBz), 7.67 (2H, d, J = 7.7 Hz, *p*-BrBz)), 7.96 (2H, dd, J = 7.6 Hz, *p*-BrBz)), 8.16 (2H, d, J = 13.6 Hz, *p*-BrBz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.1 (Ar*C*O (C-3)), 164.9 (Ar*C*O (C-16)), 149.9 (C-20), 135.8, 133.1, 133.0, 132.6, 130.1, 128.3 (*p*-BrBz), 110.3 (C-29), 82.2 (C-3), 80.6 (C-16), 55.5 (C-5), 50.0 (C-9), 48.0 (C-18), 47.7 (C-17), 47.6 (C-19), 44.5 (C-14), 41.2 (C-8), 38.5 (C-1), 38.3 (C-4), 37.9 (C-22),

37.5 (C-10), 37.2 (C-13), 34.3 (C-7), 33.6 (C-15), 29.8 (C-21), 28.2 (C-23), 24.7 (C-12), 23.8 (C-2), 21.0 (C-11), 19.4 (C-30), 18.3 (C-6), 16.9 (C-24), 16.3 (C-26), 16.2 (C-25), 16.1 (C-27), 13.2 (C-28); EIMS *m*/*z*: 606 [M-(O-*p*-Br-Bz]$^+$ (72), 604 (69), 591 (13), 589 (14), 405 (18), 390 (13), 189 (77), 187 (49), 184 (100), 182 (99). HR-EIMS *m*/*z*: 806.2475 (calcd for $C_{44}H_{56}Br_2O_4$ [M]$^+$ 806.2545).

4.10.4. Lup-20(29)-ene-3β,16β-diol dihemiadipate (20)

Following the general procedure, to a solution of **1** (30.0 mg, 0.07 mmol) in CH_2Cl_2 were added Et_3N (62 μL, 0.41 mmol) and adipoyl chloride (25 μL, 0.15 mmol). The reaction mixture was stirred under reflux for 24 h. Hydrolysis of the intermediate acyl chloride took place during the work-up in the aqueous media, to give after purification 33.6 mg (71%) of compound **20** as a white amorphous solid, mp 97–98 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 0.90 (3H, s, H-24), 0.91 (3H, s, H-23), 0.93 (3H, s, H-28), 0.94 (3H, s, H-26), 1.12 (6H, s, H-25, H-27), 1.66–1.69 (8H, m, hemiadip.), 1.74 (3H, s, H-30), 2.31–2.37 (8H, m, hemiadip.), 2.57 (1H, ddd, *J* = 6.2, 10.3, 11.1 Hz, H-19), 4.49 (1H, dd, *J* = 5.5, 10.3 Hz, H-3), 4.64 (1H, br s, H-29a), 4.76 (1H, br s, H-29b); ^{13}C NMR (75 MHz, CD_3OD) δ 177.3 (COOH × 2), 175.0 (C-1′(C-3)), 174.9 (C-1″(C-16)), 151.0 (C-20), 110.7 (C-29), 82.3 (C-3), 80.6 (C-16), 56.7 (C-5), 51.3 (C-9), 49.1 (C-18), 48.9 (C-19), 48.5 (C-17), 45.4 (C-14), 42.2 (C-8), 39.5 (C-1), 38.9 (C-4), 38.8 (C-13, C-22), 38.2 (C-10), 35.3 (C-15), 35.2 (C-5′, C-5″), 34.6 (C-7), 34.6 (C-2′, C-2″), 30.8 (C-21), 28.5 (C-23), 26.0 (C-12), 25.7 (C-3′, C-3″), 25.6 (C-4′) 25.5 (C-4″), 24.7 (C-2), 22.0 (C-11), 19.5 (C-30), 19.2 (C-6), 17.0 (C-24), 16.7 (C-26), 16.6 (C-25), 16.3 (C-27), 13.2 (C-28). HR-EIMS *m*/*z*: 589.4897 (calcd for $C_{41}H_{61}O_6$ [M−CO_2H]$^+$ 589.4985).

4.11. General procedure for the preparation of compounds 21 and 22

To a solution of **1** or **3** in acetone (3 mL) was added dropwise the Jones reagent at 0 °C, until the solution changed from colorless to orange. The reaction was stirred for 30 min and quenched with *i*-PrOH (2 mL), filtered through Florisil and washed several times with AcOEt. The solvent was removed and the residue was purified by flash chromatography on silica gel with hexane/AcOEt (9:1) to afford the desired ketone.

4.11.1. Lup-20(29)-en-3,16-dione (21)

Following the general procedure, a solution of **1** (50.0 mg, 0.11 mmol) in acetone was treated with Jones reagent to yield after purification 20.8 mg (42%) of compound **21**, mp 137–138 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (3H, s, H-27), 0.93 (3H, s, H-25), 1.01 (3H, s, H-24), 1.06 (3H, s, H-23), 1.09 (3H, s, H-28), 1.14 (3H, s, H-26), 1.65 (3H, s, H-30), 2.61 (1H, ddd, *J* = 6.3, 10.8, 10.9 Hz, H-19), 2.71 (1H, d, *J* = 13.6 Hz, H-15a), 4.62 (1H, br s, H-29a), 4.73 (1H, br s, H-29b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 217.8 (C-3), 215.8 (C16), 148.8 (C-20), 110.8 (C-29), 56.7 (C-17), 54.8 (C-5), 49.4 (C-9), 49.4 (C-18), 48.1 (C-14), 47.4 (C-4, C-19), 44.9 (C-15), 41.0 (C-8), 39.6 (C-1), 37.7 (C-13), 36.9 (C-10), 34.1 (C-2), 33.6 (C-7), 31.2 (C-22), 28.6 (C-21), 26.8 (C-23), 24.8 (C-12), 21.3 (C-11), 21.1 (C-24), 19.7 (C-6), 19.0 (C-30), 18.1 (C-28), 16.3 (C-26), 16.0 (C-25), 15.4 (C-27); EIMS *m*/*z*: 438 [M]$^+$ (74), 395 (18), 247 (72), 229 (42), 205 (30); HR-EIMS *m*/*z*: 438.3484 (calcd for $C_{30}H_{46}O_2$ [M]$^+$ 438.3498).

4.11.2. 3,16-Dioxo-lup-20(29)-en-30-al (22)

Following the general procedure, a solution of **3** (50.0 mg, 0.11 mmol) in acetone as treated with Jones reagent to yield after purification 12.9 mg (26%) of compound **22**, mp 143.0–143.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.89 (3H, s, H-27), 0.93 (3H, s, H-25), 1.02 (3H, s, H-24), 1.07 (3H, s, H-28), 1.13 (6H, s, H-23, H-26), 2.48 (1H, ddd, *J* = 8.6, 15.7, 15.9 Hz, H-19), 2.74 (1H, d, *J* = 13.8 Hz, H-15a), 5.98 (1H, br s, H-29a), 6.29 (1H, br s, H-29b), 9.52 (1H, s, H-30); ^{13}C NMR (150 MHz, $CDCl_3$) δ 217.9 (C-3), 215.4 (C-16), 194.8 (C-30), 156.2 (C-20), 133.3 (C-29), 56.9 (C-17), 54.8 (C-5), 49.2 (C-9, C-18), 47.7 (C-14), 47.4 (C-4), 44.9 (C-15), 41.0 (C-8), 39.6 (C-1), 37.3 (C-13, C-19), 36.9 (C-10), 34.2 (C-2), 33.6 (C-7, C-22), 31.3 (C-12, C-21), 26.8 (C-23), 21.2 (C-11), 21.2 (C-24), 19.6 (C-6), 18.1 (C-28), 16.3 (C-26), 15.9 (C-25), 15.4 (C-27); EIMS *m*/*z*: 452 [M]$^+$ (100), 437 (20), 434 (31), 247 (15), 205 (17); HR-EIMS *m*/*z*: 452.3284 (calcd for $C_{30}H_{44}O_3$ [M]$^+$ 452.3290).

4.12. Biological activity

4.12.1. Inhibition assay on AChE and BChE in vitro

Electric eel (*Torpedo californica*) AChE and horse serum BChE were used as source of both the cholinesterases. AChE and BChE inhibiting activities were measured in vitro by the spectrophotometric method developed by Ellman with slight modification.[22]

The lyophilized enzyme, 500U AChE/300U BChE, was prepared in buffer A (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4) to obtain 5/3 U/mL stock solution. Further enzyme dilution was carried out with buffer B (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) to produce 0.126/0.06 U/mL enzyme solution. Samples were dissolved in buffer B with 2.5% of MeOH as cosolvent, except for compounds **2** and **7**–**11** that were dissolved in buffer B without cosolvent. Enzyme solution (300 μL) and sample solution (300 μL) were mixed in a test tube and incubated for 60/120 min at room temperature. The reaction was started by adding 600 μL of the substrate solution (0.5 mM DTNB, 0.6 mM ATCI/BTCI, 0.1 M Na_2HPO_4, pH 7.5). The absorbance was read at 405 nm for 180 s at 27 °C. Enzyme activity was calculated by comparing reaction rates for the sample to the blank. All the reactions were performed in triplicate. IC_{50} values were determined with GraphPad Prism 5. Eserine (99%) and tacrine (99%) were used as reference AChE/BChE inhibitors.

4.12.2. Kinetic characterization of AChE inhibition

The enzyme reaction was carried out at three fixed inhibitor (compound **10**) concentrations (0, 49 and 125 μM). In each case the initial velocity measurements were obtained at varying substrate (*S*) (acetylthiocholine) concentrations and the reciprocal of the initial velocity ($1/v$) was plotted as a function of the reciprocal of [*S*]. The double-reciprocal (Lineweaver–Burk) plot showed a pattern of parallels lines with the same slopes, characteristic of an uncompetitive inhibitor. The data of the enzyme activity at different fixed substrate concentrations with increasing inhibitor concentrations were analyzed with GraphPad Prism 5. The nonlinear regression of these data fitted with uncompetitive inhibition with a R^2 = 0.9859. The calculated K_i was 144.4 μM.

4.12.3. Molecular docking determinations

Torpedo californica AChE crystal structure was chosen to perform the docking studies given that this was the enzyme used in the in vitro assays. Structure of Protein Data Bank (PDB) entry 2ACE—complexed with acetylcholine—was used for the docking simulations of compound **10**.[29] Geometry optimization was performed with semiempirical calculations (AM1) and the Hartree–Fock method and the 6-31+G (d) basis set incorporated in the Gaussian 03 program.[30–32] The charges of the ligand were obtained using the standard RESP procedure.[33]

Docking studies were performed with version 4.2.5.1 of the program AutoDock, using the implemented empirical free energy function.[34] The graphical user interface program AutoDock Tools was used to prepare, run and analyze the docking simulations. The simulation space was defined as a 26.25 × 24 × 34.5 Å box which included the active site and the peripheral site. Atomic interaction energy on a 0.375 Å grid was calculated with the

auxiliary program Autogrid 4 using probes corresponding to each map type found in the inhibitor. All rotatable dihedrals in **10** were allowed to rotate freely. The starting position of the inhibitor was outside the grid on a random position.

The triterpenoid was docked by the Lamarckian genetic algorithm protocol. A total of 256 independent simulations with a population size of 150 members were run for **10** using AutoDock 4.2.5.1 with default parameters (random starting position and conformation, translation step of 2.0 Å, mutation rate of 0.02, crossover rate of 0.8, local search rate 0.06 and 2,500,000 energy evaluations). After docking, the 256 conformers generated for the inhibitor were assigned to clusters based on a tolerance of 2.0 Å all atom root-mean-square deviation (rmsd) in position from the lowest-energy solution. The clusters were also ranked according to the energies of their representative conformations, which were the lowest-energy solutions within each cluster.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.04.050. These data include MOL files and InChiKeys of the most important compounds described in this article.

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