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Synthesis and acetylcholinesterase inhibitory activity of 2β,3α-disulfoxy-5α-cholestan-6-one

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ABSTRACT

Disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**8**) has been synthesized using cholesterol (**1**) as starting material. Sulfation was performed using trimethylamine–sulfur trioxide complex in dimethylformamide as the sulfating agent. The acetylcholinesterase inhibitory activity of compound **8** was evaluated and compared to that of disodium 2β,3α-dihydroxy-5α-cholestane disulfate (**10**) and diols **7** and **9**. Compounds **8** and **10** were active with IC_{50} values of 14.59 and 59.65 μM, respectively. Diols **7** and **9** showed no inhibitory activity (IC_{50} > 500 μM).

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1. Introduction

Sulfated polyhydroxysteroids have been described from marine sources, especially from Porifera and Ophiuroidea (Echinodermata) [1–4]. These polar compounds have received considerable attention due to their broad spectrum of biological activities, such as anti-HIV effects [5], inhibition of protein tyrosine kinases [6,7], and antiviral activities [8]. In 2000, six new sulfated antimicrobial aminosterols were isolated from the dogfish shark *Squalus acanthias* [9]. Recently, two new disulfated steroids were isolated from the sea lamprey *Petromyzon marinus* migratory pheromone [10].

Due to the low concentration of these natural products in marine organisms and the constant need for new bioactive compounds, the synthesis of analogs poses an alternative for the development of compounds with interesting biological activities. In 2006, Murphy et al. [11] synthesized 2β,3α,6α-cholestanetrisulfate, an analog of the natural sokotrasterol sulfate [12] and demonstrated the importance of the sulfate groups for the angiogenic activity. Recently, three new cytotoxic disulfated steroids with sulfate groups located at C-3 and C-6 of ring A and B were prepared [13]. In a previous work [14], we have synthesized five new sulfated and acetylated derivatives of 2β,3α-dihydroxy-5α-cholestane and tested their antiviral activity against herpes simplex virus type 2 (HSV-2). Disodium 2β,3α-dihydroxy-5α-cholestane disulfate (**10**) showed the best selectivity index (CC_{50}/IC_{50}) while its desulfated analog was inactive. These results were indicative of the role of the sulfate groups in the antiviral activity of **10** and prompted us to synthesize a new disulfated steroid, disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**8**), which differs from **10** in the presence of a carbonyl group at C-6. The aim of this work was the synthesis of this new disulfated analog and the evaluation of its acetylcholinesterase activity.

Acetylcholine serves as a neurotransmitter in the central and peripheral nervous system. Acetylcholinesterase (AChE) stops the function of acetylcholine by its hydrolytic destruction in the cholinergic synapses [15]. According to the cholinergic hypothesis, the selective and irreversible deficiency of cholinergic functions leads to memory impairment in Alzheimer's disease (AD) [16]. Enhancement of acetylcholine level in the brain is considered one of the most promising approaches for treating AD [17]. Retaining the neurotransmitters especially at the synaptic terminals via the inhibition of the hydrolytic enzymes, i.e., the cholinesterases, in order to compensate the deficiency of the cholinergic neurotransmitters would lead to the improved cognitive activities of the patient [18]. Recently, we have found that the sulfation of calenduladiol, a pentacyclic triterpene isolated from *Chuquiraga erinacea*, enhanced the inhibitory activity against AChE from 31% for calenduladiol (0.5 mM) to 94% for disodium calenduladiol disulfate (0.5 mM) [19]. These results prompted us to test the acetylcholinesterase inhibitory activity of sulfated compounds **8** and **10** and compare it to their corresponding diols (**7** and **9**).

Molecular docking studies of an active steroid into the peripheral cavity of AChE were performed in order to explain the inhibitory activity the steroids showed and to corroborate the

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inhibition kinetic data. The determination of the inhibition type is critical for identification of the mechanism of inhibition and the site of inhibitor binding.

2. Experimental

2.1. General methods

Melting points (m.p.) were determined on a Fisher Johns apparatus and are uncorrected. ^{1}H NMR, ^{13}C NMR, HSQC-DEPT, HMBC, COSY and NOESY spectra were recorded on a Bruker AM 500 spectrometer. Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. 2D NMR spectra were obtained using standard Bruker software. High resolution mass spectra were determined on a Bruker micrOTOF-Q II mass spectrometer with ESI as ionization source. IR spectra were acquired on a FT-IR Nicolet Magna 550 spectrometer. UV spectra were recorded on a GBC Spectral UV–VIS spectrophotometer. Elemental analysis was performed on an EAI Exeter Analytical, Inc. CE-440 apparatus. Microwave assisted reactions were carried out in a CEM Discover reactor. Analytical thin layer chromatography (TLC) was performed on pre-coated silica plates (Merck F_{254}, 0.2 mm thickness); TLC of sulfated steroids was performed on silica gel F_{254} (*n*-BuOH/AcOH/H_2O (12:3:5)) and C_{18} reversed-phase plates (MeOH/H_2O (80:20 v/v)) and detected by spraying with sulfuric acid (10% EtOH). Flash column chromatography was performed with silica gel Merck 60 G (90% < 45 μm). Solid phase extraction tubes of silica gel (55 μm) were purchased from Phenomenex. Reversed-phase chromatography was carried out on octadecyl-functionalized silica gel (Aldrich). All chemicals and solvents were analytical grade and solvents were purified by general methods before being used.

The commercially available trimethylamine–sulfur trioxide complex and cholesterol (**1**) were purchased from Aldrich. Cholesterol was used as starting material for the synthesis of compounds **2**-**8**. Compound **1**: ^{1}H NMR δ ($CDCl_3$): 0.68 (s, 3H, H-18), 0.87 (d, J = 6.9 Hz, 6H, H-26, H-27), 0.92 (d, J = 6.9 Hz, 3H, H-21), 1.01 (s, 3H, H-19), 3.52 (m, 1H, H-3β), 5.34 (dd, J = 1.9, 3.3 Hz, 1H, H-6). Acetylcholinesterase (electric eel), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI) and eserine were purchased from Sigma.

2.2. Cholesterol mesylate (**2**)

A solution of 1.16 mL (15 mmol) of methanesulfonyl chloride in 30 mL of MEK was added dropwise over 30 min to a solution of cholesterol (**1**) (3.8683 g, 10 mmol) and triethylamine (2.8 mL) in MEK (70 mL) at 5 °C and then stirred for 30 min at room temperature. The solution was washed successively with 15% NaCl solution, saturated $NaHCO_3$ solution and 15% NaCl solution, dried over anhydrous $MgSO_4$ and evaporated in vacuo to give 4.51 g (97%) of **2**, m.p. 120 °C (acetone–H_2O). ^{1}H NMR δ ($CDCl_3$): 0.67 (s, 3H, H-18), 0.86 (d, J = 6.6 Hz, 6H, H-26, H-27), 0.91 (d, J = 6.6 Hz, 3H, H-21), 1.02 (s, 3H, H-19), 3.01 (s, 3H, C*H*$_3$–SO_3–), 4.52 (m, 1H, H-3β), 5.41 (m, 1H, H-6). ^{13}C NMR ($CDCl_3$): 138.64 (C-5), 123.82 (C-6), 82.06 (C-3), 38.75 (–OSO_2–Me), 22.80 (C-26), 22.54 (C-27), 18.69 (Me-21), 19.18 (Me-19), 11.8 (Me-18). HREIMS (ESI+), calculated for $C_{28}H_{52}NO_3S$ [M+NH_4]$^+$: 482.3662, found m/z = 482.3679. FT-IR (NaBr, film, cm^{-1}) 3050 (δ C = C–H), 1665 (δ C=C), 1465 (δ_{as} CH_3), 1352 (δ_s CH_3), 1177 (δ_s O=S=O).

2.3. 3α,5-cyclo-cholestan-6β-ol (**3**)

A solution of 3.02 g (6.49 mmol) of the mesyl ester **2** in acetone (30 mL) was added to a solution of $NaHCO_3$ (6.15 g) in water (12.1 mL). It was stirred vigorously and heated under reflux at 85–95 °C for 7 h. Then, a mixture of ethyl acetate and water (1:1) was added and the organic layer was separated and then washed with saturated NaCl solution. It was dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was purified by dry column flash chromatography on silica gel using hexane/AcOEt (97:3) to give 1.83 g (73%) of **3** as a white crystalline solid, m.p. 65–66 °C (acetone–H_2O). ^{1}H NMR δ ($CDCl_3$): 0.29 (dd, J = 4.9 Hz, 8.1 Hz, 1H, H-4α), 0.52 (t, J = 4.5 Hz, 4.5 Hz, 1H, H-4β), 0.72 (s, 3H, H-18), 0.86 (d, J = 6.6 Hz, 6H, H-26, H-27), 0.91 (d, J = 6.6 Hz, 3H, H-21), 1.05 (s, 3H, H-19), 1.08 (m, 1H, H-3α), 3.25 (m, 1H, H-6α). ^{13}C NMR δ ($CDCl_3$): 33.34 (C-1), 25.17 (C-2), 24.35 (C-3), 11.72 (C-4), 39.03 (C-5), 73.95 (C-6), 37.31 (C-7), 29.99 (C-8), 47.80 (C-9), 43.03 (C-10), 22.96 (C-11), 40.35 (C-12), 42.87 (C-13), 56.64 (C-14), 24.39 (C-15), 28.45 (C-16), 56.50 (C-17), 12.33 (C-18), 20.36 (C-19), 35.94 (C-20), 18.85 (C-21), 36.31 (C-22), 24.00 (C-23), 39.65 (C-24), 28.16 (C-25). 22.71 (C-26), 22.85 (C-27). Anal. calcd for $C_{27}H_{46}O$, C 83.87, H 11.99, O 4.14. Found, C 83.61, H 11.83. HREIMS (ESI+), calculated for $C_{27}H_{46}NaO$ [M+Na]$^+$: 409.3441, found m/z = 409.3427. FT-IR (NaBr, film, cm^{-1}) 3431 (ν O–H), 1493 (δ_{as} CH_3), 1377 (δ_s CH_3).

2.4. 3α,5-cyclo-cholestan-6-one (**4**)

Jones reagent (4 mL) was added dropwise to a stirred and cooled solution of 1.83 g of **3** (4.73 mmol) in 46 mL of MEK until the mixture remained reddish. Then, a solution of diluted $NaHSO_3$ was added until the solution remained green. The organic layer was separated and then washed with 15% NaCl solution, dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was purified by dry column flash chromatography on silica gel using hexane/AcOEt (95:5) to give 1.36 g (75%) of **4** as a white crystalline solid, m.p. 96.5–97 °C (acetone–H_2O). ^{1}H NMR δ ($CDCl_3$): 0.72 (s, 3H, H-18), 0.73 (t, J = 5.1 Hz, H-4α), 0.86 (d, J = 6.6 Hz, 6H, H-26, H-27), 0.91 (d, J = 6.6 Hz, 3H, H-21), 1.05 (s, 3H, H-19), 1.52 (m, 1H, H-3α), 1.69 (t, H-4β), 1.88 (m, H-7α), 2.43 (d, J = 12.3 Hz, H-7β). ^{13}C NMR δ ($CDCl_3$): 33.60 (C-1), 26.05 (C-2), 35.45 (C-3), 11.79 (C-4), 46.47 (C-5), 209.97 (C-6), 44.96 (C-7), 34.94 (C-8), 46.22 (C-9), 46.90 (C-10), 22.70 (C-11), 39.88 (C-12), 42.90 (C-13), 57.14 (C-14), 24.20 (C-15), 28.30 (C-16), 56.25 (C-17), 12.18 (C-18), 19.83 (C-19), 35.84 (C-20), 18.82 (C-21), 36.25 (C-22), 23.97 (C-23), 39.62 (C-24), 28.15 (C-25). 22.70 (C-26), 22.96 (C-27). Anal. calcd for $C_{27}H_{44}O$, C 84.31, H 11.53, O 4.16. Found, C 84.51, H 11.36. HREIMS (ESI+), calculated for $C_{27}H_{44}NaO$ [M+Na]$^+$: 407.32844, found m/z = 407.32837. FT-IR (NaBr, film, cm^{-1}) 1679 (ν C=O), 1443 (δ_{as} CH_3), 1363 (δ_s CH_3).

2.5. 5α-cholest-2-en-6-one (**5**)

p-Toluensulfonic acid (146.7 mg) and sodium bromide (172.6 mg) were added to a solution of 1.31 g (3.41 mmol) of **4** in 14 mL of dry dimethylformamide. The mixture was stirred and heated under reflux for 4 h. Ethyl acetate (40 mL) was added to the cooled reaction mixture, the organic layer was washed successively with water (3 × 40 mL), then dried over anhydrous $MgSO_4$ and evaporated to dryness. The reaction product was isolated by dry column flash chromatography on silica gel using hexane/AcOEt (96:4) to give 1.04 g (79%) of **5** as a white crystalline solid, m.p. 99.5–100.5 (acetone–H_2O). ^{1}H NMR δ ($CDCl_3$): 0.66 (s, 3H, H-18), 0.70 (s, 3H, H-19), 0.86 (d, J = 6.6 Hz, 6H, H-26, H-27), 0.91 (d, J = 6.5 Hz, 3H, Me-21), 1.97 (m, H-7α), 2.35 (m, H-7β), 5.56 (m, H-2), 5.68 (m, H-3). ^{13}C NMR δ ($CDCl_3$): 39.61 (C-1), 124.68 (C-2), 125.01 (C-3), 21.87 (C-4), 53.99 (C-5), 212.24 (C-6), 47.17 (C-7), 37.87 (C-8), 53.58 (C-9), 39.65 (C-10), 21.26 (C-11), 39.51 (C-12), 42.96 (C-13), 56.91 (C-14), 24.10 (C-15), 28.17 (C-16), 56.26 (C-17), 12.07 (C-18), 13.83 (C-19), 35.85 (C-20), 18.80 (C-21), 36.24 (C-22), 23.95 (C-23), 39.51 (C-24), 28.14 (C-25). 22.69 (C-26), 22.95 (C-27). Anal. calcd for $C_{27}H_{44}O$, C 84.31, H 11.53, O 4.16. Found, C 84.37, H 11.61. HREIMS (ESI+), calculated for $C_{27}H_{44}NaO$ [M+Na]$^+$: 407.32844, found m/z = 407.32861. FT-IR (NaBr, film, cm^{-1}) 3025

(ν C=C–H), 1710 (ν C=O), 1657 (ν C=C), 1459 (δ_{as} CH_3), 1380 (δ_s CH_3).

2.6. 2,3α-epoxy-5α-cholestan-6-one (**6**)

To a solution of 5α-cholest-2-ene-6-one (**5**) (246 mg, 0.64 mmol) in CH_2Cl_2 (8.6 mL) were added 10% Na_2CO_3 solution (9.8 mL). The reaction mixture was stirred vigorously and *m*-chloroperbenzoic acid (359 mg, 2.08 mmol) in 3.7 mL of CH_2Cl_2 was added slowly at 5 °C. The mixture was stirred for 4 h at 5 °C, and then the aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined CH_2Cl_2 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 dry column flash chromatography on silica gel using hexane/AcOEt (95:5) to give 169.2 mg (66%) of **6** as a white powder, m.p. 141-142 °C (acetone). ^{1}H NMR δ ($CDCl_3$): 0.64 (s, 3H, H-18), 0.70 (s, 3H, H-19), 0.85 (d, *J* = 6.6 Hz, 3H, H-26), 0.86 (d, *J* = 6.6 Hz, 3H, H-27), 0.90 (d, *J* = 6.5 Hz, 3H, Me-21), 1.92 (m, H-7α), 2.31 (dd, *J* = 13.2 Hz, 4.1 Hz, H-7β), 3.11 (dd, *J* = 5.7 Hz, 4.1 Hz, 1H H-2β), 3.26 (m, 1H, H-3β). ^{13}C NMR δ ($CDCl_3$): 38.00 (C-1), 50.31 (C-2), 52.53(C-3), 21.00 (C-4)*, 50.02 (C-5), 211.68 (C-6), 47.07 (C-7), 37.63 (C-8), 53.25 (C-9), 38.41 (C-10), 21.03 (C-11)*, 39.50 (C-12), 42.87 (C-13), 56.68 (C-14), 24.06 (C-15), 28.13 (C-16), 56.17 (C-17), 12.02 (C-18), 15.15 (C-19), 35.81 (C-20), 18.77 (C-21), 36.19 (C-22), 23.91 (C-23), 39.59 (C-24), 28.13 (C-25), 22.68 (C-26), 22.94 (C-27). (*) Signals might be interchangeable. Anal. calcd for $C_{27}H_{44}O_2 \cdot 1/2H_2O$, C 79.16, H 11.07, O 9.77. Found, C 78.88, H 10.71. HREIMS (ESI+), calculated for $C_{27}H_{44}NaO_2$ $[M+Na]^+$: 423.32335, found *m*/*z* = 423.32413. FT-IR (NaBr, film, cm^{-1}) 1704 (ν C=O), 1469 (δ_{as} CH_3), 1382 (δ_s CH_3), 1254 (ν_s C–O–C), 802 (ν_{as} C–O–C).

2.7. 2β,3α-dihydroxy-5α-cholestan-6-one (**7**)

A solution of epoxide **6** (85.7 g, 0.21 mmol) in THF (4 mL) was treated with 1 N H_2SO_4 (0.20 mL) and stirred for 24 h at room temperature. After neutralization with saturated $NaHCO_3$ solution the mixture was evaporated to fifth initial volume, diluted with water (10 mL), and extracted with ethyl acetate (3 × 10 mL). The combined organic extracts were washed with water, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The crude diol **7** was submitted to column chromatography on silica gel (0.063–0.200 mm), eluted with CH_2Cl_2/MeOH (95:5 v/v) and evaporated under reduced pressure to afford pure 2β,3α-dihydroxy-5α-cholestan-6-one (**7**) (66.82 mg, 76%), m.p. 181-182 °C (acetone–H_2O). ^{1}H NMR δ (CD_3OD): 0.71 (s, 3H, H-18), 0.88 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.94 (s, 3H, H-19), 0.95 (d, *J* = 6.6 Hz, 3H, Me-21), 2.09 (m, H-7α), 2.20 (dd, *J* = 13.1 Hz, 4.8 Hz, H-7β), 3.81 (m, 1H, H-2α), 3.85 (m, 1-H, H-3β). ^{13}C NMR δ (CD_3OD): 40.07 (C-1), 70.94 (C-2), 70.09 (C-3), 24.00 (C-4), 52.77 (C-5), 215.73 (C-6), 47.47 (C-7), 38.82 (C-8), 55.78 (C-9), 42.16 (C-10), 24.93 (C-11), 40.88 (C-12), 44.17 (C-13), 57.87 (C-14), 24.93 (C-15), 29.15 (C-16), 57.46 (C-17), 12.46 (C-18), 15.56 (C-19), 37.04 (C-20), 19.18 (C-21), 37.30 (C-22), 22.27 (C-23), 40.67 (C-24), 29.15 (C-25), 23.20 (C-26), 22.95 (C-27). Anal. calcd for $C_{27}H_{46}O_3 \cdot 1/2H_2O$, C 75.83, H 11.07, O 13.10. Found, C 75.72, H 11.07. HREIMS (ESI+), calculated for $C_{27}H_{47}O_3$ $[M+H^+]$: 419.35197, found *m*/*z* = 419.35174. FT-IR (NaBr, film, cm^{-1}) 3431 (ν O–H), 1690 (ν C=O), 1450 (δ_{as} CH_3).

2.8. Disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**8**)

Trimethylamine–sulfur trioxide complex (36 mg, 0.26 mmol) was added to a solution of 2β,3α-dihydroxy-5α-cholestan-6-one (**7**) (14 mg, 0.033 mmol) in DMF (0.45 mL). The reaction mixture was irradiated and stirred at 150 °C for 7 min in a sealed tube in a microwave reactor and then quenched with water (0.5 mL). After evaporation to dryness the residue was eluted through Amberlite CG-120 (sodium form) with methanol, evaporated under reduced pressure and purified by solid phase extraction over silica gel (55 μm). Fractions eluted with CH_2Cl_2/MeOH (98:2) afforded pure disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**8**) (19 mg, 92%), m.p. 184.5-185 °C (decomp). ^{1}H NMR δ (CD_3OD): 0.71 (s, 3H, H-18), 0.88 (d, *J* = 6.7 Hz, 3H, H-27), 0.89 (d, *J* = 6.7 Hz, 3H, H-26), 0.94 (s, 3H, H-19), 0.95 (d, *J* = 6.7 Hz, 3H, Me-21), 2.12 (m, H-7α), 2.19 (dd, *J* = 13.3 Hz, 5.1 Hz, H-7β), 4.73 (m, 1H, H-2α), 4.77 (m, 1-H, H-3β). ^{13}C NMR δ (CD_3OD): 38.35 (C-1), 75.17 (C-2), 75.11 (C-3), 22.45 (C-4), 52.65 (C-5), 214.83 (C-6), 47.32 (C-7), 38.89 (C-8), 55.63 (C-9), 41.81 (C-10), 24.88 (C-11), 40.84 (C-12), 44.16 (C-13), 57.82 (C-14), 24.93 (C-15), 29.14 (C-16), 57.42 (C-17), 12.43 (C-18), 15.24 (C-19), 37.03 (C-20), 19.14 (C-21), 37.29 (C-22), 22.25 (C-23), 40.66 (C-24), 29.14 (C-25), 23.17 (C-26), 22.92 (C-27). HREIMS (ESI+), calculated for $C_{27}H_{44}NaO_9S_2$ $[M-2H+Na]^-$: 599.23299, found *m*/*z* = 599.23265. FT-IR (KBr, cm^{-1}) 1699 (ν C=O), 1463(δ_{as} CH_3), 1382 (δ_s CH_3), 1254 and 1221 (δ_s S=O).

2.9. Acetylcholinesterase inhibition assay

Electric eel (*Torpedo californica*) AChE activity was measured in vitro by a modified spectrophotometric method developed by Ellman et al. [20]. The lyophilized enzyme (425 U/mg solid) was prepared in buffer phosphate (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4) to obtain 5 U/mL stock solution. Further enzyme dilution was carried out with buffer phosphate (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 U/mL enzyme solution. Samples were dissolved in the same buffer. AChE solution (300 μL) and 300 μL of sample solution were mixed in a test tube and incubated for 30 min at room temperature. The reaction was started by adding 600 μL of the substrate solution (0.1 M Na_2HPO_4, 0.5 mM (DTNB, 0.6 mM ATCI, 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 probit analysis (EPA Probit 1.4). Eserine (99%) was used as the reference AChE inhibitor.

2.10. Graphic determination of inhibitor type

The enzyme reaction was carried out at three fixed inhibitor (compound **8**) concentrations (0, 52 and 104 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 $R^2 = 0.9703$. The calculated αKi was 121.1 μM.

2.11. Molecular docking determinations

Structure of Protein Data Bank (PDB) entry 2ACE [21], *T. californica* AChE crystal structure, complexed with acetylcholine, was used for the docking simulations of disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**8**). Geometry optimization was performed with the Hartree-Fock [22] method and the 6–31 + G (d) basis set and semiempirical calculations (AM1) [23] incorporated in the Gaussian 03 program [24]. The charges of the ligands were obtained using the standard RESP procedure.

Docking studies were performed with version 4.0 of the program AutoDock [25], using the implemented empirical free energy function. The graphical user interface program AutoDock Tools was used to prepare, run and analyze the docking simulations. The simulation space was defined as a 21.28 Å × 31.24 Å × 20.37 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 the steroids were allowed to rotate freely but those of the side chain. The starting positions of the steroids were outside the grid on a random position.

The steroids were docked by the Lamarckian genetic algorithm protocol. A total of 256 independent simulations with a population size of 150 members were run for the steroid using Autodock 4.0 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 each compound 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.

3. Results and discussion

The first reported synthesis of disulfated steroid **8** (Scheme 1) starts from commercially available cholesterol (**1**). This reacted with methanesulfonyl chloride to give cholesterol mesylate (**2**). Following the procedure of Mori et al. [26] a rearrangement and subsequent oxidation with Jones reagent converted the mesylate steroid **2** to the 3α,5-cyclo-6-one steroid **4**. The ^{1}H NMR spectrum of **4** showed two signals at δ 0.73 (H-4α) and 1.69 (H-4β) ppm ascribed to the methylene protons of the cyclopropane ring. This is in accordance with the chemical shift observed at δ 11.79 ppm in the ^{13}C NMR spectrum of **4** and assigned to C-4 on the basis of HSQC and HMBC spectra. Rearrangement of compound **4** generated the Δ^2 double bond in **5**. Multiplets at δ 5.56 (H-2) and 5.68 (H-3) ppm in the ^{1}H NMR spectrum together with the corresponding signals at δ 124.68 (C-2) and 125.68 (C-3) ppm as well as the carbonyl signal at 212.24 (C-6) ppm confirmed the structure of compound **6**. Further epoxidation and acid aperture of epoxide **6** [14] rendered diol **7**. The ^{1}H NMR spectrum of **7** showed two multiplets at δ 3.81 (H-2β) and 3.85 (H-3α) ppm, geminal to hydroxyl groups at C-2 (70.94) and C-3 (70.09), as determined by analysis of ^{13}C NMR, HSQC, HMBC and NOESY spectra.

Compound **7** was purified by column chromatography on silica gel and used as starting material for the synthesis of compound **8**. Trimethylamine–sulfur trioxide complex was selected as the sulfating reagent instead of the triethyl complex used in our previous work [14] owing to the better yields obtained. A microwave based protocol was chosen to enhance the rate of sulfation, dramatically decreasing the reaction time from 13 h to 7 min [27]. Treatment of diol **7** with 8 equiv. of trimethylamine–sulfur trioxide complex for 7 min at 150 °C at a microwave reactor afforded the ammonium sulfate of compound **8**, which was transformed via ion exchange into the disodium salt **8** (Scheme 1). Disodium 2β,3α-dihydroxy-5α-cholestan-6-one (**8**) showed two methine signals at δ 4.73 (H-2) and 4.77 (H-3), characteristic of the presence of two sulfate groups at C-2 and C-3 [14]. This is in accordance with the chemical shifts observed for C-2 (72.0 ppm) and C-3 (71.8 ppm) in the ^{13}C NMR spectrum, as determined from the HSQC spectrum. Both methine signals are broad singlets according to their equatorial position in the ring supporting a 2β,3α configuration, as determined by NOESY.

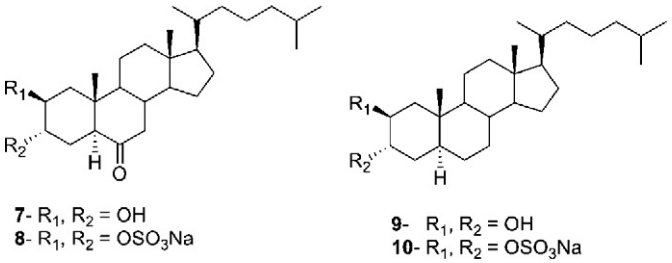


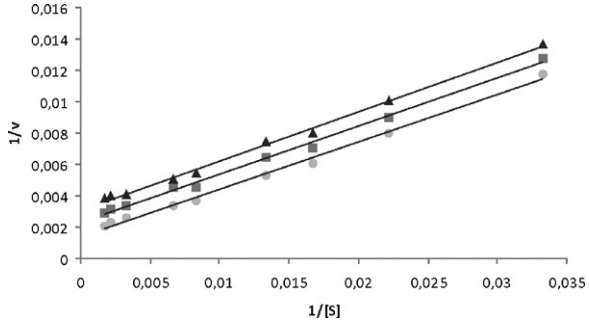
Fig. 1. Chemical structure of diols (**7**, **9**) and sulfated steroids (**8**, **10**) tested against AChE.

Table 1
Summary of the in vitro antiacetylcholinesterase activities.

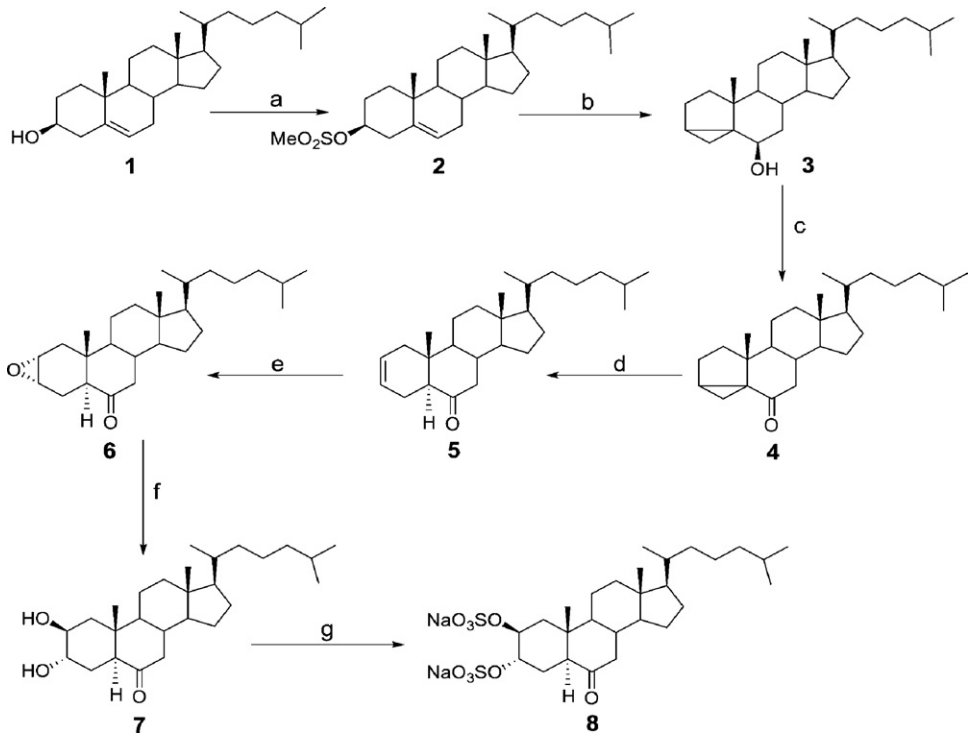
Compound	7	8	9	10
IC_{50} (μM)	>500	14.59 ± 0.88	>500	59.65 ± 2.30

The acetylcholinesterase inhibitory activity of compounds **7** and **8** was evaluated and compared to that of analogs **9** and **10** (Fig. 1) previously synthesized by our group [14]. From the data shown in Table 1, disulfated compounds **8** and **10** were the most active with IC_{50} values of 14.59 and 59.65 μM, respectively. Diols **7** and **9** showed no inhibitory activity (IC_{50} > 500 μM). These results are indicative of the role of the sulfate groups in the AChE inhibitory activity of **8** and **10** and suggest that the presence of a carbonyl group at C-6 in **8** enhances its activity.

Disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**8**), the most active compound in the tested set, 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 (Graphic 1). The double-reciprocal plots show that with increasing concentration of **8** the values of both K_m and V_{max} are enhanced, 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 **8** does not bind to the free enzyme but binds reversibly to the enzyme–substrate complex, yielding an inactive complex. This inhibition is an example of the sequential order of binding of two ligands to the enzyme in an obligate order. The substrate binding is considered necessary to produce conformational changes in the enzyme, which creates or opens the inhibitor binding site [28]. Therefore, molecular docking studies were performed with the AChE complexed with acetylcholine. Table 2 summarizes the docking results of 2β,3α-



Graphic 1. Lineweaver–Burk plots of the inhibition of AChE by 2b,3a-dihydroxy-5a-cholestane-6-one disulfate (**8**) with acetylthiocholine (S) as a substrate. Concentrations of compound **8**: (circle): 0 μM; (square): 52 μM; (triangle): 104 μM. Linear regression equations: $y = 0.3004x + 0.0014$ ($R^2 = 0.9953$); $y = 0.3066x + 0.0023$ ($R^2 = 0.9959$); $y = 0.3142x + 0.0031$ ($R^2 = 0.9963$) for 0, 52 and 104 μM respectively.



Scheme 1. Conditions: (a) MsCl, TEA, MEK, (b) $NaHCO_3$, H_2O, MEK, (c) CrO_3, H_2SO_4, MEK, (d) LiBr, p-TsOH, DMF (e) *m*-ClPBA, Na_2CO_3, H_2O–Cl_2CH_2, (f) H_2SO_4, THF and (g) 8 equiv. $Et_3N.SO_3$, DMF, Amberlite CG-120 (MeOH).

dihydroxy-5α-cholestan-6-one disulfate (**8**). 256 docking runs with **8** generated six clusters. All of them showed the steroid buried into the aromatic gorge and five of the six clusters showed that the steroid penetrates the peripheral site through the side chain, which might be due to its aliphatic character in comparison with that of the disulfated six-membered ring A (Fig. 2). The main hydrophobic interactions between the hydrocarbon skeleton of the inhibitor and the protein were observed with the residues: TYR 70, ASP 72, TRP 84, TYR 124, TYR 121, TRP 279, PHE 330 and TYR 334 (Fig. 3). These results are consistent with molecular modeling studies of natural cholinesterase-inhibiting steroidal alkaloids isolated from *Sarcococca saligna* [28]. As for steroid **8**, the steroidal alkaloids penetrate the aromatic gorge of the enzyme through the most hydrophobic side of the molecule [29].

The docking studies also showed that the affinity of steroid **8** for the complex enzyme–substrate is favoured by hydrogen bonding interactions, which involve both sulfates at ring A. Sulfate groups at C-2 and C-3 come close to TYR 70 and TRP 279, respectively. Hydrogen bonding between the sulfate group at C-3 of the inhibitor and the amidic hydrogen of TRP 279, and between the sulfate group at C-2 and the hydroxyl of TYR 70 may occur as the distances and angles observed are within a suitable range.

Table 2
Summary of the docking results of 2(,3(-dihydroxy-5(-cholestan-6-one disulfate (**8**).

Compound	Total number of clusters	Docking statistics	
		Cluster rank	Number of runs in the cluster
8	6	1	33
		2	140
		3	15
		4	1
		5	65
		6	2

The docking studies allowed us to establish the binding mode of steroid **8** to the enzyme–substrate complex and identify hydrophobic interactions inside the aromatic gorge as well as hydrogen bonding interactions as the 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 and to determinate if the enzyme undergoes structural rearrangements.

Taking into account the docking results and the AChE inhibitory activity of steroid **8**, this disulfated compound is a good model for a rationale design of analogs by introducing additional functional groups at the steroidal skeleton or its side chain in order to evaluate structure–activity correlations in the acetylcholinesterase inhibitory activity.

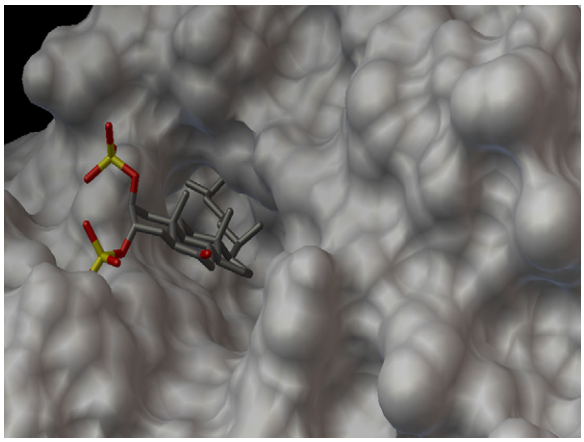


Fig. 2. Docking result for disodium 2β,3α-dihydroxy-5α-cholestan-6-one disulfate (**8**).

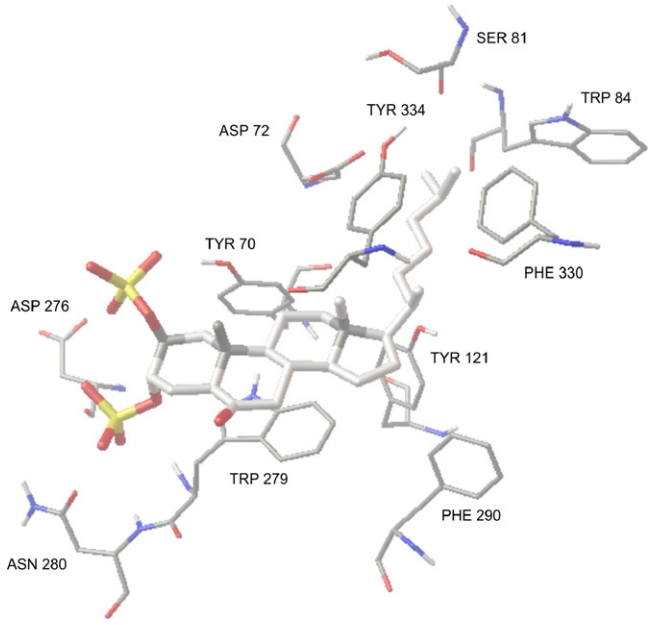


Fig. 3. Docking of compound **8** showing the interactions with the enzyme.

Acknowledgments

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